Molecular Genetic Characterization of Endemic Yellow Catfish, *Horabagrus brachysoma* (Gunther)

Thesis submitted in partial fulfilment of
the requirements for the degree of

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April 2005

Declaration

I hereby declare that this Ph. D thesis entitled "**Molecular genetic characterization of endemic yellow catfish, *Horabagrus brachysoma* (Gunther)**" is the authentic and bonafide record of the research work done by me at National Bureau of Fish Genetic Resources (NBFGR) Cochin Unit, at Central Marine Fisheries Research Institute, Cochin and it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

Cochin
15th April 2005

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Certificate

This is to certify that the thesis entitled, "**Molecular genetic characterization of endemic yellow catfish, *Horabagrus brachysoma* (Gunther)**" is an authentic record of original and bonafide research work carried out by **Mr. Abdul Muneer P.M. (Reg. No. 2472)** at National Bureau of Fish Genetic Resources (NBFGR) Cochin Unit, at Central Marine Fisheries Research Institute, under my supervision and guidance for the award of the degree of **Doctor of Philosophy** in the Faculty of Marine Sciences, **Cochin University of Science and Technology, Cochin,** Kerala. It is also certified that no part of the work presented in this thesis has been submitted earlier for the award of any other degree, diploma or any other similar title.

A. Gopalakrishnan

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Abdul Muneer P.M.

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Contents

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i. List of Abbreviations

µg	:	Micro grams
µl	:	Micro litre
AAT	:	Aspartate amino transferase
ACP	:	Acid phosphatase
ADH	:	Alcohol dehydrogenase
AFLP	:	Amplified Fragment Length Polymorphism
AGE	:	Agarose Gel Electrophoresis
AK	:	Adenylate kinase
ALP	:	Alkaline phosphatase
APS	:	Ammonium persulphate
bp	:	Base pairs
BPB	:	Bromo phenol blue
CK	:	Creatine kinase
CMFRI	:	Central Marine Fisheries Research Institute
Da	:	Dalton
DNA	:	Deoxyribo Nucleic Acid
dNTPs	:	Deoxynucleoside tri phosphates
EDTA	:	Ethylene Diamine Tetra Acetic acid
EST	:	Esterase
F_{IS}	:	Co-efficient of inbreeding
F_{ST}	:	Co-efficient of genetic differentiation
FUM	:	Fumerase
G_6PDH	:	Glucose-6-phosphate dehydrogenase
GAPDH	:	Glyceraldehyde-3-Phosphate dehydrogenase
αG_3PDH	:	α-Glycerophosphate dehydrogenase
GDH	:	Glutamate dehydrogenase
GLDH	:	Glucose dehydrogenase
GPI	:	Glucose phosphate isomerase
H	:	Average gene diversity or heterozygosity
HK	:	Hexokinase
Hobs	:	Observed heterozygosity
Hexp	:	Expected heterozygosity
ICDH	:	Isocitrate dehydrogenase
HWE	:	Hardy-Weinberg Equilibrium
IUCN	:	International Union for Conservation of Nature and Natural Resources

LDH	:	Lactate dehydrogenase
MDH	:	Malate dehydrogenase
ME	:	Malic enzyme
MFRs	:	Microsatellite Flanking Regions
Mol. wt.	:	Molecular weight
mtDNA	:	Mitochondrial DNA
MW	:	Molecular weight
NAD	:	Nicotinamide adenine dinucleotide
NADP	:	Nicotinamide adenine dinucleotide phosphate
NATP	:	National Agricultural Technology Project
NBFGR	:	National Bureau of Fish Genetic Resources
NCBI	:	National Centre for Biotechnology Information
nDNA	:	Nuclear DNA
na	:	Observed number of alleles
ne	:	Effective number of alleles
Ne	:	Effective population size
ng	:	Nano grams
Nm	:	Rate of gene flow
ODH	:	Octonol dehydrogenase
PAGE	:	Poly Acrylamide Gel Electrophoresis
PCR	:	Polymerase Chain Reaction
6PGDH	:	Phosphogluconate dehydrogenase
PGM	:	Phosphoglucomutase
PK	:	Pyruvate kinase
RAPD	:	Random Amplified Polymorphic DNA
RARS	:	Regional Agricultural Research Station
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribo Nucleic Acid
rpm	:	Revolutions per minute
SDS	:	Sodium Dodecyl Sulphate
SOD	:	Superoxide dismutase
SSRs	:	Simple Sequence Repeats
STRs	:	Short Tandem Repeats
TEMED	:	N.N.N'.N'. Tetra Methyl Ethylene Diamine
T_a	:	Annealing Temperature
T_m	:	Melting Temperature
VNTRs	:	Variable Number of Tandem Repeats
XDH	:	Xanthine dehydrogenase

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INTRODUCTION

Fishes are valuable sources of high-grade protein and other organic products. They occupy a significant position in the socio-economic fabric of the South Asian countries by providing the population with not only nutritious food but also income and employment opportunities. Of the 21,723 fish species known to science, over 40% live in freshwater and the majority of them live in the tropics between latitudes $23^{0}5'$ N and $23^{0}5'$ S. Nowhere in the world is a zoogeographic region so blessed as the Indian subcontinent (India, Pakistan, Nepal, Myanmar, Sri Lanka and Bangladesh) in respect of the diversity of fish wildlife that dwells in the inland waters. India is endowed with a vast expanse of open inland waters in the form of rivers, canals, estuaries, natural and man-made lakes, backwaters, brackishwater impoundment and mangrove wetlands. The freshwater fishes are closely bound to the landmasses and are inescapably confined to their own drainage systems. Their dispersal from one system to another can only be effected through the hydrographic changes caused by the geological and climatic factors, with the exceptions of human interference (Talwar and Jhingran, 1991). In this study, a fish species found in rivers originating from the Western Ghat region was taken for population genetic analysis.

The Western Ghat Mountains along the west coast of Indian Peninsula constitute one of the unique biological regions of the world. Geographically the Western Ghats lie along the west coast from Kanyakumari up to the Tapti River in Gujarat ($8^{0}N$ – $21^{0}N$). It has been rightly recognized as one of the 25 globally identified 'hot spot' areas of mega bio- diversity for conservation and one of the two such areas in the country. With respect to freshwater fish species, the streams and rivers originating from Western Ghats have been identified as one of the few sites in

the world exhibiting high degree of endemism and exceptional biodiversity (Myers *et al.*, 2000).

In spite of its rich piscine diversity, practically no attention has been paid to stock assessment, sustainable utilization and conservation of the Western Ghats species. The National Bureau of Fish Genetic Resources (NBFGR) has recently identified 295 species from the region of which approximately 195 are endemic to the Western Ghats (Shaji *et al.,* 2000). Several endemic food fishes of the region have been enlisted as endangered, either due to over exploitation, wanton destruction of spawners, dynamiting or construction of dams (Anon, 1998). Attempts to promote aquaculture practices in the area using transplanted Indian major carps and other exotic species have led to further deterioration in the situation. These waters are also considered as the gold mine for nearly 110 endemic ornamental fishes like loaches, cyprinids and bagrid catfishes. But, recent surveys report an alarming rate of depletion due to over-exploitation and clandestine export (Ponniah and Gopalakrishnan, 2000).

1.1. Scope of the study

The water bodies in the form of oceans, rivers, lakes etc., have been exploited by man since time immemorial for the augmentation of food production. The heavy and sometimes ruthless exploitation has even caused extinction of many of the aquatic flora and fauna. There is an urgent need for the development of apt management strategies to exploit resources judiciously. One of the strategies thus developed for the scientific management of these resources was to identify the natural units of the fishery resources under exploitation (Altukov, 1981). Natural units of a species can otherwise be called 'stocks'. A stock can be defined as "a panmictic population of related individuals within a single species that is genetically distinct from other such populations" (Shaklee *et al.,* 1990).

Genetic variation in fishes has proven valuable in aquaculture and fisheries management, for identification of stocks, in selective breeding programmes and for estimating contributions to stock mixtures. Moreover, an efficient use of biological resources requires a thorough knowledge of the amount and distribution of genetic

variability within the species considered. Generally, individuals with greater genetic variability have higher growth rates, developmental stability, viability, fecundity, and resistance to environmental stress and diseases (Carvalho, 1993). It is believed that a species may undergo microevolutionary processes and differentiate into genetically distinct sub-populations or stocks in the course of time, if reproductively and geographically isolated. In recent times, there has been a widespread degradation of natural aquatic environment due to anthropogenic activities and this has resulted in the decline and even extinction of some fish species. In such situations, evaluation of the genetic diversity of fish resources assumes importance. A proper knowledge of the genetic make-up and variability of fish stocks will help us in the management, conservation of endangered species and improvement of stocks of cultivable species. If the population genetic structure of a species is known, the distribution of subpopulation in mixed fisheries can also be estimated easily. A lack of knowledge about the genetic structure of these populations may result in the differential harvest of the populations that will ultimately have a drastic and long-term effect. To overcome this, there is always a need for investigations encompassing the genetic variations at the intra and inter-population levels as well as at the intra and inter-specific levels of the fish and shellfish resources of any nation (Allendorf and Utter, 1979).

For the accomplishment of above objectives, scientists all over the world have developed different methodologies to distinguish and characterize fish stocks and to evaluate genetic variation. One of the traditional methods for distinguishing fish stocks has been the comparative examination of morphological characters (Hubbs and Lagler, 1947). But the conventional morphometric measurements are often inefficient and biased, as they may produce uneven areal coverage of the body form. Most of the landmarks were repetitive and unidirectional lacking information of depth and breadth of the body forms (Strauss and Bookstein, 1982; Sathianandan, 1999). This had led to the development of a new method called a 'truss network analysis', where the shape of the body forms of fish or shellfish is also taken into account along with the size (Humphries *et al.*, 1981; Winans, 1984). However, the application of truss network analysis for the identification of stocks is as complicated as the morphometric

measurement. The reason for this is the role of non-genetic factors in determining the variability of morphological characters. In the mid fifties, protein electrophoresis (Smithies, 1955) and histochemical staining methods (Hunter and Markert, 1957) gained advantage over morphological studies by providing rapidly collected genetic data. This method is capable of unveiling the invisible differences at the molecular level as visible biochemical phenotypes through allozyme electrophoresis. Allozymes are the direct gene products, coded by a single locus, and often appear in different molecular forms. Any detectable change at the allozyme level reflects the genetic change in the nucleotide sequence of DNA. This genetic change is heritable in Mendelian fashion and the pattern of allozyme gene expression is of co-dominant type (Ayala, 1975). These characteristics make allozymes superior markers over morphological characteristics. Stock identification of several species has been carried out using the above-mentioned techniques (Ferguson, 1980; Shaklee *et al.*, 1990; Ferguson *et al.*, 1995; O'Connell and Wright, 1997; Rossi *et al.*, 1998). Allozymes were also found to be helpful in generating species-specific profiles and resolving taxonomic ambiguities in several species (Rognon *et al.*, 1998; Gopalakrishnan *et al.*, 1997; Menezes, 1993; Low *et al.*, 1992; Menezes *et al.*, 1992; Menezes and Taniguchi, 1988; Pouyaud *et al.*, 2000).

The amino acid substitutions of protein detected by electrophoresis are indirect reflections of the actual base substitutions in base sequences. Furthermore, all base substitutions do not necessarily result in change of amino acids and all amino acid substitutions do not result in protein change that are electrophoretically detectable. It has been estimated that only about one third of the amino acid substitutions are detected under the conditions used to collect electrophoretic data in most laboratories (Lewontin, 1974). It is apparent from the above facts that the electrophoretic identity of proteins does not necessarily mean identity of base sequences in DNA. The vast majority of DNA within the nucleus does not code for protein products and therefore, probably do not affect the fitness of an individual fish. Thus, these non-coding DNA sequences are under relaxed selective constraints and may be freer to evolve much more rapidly than the coding sequences.

With the advent of thermocyclers the amplification of small fragments of DNA through Polymerase Chain Reaction (PCR) has gained popularity. This enabled the users to screen polymorphisms in the DNA of individuals without sacrificing them. One such technique (Williams *et al.*, 1990 and Welsh and McClelland, 1990) is Random Amplified Polymorphic DNA (RAPD) based on PCR using short single primers of arbitrary nucleotide sequence typically of length of ten nucleotides that amplified random segments of the genome. Like allozyme markers, the amplified fragments are also often inherited in Mendelian fashion (Williams *et al.*, 1993; Bardakci and Skibinski, 1994; Appleyard and Mather, 2000). RAPD fingerprinting has been used recently in many studies for the analysis of phylogenetic and genetic relationship among organisms (Stiles *et al.*, 1993; Bardakci and Skibinski, 1994; Orozco-castillo *et al.*, 1994; Van Rossum *et al.*, 1995).

Amplified fragment length polymorphism (AFLP) is another advanced technique suitable for finger printing simple and complex genomes from different species (Vos *et al.*, 1995; Felip *et al.*, 2000). In AFLP, genomic DNA is digested by restriction endonucleases and amplified by PCR using primers that contain common sequences of the adapters and one to three arbitrary nucleotides as selective sequences (Lin and Kuo *et al.*, 2001).

Variable Number of Tandem Repeat (VNTR) includes minisatellites and microsatellites. Minisatellites are DNA sequences usually 10-200 bp long that are repeated in tandem in variable numbers. Microsatellites are the tandemly repeated DNA sequences with repeat size of 1-6 bp repeated several times flanked by regions of non-repetitive DNA (Tautz, 1989). These are highly polymorphic in nature and can be analyzed with the help of Polymerase Chain Reaction (PCR). They are another type of powerful DNA marker used for quantifying genetic variations within and among populations of species (O'Connell *et al.*, 1997).

The mitochondrial DNA (mtDNA) is another type of molecular marker, which can reveal high levels of sequence diversity at the species and lower levels, despite great conservation of gene function and arrangement (Avise and Lansman,

1983; Brown, 1985). MtDNA is small, double-stranded and is typically made up of only 16000-20000 nucleotides (Brown, 1983). Initial surveys to detect informative polymorphisms may involve the use of a large number (10-30) of restriction enzymes, but once diagnostic polymorphisms have been identified, only those informative enzymes need be used in subsequent screening. As it is maternally inherited, the analysis of maternal lineage can be done with ease. The use of mtDNA proteins and more recently PCR amplifications of selected regions have made the examination of mtDNA variations considerably easier and faster. Universal vertebrate primers can be used to amplify various mtDNA regions and with the advent of recent sample mtDNA sequences for several fish species being available, more fish specific primers can be designed.

In brief, the techniques available to screen variability at different levels of the species organization are many, ranging from simple morphometric to molecular genetic methods than can reveal polymorphism at the DNA level. The species that was selected in the present investigation for applying three molecular genetic markers (allozymes, RAPDs and microsatellites) was the yellow catfish, *Horabagrus brachysoma* from 3 rivers originating from the Western Ghats. The major reasons for selecting this particular species are given below.

Horabagrus brachysoma (Gunther) (Fig.1) is a cultivable catfish belonging to family Bagridae, endemic to the rivers originating from southern part of the biodiversity hotspot – Western Ghats. This species grows to 60 cm and enjoys a good market value as food fish. Its attractive golden yellow colour makes it a much sought after species for aquarium keeping in India and abroad. To date, stock estimates of the species have not been made in different rivers, hence there is no information about the current exploitable potential of yellow catfish. However, there has been a massive hunt for the species from the wild for the aquarium trade recently and a drastic decline of yield of the species from rivers was recorded in 1997. The workshop on Conservation Assessment Management Plan (CAMP) to evaluate the status of freshwater species of India, held in 1997 categorized this species as "endangered" based on IUCN criteria due to restricted distribution, loss of habitat, over exploitation,

destructive fishing practices and trade (Anon., 1998). The species is shortlisted for development of a taking up stock-specific propagation assisted rehabilitation programme in rivers where it is naturally distributed. In connection with this, captive breeding and milt cryopreservation techniques have been developed for the species by National Bureau of Fish Genetic Resources (NBFGR) in collaboration with Regional Agricultural Research Station (RARS) of Kerala Agricultural University at Kumarakom. However, there is no information on stock structure and basic genetic profile of the species, which is essential for the fishery management, conservation and rehabilitation of this species. In view of the above facts and reasons, the present work was undertaken to identify molecular genetic markers like allozymes, RAPD and microsatellites in *H. brachysoma* and to use them in population genetic structure analysis of this species collected from three geographically isolated environments.

1.2. Objectives of the study

1. Identification of allozyme and RAPD markers to be used for stock discrimination of *H. brachysoma.*

2. Identification of microsatellite markers by cross-species amplification of primer sequences from other closely related fish species (derived from available accessions in gene banks) for use as potential genetic markers in *H. brachysoma.*

3. Optimization of PCR conditions for both RAPD and microsatellites.

4. Cloning and sequencing of microsatellite loci developed in *H. brachysoma,*

5. Population genetic structure analysis of *H. brachysoma* from three geographically isolated river systems by using allozyme, RAPD and microsatellite markers.

1.3. Description of the species

1.3.1. Taxonomic status

Horabagrus brachysoma (Fig. 1) is a large freshwater catfish described by Gunther in 1864 from South Indian Rivers as *Pseudobagrus brachysoma.* The species has the following synonyms: *Pseudobagrus chryseus* Day, 1865; *Macrones chryseus* Day, 1877. The current taxonomic position of *H. brachysoma* according to Talwar and Jhingran (1991) and Jayaram (1999) is given below.

Phylum	:	Vertebrata
Subphylum	:	Craniata
Superclass	:	Gnathostomata
Series	:	Pisces
Class	:	Teleostei
Subclass	:	Actinopterygii
Superorder	:	Acanthopterygii
Order	:	Siluriformes
Family	:	Bagridae
Genus	:	*Horabagrus*
Species	:	*brachysoma*

(Arratia *et al.*, (2003) reported absence of certain bagrid morphological and osteological characters in the genus *Horabagrus*. Hence, it is being excluded from the family Bagridae and may be placed in a new family "Horabagridae").

1.3.2. Distinguishing Characters

D I 6-7; A iii 20-25; P I 8-9; V I 5.

Body is moderately elongated, compressed body with a large head and wide sub terminal mouth. Teeth in villiform bands on jaws; occipital process exposed, extending to predorsal plate; eyes are large, inferior and ventro- lateral in position, visible from under side of head. The dorsal fin, consisting of the rayed fin with 5-7 rays, possesses a hard spine and is serrated from the softer smaller adipose dorsal fin.

Adipose fin short, commencing over the last fourth of the anal. Pectoral just reaching or not reaching pelvic; its spine stronger than that of dorsal and as long as the head, excluding snout, serrated externally and with 16-18 strong teeth internally. Ventral fin about half as long as the pectoral and reaches the anal; the base of the anal fin almost equals the length of the head. Caudal fin slightly or deeply lunated or even forked.

It has 4 pairs of barbels: one nasal, two mandibulars and one maxillary. Maxillary barbels extend posterior to pectoral fin base, outer mandibulars an eye diameter shorter than maxillaries; inner mandibulars, 1/4 shorter than outer mandibulars, nasals nearly 1/2 as long as head.

1.3.4. Colour

In life, greenish yellow above, the flanks brilliant golden, belly white, with a large round black mark on shoulder surrounded by a light yellow ring. Dorsal and anal fins yellowish orange stained darker at their margins. Caudal fin yellow with black base and dark edges.

1.3.5. Common names

The species is commonly known as "Gunther's catfish" or "yellow catfish" or "suncatfish" in English and locally called as "Manjakoori" or "Manjaletta" in Malayalam.

1.3.6. Habitat and distribution

H. brachysoma is distributed in selected west flowing rivers originating from the Western Ghats in the states of Kerala and Karnataka. The species once found in abundance has recorded a sharp decline in the catches due to over-exploitation for the ornamental fish trade and for human consumption and is now restricted to a few rivers of Kerala and Canara *viz.*, Nethravathi, Chaliyar, Chalakkudy, Periyar, Meenachil and Pampa Rivers. It is confined to the lower stretches of the river (approximately within 30 kilometers distance) and is not migratory. It has a group synchronous ovary and

breeds once in a year during the southwest monsoon time (June-August). The fish though a freshwater inhabitant, is also reported from brackishwater during the southwest monsoon (Unnithan, 2001). Recently, Anuradha Bhat (2001) reported rare occurrence of this species in Sharavati River in Uttara (North) Kannada, Karnataka.

Fig. 1. *Horabagrus brachysoma* (Gunther, 1864)

2

REVIEW OF LITERATURE

Population genetics is the application of Mendel's laws and other genetic principles to entire populations of organisms (Hartl and Clark, 1989). It includes the study of genetic variation within and between species and attempts to understand the processes that result in adaptive evolutionary changes in species through time. Population genetics deals with phenotypic diversity with respect to height, weight, body confirmation, hair colour and texture, skin colour, eye colour among human beings and especially with that portion of the diversity that is caused by differences in genotype. In particular, the field of population genetics has set for itself the tasks of determining how much genetic variation exists in natural populations and of explaining its origin, maintenance and evolutionary importance. Population sub-structure is almost universal among organisms. Many organisms naturally form sub-populations as herds, flocks, schools, colonies or other types of aggregations. Where there is population sub-division, there is almost inevitably some genetic differentiation that may result from natural selection, favoring different genotypes in different sub-populations (Hartl and Clark, 1989).

Organisms are incessantly undergoing micro and macro evolutionary processes both at molecular and organismal levels. Actually, the process of evolution starts at the molecular level, more precisely from a single base of the DNA molecule and ends up in variations at the organismal level. Genes are the factors, which determine the phenotypic characters of any organism. Thus, the variations that happen to the genes in turn produce individuals, which are different either at the molecular level or at the organismal level. These individuals may form separate groups within the species itself and such groups are the fundamental genetic units of evolution. These intraspecific groups were called as 'stocks' and fishery biologists started using these stocks as a basis to manage commercially important marine organisms. Shaklee *et al.* (1990) defined a stock as "a panmictic population of related individuals within a

single species that is genetically distinct from other such populations". Therefore, in any management regime, identification of discrete stocks becomes a critical element (Ihssen *et al.*, 1981a; Fetterolf, 1981).

Genetic variation in populations became a subject of scientific enquiry in the late nineteenth century prior even to the rediscovery of Mendel's paper in 1900. Genetic variation, in the form of multiple alleles of many genes, exists in most natural populations. In most sexually reproducing populations, no two organisms (barring identical twins or other multiple identical births) can be expected to have the same genotype for all genes (Hartl and Clark, 1989). For the identification of stock structure and genetic variation in a population, Ihssen *et al.* (1981b) suggested that population parameters and physiological, behavioral, morphometric, meristic, calcareous, cytogenetic and biochemical characters are useful.

Of these, morphometric investigations are based on a set of measurements of the body form (Hubbs and Lagler, 1947). Study on the life history, morphology and electrophoretic characteristics of five allopatric stocks of lake white fish showed that morphometry can be used to distinguish individuals of different stocks (Ihssen *et al.*, 1981b), although the branching patterns for morphometrics versus biochemical variation were different. For selection of brood stock in genetic improvement programmes of certain penaeids, one or two morphometric variables could be identified, giving accurate estimate of tail weight (Lester, 1983; Goswami *et al.*, 1986). A study of Pacific white shrimp, *Penaeus vannamei* from different commercial hatcheries found significant differences in all morphometric traits between sites, indicating that environmental differences affected growth as well as shape of the shrimps (Chow and Sandifer, 1992). But, in a study using canonical discriminant analysis of morphometric and meristic characters to identify cultured tilapias, the results did not support the use of morphometric characters for differentiating tilapia strains and introgressed hybrids (Pante *et al.*, 1988). These conventional data sets can be biased and they have got several weaknesses too. (i) They tend to be in one direction only (longitudinal) lacking information of depth and breadth, (ii) they often produce uneven and biased areal coverage of the body form, (iii) repetition of

landmarks often occur, (iv) many measurements extends over much of the body and (v) the amount of distortion due to preservation cannot be easily estimated in case of soft bodied organisms (Sathianandan, 1999). To overcome these problems, a new method called the "truss network" was developed in which an even areal coverage over the entire fish form was possible (Humphries *et al.,* 1981). This method can discriminate stocks of fishes and prawns on the basis of size free shape derived from distance measures. Here, the forms may be standardized to one or more common reference sizes by representing measured distances on some composite measure of body size and reconstructing the form using the distance values predicted at some standard body size. The composite mapped forms are suitable for biorthogonal analysis of shape differences between forms (Sathianandan, 1999). Truss network analysis on chinook salmon demonstrated shape differences among the three naturally occurring populations (Winans, 1984). This method was introduced among prawns to study the shape differences among them (Lester and Pante, 1992) and a machine vision system was developed for the selection of brood stock using the truss network (Perkins and Lester, 1990). A comparison of the conventional morphometrics and truss network analysis done on the blunt snout bream, finally described the truss network analysis as a better tool for probing evolutionary processes or elucidating relationships among populations (Li *et al.,* 1993).

But the application of the above said techniques in stock identification, however, is complicated by the fact that phenotypic variation in these characters are often influenced by environmental factors and has not always been directly related to particular differences in the genome (Clayton, 1981). Therefore, several new techniques using genetic markers were developed to detect the stock structure and genetic variation of the organism.

2.1. Genetic Markers

The need to detect genetic variation has fueled the development of novel genetic marker systems in fisheries biology. The detection of genetic variation among individuals is a requirement in all application of genetic markers. A genetically

inherited variant in which the genotype can be inferred from the phenotype during genetic screening is known as a genetic marker. The most common use of genetic markers in fisheries biology is to determine if samples from culture facilities or natural populations are genetically differentiated from each other. They are also used to identify different species in the event of taxonomic disputes and to detect genetic introgression in a species. The detection of genetic differentiation would imply that the source groups comprise different stocks (Carvalho and Hauser, 1994) and should be treated as separate management units or stocks (Moritz, 1994). A common objective of molecular genetic analyses is to find diagnostic differences among presumed stocks in either nuclear allelic types or mtDNA haplotypes. Most often, however, stocks differ in frequencies of the same alleles or haplotypes (Danzmann and Ihssen, 1995). Polymorphic DNA markers can provide fisheries researchers with new insights into the behavior, ecology and genetic structure of fish populations, levels of inbreeding, disassortive mating success of alternative reproductive strategies and life histories and the intensity of natural and sexual selection (Ferguson and Danzmann, 1998).

The various marker types available for fisheries and conservation applications (Park and Moran, 1994) represent a bewildering array of choices for the uninitiated. The development of new markers has been necessary for species with little detectable variation among individuals using the old markers. However, relative novelty and not the attributes of the markers themselves have often dictated marker choice (Utter *et al.*, 1991). There is no single marker type that is appropriate for all applications and a genetic marker system should be based on the characteristics of a particular species (interacting with the attributes of the marker type) rather than how recently they have been developed (Ferguson and Danzmann, 1998). In fact, a combination of mitochondrial and nuclear markers is the most powerful approach (Ward and Grewe, 1994). Attributes of the species (genetic effective population size-contemporary and historical (Ne)), amount of gene flow (migration) in combination with those of the marker loci themselves could be used to choose an appropriate marker system. Other important factors influencing marker choice are cost and sampling requirements (Ferguson *et al.*, 1995).

Genetic markers are basically of 2 types - protein and DNA. In 1960's initial studies involved proteins such as haemoglobin and transferrin. However, very soon the attention was turned to enzymatic protein (allozyme) variation on which most subsequent studies have been based (Ferguson *et al.*, 1995). A new technique based on molecular characters to identify the stocks was also developed in the early nineties (Williams *et al.*, 1990, Welsh and McClelland, 1990; Penner *et al.*, 1993; Jeffreys *et al.*, 1985; Tautz, 1989).

2.1.1. Allozyme markers

Electrophoretic studies in fish populations at the protein level commenced around 50 years back with the development of starch gel electrophoresis (Smithies, 1955). Studies on the biochemical genetics of fish/shellfish populations evolved from early descriptions of simple polymorphism at one or a few general protein/enzyme loci as reported in the haemoglobin polymorphisms in fishes (Sick, 1965). The application of these techniques in fisheries science also revealed a wide range of genetic variability in many species of fishes and shellfishes (Ligny, 1969). From 1964, electrophoretic examination of protein variants became the method of choice for studying genetic variations in natural and cultured fish populations (Utter, 1991). The proficiency of the electrophoretic techniques was enhanced by the application of histochemical staining methods of Hunter and Markert (1957). These methods could uncover a wealth of genetic variation at the molecular level, which were reflected either as multilocus isozymes or as allelic isozymes. The isozyme is considered as advantageous over the morphological and classical variables as (i) the biochemical phenotype is essentially unaffected by the environment, (ii) the biochemical phenotype of each individual is stable through time and (iii) the observed genetic variation is usually caused by a single gene whose alleles are co-dominantly expressed and inherited in a Mendelian fashion (Ayala, 1975). A comprehensive review by de Ligny (1969, 1972) shows that the use of isozyme or allozyme study has become essential for the analysis of population genetic structure of many fishes.

An enzyme coded by a single locus often appears in different molecular forms and these multiple molecular forms of enzymes were called "allozymes" by Markert

and Mollier (1959). Allozymes are functionally similar, several different forms of enzyme catalyzing the same reaction within a single species. These could differ from one another in terms of amino acid sequences, some covalent modifications, or possibly in terms of three-dimensional structure (conformational changes) etc. Allozymes are formed generally due to genetic causes. Sometimes non-genetic causes like post-translational modification and conformational changes also lead to a change in pattern of isozymes (Padhi and Mandal, 2000). Investigations in the last 25 years have used allozyme analysis to measure parameters such as genetic variability in natural populations, gene flow among populations, process of natural hybridization, species dispersion and phylogenetic analysis in many animals, plants and microorganisms (Ferguson *et al.*, 1995). Allozyme electrophoresis can give independent estimates of levels of variation between different populations without an extensive survey of morphological and other quantitative traits (Menezes *et al.*, 1993). There are also many reports of the efficiency of biochemical genetic techniques in revealing intraspecies allozyme polymorphism and existence of heterogeneous or homogeneous stocks in various species including teleosts (Richardson *et al.*, 1986).

Studies have been successfully carried out to assess levels of genetic differentiation and gene flow at the intra specific level in several important fish species using allozyme/isozyme electrophoresis (Richardson, 1982; Menezes *et al.*, 1992; Begg *et al.*, 1998; Appleyard and Mather, 2000; McGlashan and Hughes, 2000; Cook *et al.*, 2002; Salini *et al.*, 2004) and the taxonomic uses of enzyme electrophoresis are also well known (Avise, 1974; Ferguson, 1980). Many workers have already demonstrated the use of allozymes and other proteins as genetic markers for the identification of fish stocks or species (Simonarsen and Watts, 1969; Fujio and Kato, 1979; Mulley and Latter, 1980; Grand and Utter, 1984) and in fish breeding (Moav *et al.*, 1978). Significant differences in the allelic frequencies among populations of a species clearly indicate that these were not interbreeding but isolated populations (Ayala and Keiger, 1980; 1984). The significance of similar worldwide reports of genetic diversity in fishes and shellfishes was well evaluated in the international symposia held in 1971 (Ligny, 1971). Later, the special significance of the genetic stock concept at various levels of fisheries management and various

techniques for detection of genetic stocks were re-evaluated in the international symposia held in 1981, the proceedings of which were published as a special issue [*Can. J. Fish. Aquat. Sci.* Vol., 38 (12), 1981]. Using allozyme genetic tags, six genetically heterogeneous stocks were detected in the flounder populations of Newfoundland region (Fairbairn, 1981). Ridgway *et al.* (1970) reported the esterase polymorphism in the Atlantic herring and Shaklee and Salini (1985) in barramundi, *Lates calcarifier*. These studies are relevant not only to evolutionary biology but also to the management of these stocks, providing information to adjust regulations according to observed stock structure.

Many authors have studied the extent of genetic differentiation and population structure using allozyme markers in fish species. A homogeneous stock structure was reported in European hake, *Merluccius merluccius* (Mangaly and Jamieson, 1978). Coelho *et al.* (1995) studied the genetic structure and differentiation among populations of two cyprinids *Leuciscus pyrenaicus* and *L. caroliterti.* Some investigators made a comparative study of different populations of chum salmon: Wilmot *et al.* (1994) compared Western Alaskan and Russian Far East stocks; Winans *et al.* (1994) studied Asian stocks; Phelps *et al.*(1994) the Pacific North West populations; Kondzela *et al.* (1994) compared stocks of South East Alaska and Northern British Columbia. Allozyme markers have been employed in other salmons by different groups: In sockeye salmon (Wood *et al.,* 1994, in Canada; Varnavskaya *et al.*, 1994a, in Asia and North America; 1994b in Alaska, British Colombian and Kamchatka lake in Russia; in Atlantic salmon (Cross and Challanin, 1991; Skaala *et al.,* 1998); in odd year pink salmon, *Oncorhynchus gorbuscha* (Shaklee and Varnavskaya, 1994); and in Chinook salmon, *Oncorhynchus tshawytscha* (Verspoor *et al.,* 1991; Youngson *et al.*, 1991; Adams, 1994). Using allozyme markers, distinct genetic stocks of cultured tilapia in Fiji were identified by Appleyard and Mather (2000). Similarly, significant genetic differentiation was detected in North Australian mackerel (Begg *et al.*, 1998); in *Barbus callensis* (Berrebi *et al.*, 1995); in African and Iberian populations of *Cobitis* (Perdices, 1995) and in North Atlantic tusk, *Brosme brosme* (Johansen and Naevdal, 1995). Recently, Peres *et al.* (2002) reported genetic variability patterns in *Hoplias malabaricus* in fluvial and lacustrine environments in

the upper Paranas flood plain. Musyl and Keenan (1996) found small genetic differences in the Australian catfish, *Tandanus tandanus* between a Brisbane River (east flowing) and a Condamine River site (west flowing). They also found lower than expected levels of genetic divergence among some eastern and western Australian populations of the perch, *Macquaria ambigua.* McGlashan and Hughes (2000) reported significant levels of genetic subdivision among 16 populations of the Australian freshwater fish, *Craterocephalus stercusmuscarum* using 7 polymorphic allozyme loci and sequence information on the ATPase gene of mictochondrial DNA. McGlashan and Hughes (2002) also showed that populations of subspecies *Craterocephalus stercusmuscarum fulvus* separated by a mountain range in Australia were genetically more similar than populations of *C.s.fulvus* and *C.s. stercusmuscarum* which inhabit a contiguous coastal margin. The same authors in 2002 reported extensive genetic subdivisions across the range of the Australian freshwater fish, *Pseudomugil signifier* using 6 polymorphic allozyme loci. Cook *et al.* (2002) reported large and significant genetic variation in *Macrobrachium australiense* among the 4 major catchments in Western Queensland, Australia, using 6 polymorphic allozymes. Genetic variation throughout the geographic range of the tropical shad, hilsa *Tenualosa ilisha* was analysed using allozyme marker by Salini *et al.* (2004).

Review of the relevant literature reveals that work on biochemical genetics of Indian fishes is scanty in comparison to the work done in many parts of the rest of the world. Chandrasekhar (1959) has studied the profile of blood proteins of five Indian carps. Krishnaja and Rege (1977, 1979) undertook electrophoretic studies on the genetics of two species of Indian carp and their fertile hybrids. Sarangi and Mandal (1996) reported isozyme polymorphism in diploid and tetraploid Indian major carps, *Labeo rohita.* Goapalakrishnan *et al.* (1997) identified species-specific esterase markers in rohu and mrigal, while Singh *et al.* (2004) identified allozyme markers helpful in population genetic analysis of *Cirrhinus mrigala.* Examples of other important biochemical genetic studies at the intraspecific level in fish from Indian waters are that of mullet, *Mugil cephalus* (Vijayakumar, 1992; Menezes *et al.,* 1990); oil sardine, *Sardinella longiceps* (Venkitakrishnan, 1992; Menezes, 1994a; 1994b);

mackerel (Menezes *et al.*, 1990); Pomfret (Menezes, 1993) hilsa from the Ganges River (above and below Farakka barrage) and Brahmaputhra River (Lal *et al.*, 2004a) and *Lactarius lactarius* (Gopalakrishnan *et al.*, 2004c). The above-mentioned investigations identified distinct genetic stocks of *M. cephalus* and *L. lactarius* from Indian waters while low genetic divergence was reported in sardines, mackerel, hilsa and pomfrets. The above examples reveal that biochemical genetic techniques are efficient in differentiating genetic variation in natural stock of fish/shellfish species. The phenomenon of the very low-level genetic variation and close genetic homogeneity was reported even in distant geographic populations in species of penaeidae from Indian waters using allozymes as reported in other parts of the world (Bindhu Paul, 2000; Rebello, 2002).

The electrophoretic techniques used for separation of allozymes have their own limitations even though the technique is less expensive compared to modern molecular genetic (DNA) analyses. First of all, the numbers of polymorphic enzyme loci examined are always much less than the hundreds of protein loci present in each species. Probably, less than 25% of estimated amino acid substitutions are detectable by gel electrophoresis (Bye and Ponniah, 1983; Powers, 1993). Besides, not all protein variants can be detected by electrophoresis unless such variants also produce electrophoretically detectable level of electric charge differences. Moreover, all the differences in the DNA sequences are not translated directly to protein polymorphism detected by electrophoretic methods. On the other hand, modern DNA techniques can reveal and measure variations in nucleotide sequences even in very small samples of DNA fragments (Ayala and Keiger, 1984). Hence, the analysis of base sequences of the DNA is often considered a better alternative for the study of population genetics. Thus, DNA results may have greater implications for fisheries management and conservation of the genetic resources than that provided by biochemical genetic method.

2.1.2. Molecular markers

Molecular markers can be categorized into two *viz.*, nuclear DNA and mitochondrial DNA (mtDNA) markers based on their transmission and evolutionary

dynamics (Park and Moran, 1994). Nuclear DNA markers such as Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Variable Number of Tandem Repeats loci (VNTRs: minisatellites, microsatellites) and Single Nucleotide Polymorphisms (SNPs) are biparently inherited. Mitochondrial DNA markers are maternally inherited; exhibit high rates of mutation and are non-recombining such that, they have one quarter the genetic effective population size (Ne) of nuclear markers (Ferguson and Danzmann, 1998). Using restriction enzymes mtDNA sequence can be cut at specific sites to generate restriction fragment length polymorphisms (RFLPs); or sequence analysis of different genes of mtDNA can be used to detect phylogenetic relationships, undertake pedigree analysis and to assess population differentiation in many species.

Detection of polymorphisms at the nucleotide sequence level represents a new area for genetic studies, especially as technologies become available, which allow routine application with relative ease and low cost. From the 1990's an increasing number of studies have been published making use of random parts of a genome. With the advent of thermocyclers, the amplification of small fragment of DNA through Polymerase Chain Reaction (PCR) gained popularity. The PCR technique was discovered in 1985 and the development of DNA amplification using the PCR technique has opened the possibility of examining genetic changes in fish populations over the past 100 years or more using archive materials such as scales (Ferguson and Danzmann, 1998). The advent of PCR coupled with automated DNA sequencers made feasible major technological innovations such as minisatellite variant repeat mapping (Jeffreys *et al.*, 1991) and assessment of the variations at microsatellite loci (Weber and May, 1989). The PCR based techniques have the added attraction of needing only extremely small amounts of DNA that has led to wide usage of this technique in aquaculture and fisheries.

2.1.2.1. Random amplified polymorphic DNA (RAPD)

From 1990's, an increasing number of studies have been published making use of random parts of a genome. One such approach involves PCR amplification of

anonymous DNA fragments commonly known as Random Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1993; Welsh and Mc Clelland, 1990) to amplify stretches of DNA identified by random primers. A single short primer (10 base pairs) and low annealing temperature are combined to obtain specific amplification patterns from individual genomes. Priming sites are randomly distributed throughout a genome and polymorphisms in such sites result in differing amplification products, detected by the presence and absence of fragments. Such polymorphisms are generally inherited in a Mendelian fashion and can be used as genetic markers (Bardakci and Skibinski, 1994; Liu *et al.*, 1999a; Appleyard and Mather, 2002). This technique is able to provide a convenient and rapid assessment of the differences in the genetic composition of related individuals (Kazan *et al.*, 1993). RAPD fingerprinting has been used recently in many studies for the analysis of phylogenetic and genetic relationship among organisms (Stiles *et al.*, 1993; Bardakci and Skibinski, 1994; Orozco- castillo *et al.*, 1994; Van Rossum *et al.*, 1995; Hadrys *et al.*, 1992; Ward and Grewe, 1994). The technique therefore has the potential for greatly enhancing population structure studies, as it is less laborious than the currently popular mtDNA RFLP technique; and the detected polymorphisms (multiple RAPD markers) reflect variation in nuclear DNA and can presumably therefore provide a more comprehensive picture of population genetic structure.

The principle behind RAPD analysis is that at low annealing temperatures or high magnesium concentrations, a primer is likely to find many sequences within the template DNA to which it can anneal. Depending on the length and complexity of the genome of an organism, there can be numerous pairs of these sequences and they will be arranged inversely to and within about two kilobases of each other. Considering this, PCR will amplify many random fragments that can vary in size when different species, subspecies, populations or individuals are analyzed and this will constitute the basis of identification. A single primer is used to amplify the intervening region between two complementary, but inversely oriented, sequences. Suitable primers include random GC-rich 10 mers and polymers complementary to random repeats. Priming sites are randomly distributed throughout the genome and polymorphism in such sites results in differing amplification products, detected by the presence or

absence of fragments. Hence, RAPDs are treated as dominant markers. Polymorphisms result from either size changes in the amplified region or base changes that alter primer binding. The RAPD technique apart from single copy fraction, also amplifies highly repetitive regions that may accumulate more nucleotide mutations compared with those encoding allozyme variants, offering a wider potential for assessing inter-population genetic differentiation. Thus, several authors reported specific RAPD markers, useful for distinguishing intra-species population or between closely related species, in organisms where allozymes have proven to show low-resolution power to assess genetic differences (Black *et al.,* 1992 and Cognato *et al.,* 1995).

RAPD markers have also provided fisheries researchers with new insights in to behavioral ecology and genetic structure of fish populations, levels of inbreeding, disassortive mating, the success of alternative reproductive strategies and life histories (Wirgin and Waldman, 1994; Rico *et al.*, 1992; Appleyard and Mather, 2002). RAPDs have been widely used in different groups of microbes, plants and animals in recent times because of its simplicity and low cost (Hadrys *et al.*, 1992; Mailer *et al.,* 1994; Tibayrenc *et al.,* 1993; Thomas *et al.,* 2001; Menezes *et al.,* 1999; Balakrishana, 1995). RAPD-PCR technique has been shown to give high resolution especially in separating species complexes and sibling species, in detecting cryptic pairs of species and in confirming close relationships between species. Some authors have also employed this technique in studies of the systematics of numerous plant and animal species (Sultmann *et al.,* 1995; Stothard and Rollinson, 1996).

RAPDs have been used extensively in aquatic studies of organisms such as the penaeid prawn, *Penaeus monodon* as markers for breeding programs (Garcia and Benzie, 1995); in fresh water shrimp *Macrobrachium borellii* for evaluating the genetic diversity among 2 of its populations (D'Amato and Corach, 1996); in the freshwater crab, *Aegla jujuyana* for the analysis of population genetic structure (D'Amato and Corach, 1997) and in north-east Atlantic minke whale, *Balaenoptera acutorostrata* for stock identification (Martinez *et al.*, 1997). Klinbunga *et al.* (2000a and 2000b) developed species-specific markers for the tropical oyster, *Crassostrea*

belcheri and in mud crabs (*Scylla*). McCormack *et al.* (2000) reported a comparative analysis of two populations of the Brittle star (*Amphiura filiformis*) using RAPDs.

In teleosts, the RAPD method has been used for the identification of species and subspecies in tilapia (Bardakci and Skibinski, 1994; Sultmann *et al.*, 1995; Appleyard and Mather, 2002) and *Xiphophorus hellari* (Borowsky *et al.*, 1995); intraspecific genetic variation in red mullet (*Mullus barbatus*) (Mamuris *et al.*, 1998) and monitoring of genetic polymorphism in sea bass after acclimation to freshwater (Allegrucci *et al.*, 1995). In addition, a comparative study of RAPD and multilocus DNA fingerprinting on strains of *Oreochromis niloticus* revealed similar genetic relationships (Naish *et al.*, 1995; Lee and Kocher, 1996). RAPD markers were also used in hilsa shad, *Tenualosa ilisha* for discriminating 3 populations (Dahle *et al.*, 1997); in common carp, *Cyprinus carpio* for the study of heterosis (Dong and Zhou, 1998); in Spanish barb for identification of 3 endemic species (Callejas and Ochando, 1998); in the Atlantic four-wing flying fish *Hirundichthys affinis* for stock discrimination (Gomes *et al.*, 1998); in grouper *Epinephelus* for differentiating different species (Baker and Azizah, 2000; Govindaraju and Jayasankar, 2004; Christopher, 2004); in Iberian *Barbus* for molecular identification of 8 species (Callejas and Ochando, 2001); for studying variations between African and American Cichlids (Goldberg *et al.*, 1999); in the Pacific cod *Gadus macrocephalus* to identify genetic variation within 3 Japanese coastal areas (Saitoh,1998); in red mullet, *Mullus barbatus* to evaluate genetic affinities among 8 samples from the Mediterranean Sea (Mamuris *et al.*, 1998); in scombroid fishes as species specific markers (Jayasankar and Dharmalingam, 1997) and in brown trout, *Salmo trutta* for determining genetic variability among 4 populations (Cagigas *et al.*, 1999).

RAPD analysis has several advantages over other procedures. These include relatively shorter time (1-2 days) required to complete analysis after standardization, ability to detect extensive polymorphisms, low cost, simplicity, rapidity, need for minute amounts of genomic DNA (≈30ng), random primers required for analysis, simpler protocols and involvement of non - invasive sampling for tissue analysis. There is no need for molecular hybridization and the technique allows examination of

genomic variation without prior knowledge of DNA sequences (Welsh and McClelland, 1990; Williams *et al.*, 1993; Liu *et al.*, 1999a). RAPD-PCR technique has been shown to give a high resolution especially in separating species complexes and sibling species, in detecting cryptic pairs of species and in confirming close relationships between species.

However, the application and interpretation of RAPD-PCR in population genetics is not without technical problems and practical limitations. The main negative aspect of this technique is that, the RAPD patterns are very sensitive to slight changes in amplification conditions giving problems of reproducibility and necessity for extensive standardization to obtain reproducible results (Ferguson *et al.*, 1995). In addition, most of the RAPD polymorphism segregates as dominant markers and individuals carrying two copies of an allele (heterozygotes) cannot be distinguished from individuals carrying a single copy of an allele (homozygotes). In the application of RAPD analysis, it is assumed that populations conform to Hardy-Weinberg equilibrium, which may not necessarily hold true especially in threatened species. The limited sample size in each population and the specific RAPD primers utilized can also have an influence over the results (Gopalakrishnan and Mohindra, 2001).

2.1.2.2. Microsatellites

Recently, attention has turned to another type of genetic variation that of differences in the number of repeated copies of a segment of DNA. These sequences can be classified based on decreasing sizes into satellites, minisatellites and microsatellites (Tautz, 1993). Satellites consist of units of several thousand base pairs, repeated thousands or millions of times. Minisatellites consist of DNA sequences of some 9-100bp in length that are repeated from 2 to several 100 times at a locus. Minisatellites discovered in human insulin gene loci with repeat unit lengths between 10 and 64 bp were also referred to as 'Variable Number of Tandem Repeats' (VNTRs) DNA (Nakamura *et al.*, 1987). Microsatellites have a unique length of 1-6 bp repeated up to about 100 times at each locus (Litt and Luty, 1989). They are also called as 'simple sequence repeat' (SSR) by Tautz (1989) or 'short tandem repeat' (STR) DNA by Edwards *et al.* (1991). Jeffreys *et al.* (1988) and Weber (1990) opined

that length variations in tandemly arrayed repetitive DNA in mini and microsatellites are usually due to an increase or decrease in repeat unit copy numbers. Differences in repeat numbers represent the base for most DNA profiling techniques used today.

Microsatellites are short tandemly arrayed di-, tri-, or tetra- nucleotide repeat sequences with repeat size of 1-6 bp repeated several times flanked by regions of non-repetitive unique DNA sequences (Tautz, 1989). Polymorphism at microsatellite loci was first demonstrated by Tautz (1989) and Weber and May (1989). Alleles at microsatellite loci can be amplified by the polymerase chain reaction (Saiki *et al.*, 1988) from small samples of genomic DNA and the alleles separated and accurately sized on a polyacrylamide gel as one or two bands and they are used for quantifying genetic variations within and between populations of species (O'Connell *et al.*, 1997). The very high levels of variability associated with microsatellites, the speed of processing and the potential to isolate large number of loci provides a marker system capable of detecting differences among closely related populations. Microsatellites that have been largely utilized for population studies are single locus ones in which both the alleles in a heterozygote show co-dominant expression (Gopalakrishnan and Mohindra, 2001). Individual alleles at a locus differ in the number of tandem repeats and as such can be accurately differentiated on the basis of electrophoresis (usually PAGE) according to their size. Different alleles at a locus are characterized by different number of repeat units. They give the same kind of information as allozymes: distinguishable loci with codominant alleles but they are generally neutral and more variable than allozymes (Queller *et al.*, 1993). Like allozymes, microsatellites alleles are inherited in a Mendelian fashion (O'Connell and Wright, 1997). Moreover, the alleles can be scored consistently and compared unambiguously, even across different gels. An additional advantage is that they allow the use of minute or degraded DNA (Queller *et al.*, 1993).

Generally, microsatellite loci are abundant and distributed throughout the eukaryotic genome (Tautz and Renz, 1984) and each locus is characterized by known DNA sequence. These sequences consist of both unique DNA (which defines the locus) and of repetitive DNA motifs (which may be shared among loci). The repetitive

elements consist of tandem reiterations of simple sequence repeats (SSRs) and are typically composed of two to four nucleotides such as (AC)n or (GATA)n where n lies between 5 and 50 (DeWoody and Avise, 2000). Within vertebrates, the dinucleotide repeats -GT and CA- are believed to be the most common microsatellites (Zardoya *et al.*, 1996). Study of single locus microsatellites requires specific primers flanking the repeat units, whose sequences can be derived from (i) genomic DNA libraries or (ii) from available sequences in the gene banks.

The high variability, ease and accuracy of assaying microsatellites make them the marker of choice for high-resolution population analysis (Estoup *et al.*, 1993). Microsatellites with only a few alleles are well suited for population genetic studies, while the more variable loci are ideal for genome mapping and pedigree analysis and the fixed or less polymorphic microsatellite loci are used to resolve taxonomic ambiguity in different taxa (Carvalho and Hauser, 1994). Highly polymorphic microsatellite markers have great potential utility as genetic tags for use in aquaculture and fisheries biology. They are powerful DNA markers for quantifying genetic variations within and between populations of species (O'Connell *et al.*, 1998). They may prove particularly valuable for stock discrimination and population genetics due to the high level of polymorphism compared with conventional allozyme markers (Bentzen *et al.*, 1991; Wright and Bentzen, 1994). Microsatellite DNA markers are among the most likely to conform to the assumption of neutrality and have proven to be powerful in differentiating geographically isolated populations, sibling species and sub-species (Zardoya *et al.*, 1996). The qualities of microsatellites make them very useful as genetic markers for studies of population differentiation and stock identification (reviewed in Park and Moran 1994; Wright and Bentzen, 1994; O'Reilly and Wright, 1995), in kinship and parentage exclusion (Queller *et al.*, 1993; Kellog *et al.*, 1995; Hansen *et al.*, 2001) and in genome mapping (Lee and Kocher, 1996). Microsatellites are also being used as genetic markers for identification of population structure, genome mapping, pedigree analysis; and to resolve taxonomic ambiguities in many other animals besides fishes (Garcia *et al.*, 1996; Nelson *et al.*, 2002; Naciri *et al.*, 1995; Waldick *et al.*, 1999; Brooker *et al.*, 2000; Sugaya *et al.*, 2002; Ciofi *et al.*, 2002; Shaw *et al.*, 1999; Supungul *et al.*, 2000; Norris *et al.*, 2001).

Various authors have reported microsatellite polymorphisms and sequences in some marine and freshwater fish species for population genetic analysis (Estoup *et al.*, 1993; Rico *et al.*, 1993; Brooker *et al.*, 1994; Garcia de Leon *et al.*, 1995; Presa and Guyomand, 1996; Appleyard *et al.*, 2002; Han *et al.*, 2000; Ball *et al.*, 2000; Kirankumar *et al.*, 2002). The development of polymorphic microsatellite markers to determine the population structure of the Patagonian tooth fish, *Dissostichus eleginoides*, has been reported by Reilly and Ward (1998). Microsatellite polymorphisms have been used to provide evidence that the cod in the northwestern Atlantic belong to genetically distinguishable populations and that genetic differences exist between the northwestern and southeastern cod populations (Bentzen *et al.*, 1996). O'Connell *et al.* (1997) reported that microsatellites, comprising $(GT)_n$ tandemly repeated arrays, were useful in determining the patterns of differentiation in freshwater migratory populations of rainbow trout *Oncorhynchus mykiss* in Lake Ontario. Takagi *et al.* (1999) identified four microsatellite loci in tuna species of genus *Thunnus* and investigated genetic polymorphism at these loci in Northern Pacific populations. In a cichlid, *Eretmodus cyanostictus*, Taylor *et al.* (2001) determined four polymorphic microsatellite loci for studying nine populations in Lake Tanganyika. Appleyard *et al.* (2002) examined seven microsatellite loci in Patagonian Tooth fish from three locations in the Southern Ocean. Gold *et al.* (2002) analysed the population structure of king mackerel (*Scomberomorus cavalla*) along the east (Atlantic) and west (Gulf) coasts of Florida using seven microsatellite loci. O'Connell *et al.* (1998) reported the investigation of five highly variable microsatellite loci for population structure in Pacific herring, *Clupea pallasi* collected from 6 sites in Kodiak Island. Similarly, many others have reported studies of polymorphic microsatellite loci to evaluate population structure of different fish species (Beacham and Dempson, 1998; McConnell *et al.*, 1995; Reilly *et al.*, 1999; Perez-Enriquez *et al.*, 1999; Ball *et al.*, 2000; Appleyard *et al.*, 2001; Brooker *et al.*, 2000; Colihuque, 2003; Ruzzante *et al.*, 1996).

Salzburger *et al.* (2002) reported a case of introgressive hybridization between an ancient and genetically distinct cichlid species in Lake Tanganyika that led to the recognition of a new species. This is evidenced by the analysis of flanking regions of

the single copy nuclear DNA locus (Tmo M27) and by studying the parental lineages in six other microsatellite loci. Leclerc *et al.* (1999) had cloned and characterized a highly repetitive DNA sequence from the genome of the North American *Morone saxatilis* that was used to distinguish the four other species. Neff *et al.* (1999) described 10 microsatellite loci from blue gill (*Lepomis macrochirus*) and discussed their evolution within the family Centarchidae. Kellog *et al.* (1995) applied microsatellite-fingerprinting approach to address questions about paternity in cichlids. The usefulness of microsatellite markers for genetic mapping was determined in *Oreochromis niloticus* by Lee and Kocher (1996), while Brooker *et al.* (1994) reported the difference in organization of microsatellite between mammals and cold water teleost fishes. DeWoody and Avise (2000) reported microsatellite variation in marine, fresh water and anadromous fishes compared with other animals. Microsatellite DNA variation was used for stock identification in north Atlantic populations of Whiting (Rico *et al.*, 1997); *Oncorhynchus kisutch* (Small *et al.*, 1998a); Atlantic salmon (Beacham and Dempson, 1998) and Ayu, *Plecoglossus altivelis* (Takagi *et al.*, 1999). Microsatellite markers have been studied in a few cyprinids also. Naish and Skibinski (1998) studied tetranucleotide (TCTA) repeat sequences in Indian major carp, *Catla catla* as potential DNA markers for stock identification. Das and Barat (2002a, b, c) carried out characterization of dinucleotide microsatellite repeats in *Labeo rohita*. Kirankumar *et al.* (2002) reported that the complete sequence of a repeat like region in Indian rosy barb (*Puntius conchonius*).

Although microsatellite DNA analysis via PCR is an ideal technique for answering many population genetic questions, the development of species-specific primers for PCR amplification of alleles can be expensive and time-consuming, as it involves construction of genomic libraries, screening of clones with microsatellite sequences and designing microsatellite primers. However, there are reports which point to the fact that flanking sequences of some microsatellite loci are conserved among related taxa so that primers developed for one species can be used to amplify homologous loci in related species. The conservation of flanking regions of microsatellite sequences among closely related species has been reported by a number of groups (Moore *et al.*, 1991; Schlotterer *et al.*, 1991; Estoup *et al.*, 1995; Zheng *et*

al., 1995; Presa and Guyomard, 1996; Scribner *et al.*, 1996; May *et al.*, 1997; Coltman *et al.*, 1996; Pepin *et al.*, 1995). Such an approach can circumvent extensive preliminary work necessary to develop PCR-primers for individual loci that continues to stand in the way of quick and widespread application of single locus microsatellite markers. Thus, by using heterologous PCR primers the cost of developing similar markers in related species can be significantly reduced.

Schlotterer *et al.* (1991) found that homologous loci could be amplified from a diverse range of toothed (Odontoceti) and baleen (Mysticeti) whales with estimated divergence times of 35-40 million years. Moore *et al.* (1991) found microsatellites flanking regions were conserved across species as diverse as primates, artiodactyls and rodents. Microsatellite primers developed from domestic dogs were used in studies of a variety of canid species (Gotelli *et al.*, 1994). Similarly, primers developed for passerine birds were used in studies of a variety a of other bird species (Galbusera *et al.*, 2000).

A number of attempts have been made to study the cross species amplification of microsatellite loci in fishes. Scribner *et al.* (1996) isolated cloned microsatellites from Atlantic salmon genomic libraries and used them for cross-species amplification and population genetic applications in other salmon species. May *et al.* (1997) reported microsatellite genetic variation through cross species amplification in sturgeons *Acipenser* and *Scaphirhynchus*. Takagi *et al.* (1999) reported that microsatellite primers isolated from one tuna might be used to amplify microsatellite loci in other tuna species especially those of the genus *Thunnus*. Microsatellites from rainbow trout *Oncorhynchus mykiss* have been used for the genetic study of salmonids (Morris *et al.*, 1996; Small *et al.*, 1998; Beacham and Dempson, 1998). Heterologous primers have been used to characterize bull trout by using three sets of primers from sockeye salmon, rainbow trout and brook trout (Kanda and Allendorf, 2001), for several *Salvelinus* species using primers of *Salvelinus fontinali;* for Brook charr (Angers and Bernatchiz, 1996), for *Poecilia reticulata* by using primers of *Poecilia occidertalis* (Parker *et al.*, 1998) and *Oreochromis shiranus* and *O. shiranus chilwae* by using primers of Nile tilapia (Ambali, 1997). There are some reports in which the

flanking sequences are conserved between families of the same order. Primers of stickleback and cod have been used in *Merlangius merlangius* (Gadidae) (Rico *et al.*, 1997); that of rainbow trout (Family: Salmonidae) in whitefish, *Coregonus nasus* (Patton *et al.*, 1997); and primers of goldfish, *Carassius auratus* in nine species of cyprinids (Zheng *et al.*, 1995). Yue and Orban (2002) developed 15 polymorphic microsatellite loci in silver crucian carp *Carassius auratus gibelio* and reported, eleven out of 15 primer pairs cross-amplified in the genome of common carp (*Cyprinus carpio*). Zardoya *et al.* (1996) through a classical study demonstrated that microsatellite flanking regions (MFRs) contain reliable phylogenic information and they were able to recover with considerable confidence the phylogenetic relationship within Family Cichlidae and other families of the suborder Labroidei from different parts of the world including India. In India, Mohindra *et al.* (2001 a, b; 2002 a, b, c) have carried out cross-species amplification of *C. catla* G1 primer in *Catla catla* from Gobindsagar; *Labeo dero, L. dyocheilus L. rohita* and *Morulius calbasu*, and sequenced the loci in these species. Das and Barat (2002a, b, c) also carried out charecterisation of dinucleotide microsatellite repeats in *Labeo rohita*. In an endemic cyprinid of the Western Ghats (*Labeo dussumieri*), Gopalakrishnan *et al.* (2002) sequenced microsatellite loci by cross-species amplification of *C. catla* G1 primer. Kirankumar *et al.* (2002) reported the complete sequence of a repeat like region in Indian rosy barb (*Puntius conchonius*). The cross-species amplification of microsatellite in *Puntius denisonii* using primers developed for other of other cyprinid fishes was reported by Lijo John (2004). Successful identification of polymorphic microsatellite markers for *Cirrhinus mrigala* and *Gonoproktopterus curmuca* was achieved through use of primers from other cyprinid fishes (Lal *et al.*, 2004; Gopalakrishnan *et al.*, 2004a).

Advantages of microsatellites such as short size range, uninterrupted stretches of identical repeat units, high proportion of polymorphisms, insights gained in understanding the mutational process which helps in developing statistical procedures for inter-population comparisons, their abundance in fish genomes, the availability of methodologies for cloning of microsatellites, have all resulted in their abundant use in fisheries research. Tetranucleotide microsatellites are also very useful for paternity

and forensic investigations in humans. The advantageous properties of microsatellites has led to modern developments such as digital storage, automated detection and scoring systems such as automated DNA sequences, fluorescent-imaging devices *etc.* (O'Connell and Wright, 1997). Disadvantages of microsatellites include the appearance of shadow or stutter bands, presence of null alleles (existing alleles that are not observed using standard assays); homoplasy; and too many alleles at certain loci that would demand very high sample size for analysis (Mohindra *et al.*, 2001a). Also, microsatellite flanking regions (MFRs) sometimes contain length mutations which may produce identical length variants that could compromise microsatellite population level studies (and comparisons of levels of variation across species for homologous loci) and phylogenetic inferences as these length variants in the flanking regions can potentially minimize allele length variation in the repeat region (Zardoya *et al.*, 1996).

Microsatellites have become the genetic markers of choice for studies of population differentiation and parentage determination. However, several microsatellite loci are required for such studies in order to obtain an appropriate amount of genetic polymorphism (Herbinger *et al.*, 1995; Ferguson *et al.*, 1995). Fortunately, genotypic data collection has become efficient through the development of automated DNA sizing technology using fluorescent-labelled DNA and co-amplification of multiple loci in a single PCR (O'Connell and Wright, 1997; Smith *et al.*, 1997).

2.2. Genetic markers in catfishes

Genetic markers have been used to distinguish species as well as for stock structure analysis in catfishes also. Allozyme markers have been used to obtain genetic evidence for the validity of two species of African catfishes, *Clarias gareipinus* and *Clarias anguillaris,* Teugels *et al.* (1992) examined electrophoretic variation at 13 protein loci in two West African populations of both species. Agnese *et al.* (1997) described genetic variation at 25 protein loci in two sympatric samples from the Senegal River for these two species. Rognon *et al.* (1998) reported allozyme variation in both species at 25 allozyme loci. Van der Bank *et al.* (1992) carried out a

comparative biochemical genetic study of three populations of domesticated and wild African catfish, *Clarias gariepinus*. Genetic relationship of glucose phosphate isomerase-B phenotypes were analysed in channel catfish, *Ictalurus punctatus* by Goudie *et al.* (1995). Thermal stability of soluble malate dehydrogenase (sMDH) was analyzed by Monteiro *et al.* (1998) in fish belonging to the Order Siluriformes. Population genetic structure of baung, *Mystus nemurus* were analyzed in Malaysia using allozyme markers (Siraj *et al.*, 1998). Pouyaud *et al.* (2000) studied the phylogeny of the Family Pangasiidae and verified the presently used classification based on morphological data. In marine catfishes, Suzuki and Phan (1990a,b) studied intra-specific variation and inter-specific relationships of 6 ariid species from Brazil, using 10 allozymes from eye lens and skeletal muscle proteins. Gopalakrishnan *et al.* (1996) identified 7 allozyme markers to resolve a taxonomic ambiguity in *Tachysurus (Arius) maculatus, T. subrostratus* and in other species of marine catfishes.

While few authors have carried out RAPD analyzes in catfishes, inheritance of RAPD markers in channel catfish, *Ictalurus punctatus*, in blue catfish, *I. furcatus* and their F_1, F_2 and backcross hybrids has been examined (Liu *et al.*, 1998a). Liu *et al.* (1999a) evaluated the feasibility of using RAPD markers for both intraspecific mating plans and interspecific hybrid mating plans in channel catfish. Chong *et al.* (2000) identified and characterized distinct stocks of a Malaysian river catfish, *Mystus nemurus* by RAPD analysis. Yoon *et al.* (2001) analysed two different populations of cultured Korean catfish, *Silurus asotus* using RAPD analysis. Kovacs *et al.* (2001) developed a sex- specific DNA sequence in the male and female genome of African catfish, *Clarias gariepinus* by sequence characterized amplified region (SCAR) using RAPD assays. Liu *et al.* (1998b) analyzed the inheritance of AFLP markers in channel catfish, *Ictalurus punctatus,* blue catfish, *I. furcatus* and their F_1, F_2 and backcross hybrids. The same authors in 1999 (Liu *et al.*, 1999b) used AFLP markers to construct genetic maps for channel catfish, *Ictalurus punctatus* while Poompuang and Na-Nakorn (2004) constructed a genetic map for walking catfish, *Clarias macrocephalus*. Chong *et al.* (2000) identified and characterized Malaysian river catfish, *Mystus nemurus* using AFLP markers.

Several investigators in the last 15 years have made use of microsatellite markers in various catfishes. Galbusera *et al.* (1996) isolated polymorphic microsatellite markers in the genome of African catfish, *Clarias gariepinus*. Microsatellite loci were identified for *Clarius macrocephalus* and used for genetic diversity study (Na-Nakorn *et al.*, 1999). Volckaert *et al,* (1999) identified nine polymorphic microsatellite markers in the Southeast Asian catfishes, *Pangasius hypophthalmus* and *Clarias batrachus*. Krieg *et al.* (1999) isolated 10 polymorphic microsatellite loci in European catfish, *Silurus glanis*. Liu *et al.* (1999c) reported high levels of conservation at microsatellite loci among ictalurid catfishes. Tan *et al.* (1999) reported the identification of polymorphic microsatellite markers in channel catfish (*Ictalurus punctatus*) and other related catfish species. Microsatellites were used for cross-species amplification and population genetic applications using primers from channel catfish (*Ictalurus punctatus*) in blue catfish, *I. furcatus*, white catfish, *Arneiurus catus* and flathead catfish, *Pylolictus olivaris* (Liu *et al.*, 1999d). Yue *et al.* (2003) reported polymorphic microsatellite loci in *Clarias batrachus* and their cross species amplification in other catfishes. Usmani *et al.* (2001) isolated and characterized five polymorphic microsatellite loci in *Mystus nemurus*. Watanabe *et al.* (2001) isolated and characterized 20 polymorphic microsatellite loci in Japanese endangered bagrid catfish, *Psuedobagrus ichikawai*. 27 microsatellite loci were tested for amplification in five species of migratory Asian catfish, *Pangasius kremfi*, *P. bocourti*, *P. conchophilus*, *P. pleurotaemia* and *Heliophages waandersii* by Hogan and May (2002).

MtDNA and RFLP analysis have been carried out in several catfishes. Simsek et al. (1990) resolved taxonomic ambiguity of 3 species of ariid catfishes, viz. *Arius thalassinus*, *A. tenuispinus* and *A. bilineatus* from the Arabian Gulf by mtDNA RFLP. Phylogeographic structure in mtDNA of a Southeast Asian freshwater fish, *Hemibagrus nemurus* was reported by Dodson *et al.* (1995). Okazaki *et al.* (1999) studied the genetic relationships among Japanese and Korean bagrid catfishes using mtDNA analysis. The phylogeny of the Family Pangasidae was analysed by Pouyaud *et al.* (2000). Recently,

Watanabe and Nishida (2003) reported PCR-RFLP details of mtDNA control region for a population genetic structure in Japanese bagrid catfish, *Pseudobagrus ichikawai.*

All of the above-cited reports indicate that recent innovations in molecular biology have increased the potential for molecular markers to provide useful information for fisheries management and aquaculture. Markers such as microsatellites have provided increased resolution to answer questions of stock structure in fishes with relatively low levels of intra specific genetic variation. The application of DNA marker technologies in areas such as population genetics, conservation genetics, molecular systematics and molecular ecology will undoubtedly impact the aquaculture industry and fisheries sector in unforeseen ways. Already studies in population and conservation genetics are pointing out the need for evaluation of genetic attributes of many natural fish populations such as trout and salmon using molecular markers; in the light of increasing number of released fish (for augmentation and restoration of wild fish) from hatcheries (Liu and Cordes, 2004). Advances in aquaculture genomics are also likely to affect other areas utilizing molecular markers as well. Well designed studies using the above cited genetic markers will undoubtedly accelerate development in areas such as identification of genes involved in aquaculture trait loci (QTL) for marker assisted selection (MAS).

Horabagrus brachysoma, the species selected for the present study was listed as 'endangered' according to the latest IUCN categorization in the NBFGR-CAMP workshop held in 1997 (Anon., 1998). The species has been short-listed for initiating a 'stock-specific propagation assisted rehabilitation programme' in rivers where it is naturally distributed. In connection with this, captive breeding and milt cryopreservation techniques have been developed for the species by National Bureau of Fish Genetic Resources (NBFGR) in collaboration with Regional Agricultural Research Station (RARS) of Kerala Agricultural University at Kumarakom (Gopalakrishnan *et al.,* 2004b). However, no attempts have been made to study the stock structure and basic genetic profile of the species that are essential for good fishery management, conservation and rehabilitation of this species. Available information on this species relates only to length-weight relationships (Kumar *et al.,*

1999). This prompted me to take up the present work with a view to identifying polymorphic allozyme, RAPD and microsatellite markers in order to obtain a detailed picture of the population structure of the species distributed in three rivers in the Western Ghats.

3

MATERIALS AND METHODS

3.1. Collection of Fish Samples

Live yellow catfish, *Horabagrus brachysoma* (70 specimens from each population, ranging from 25 - 65 cm in total length; 150 - 500g total weight) (Fig. 1) were collected from the natural distribution range - three west flowing rivers along the Western Ghats *viz.*, 1) Meenachil River at Kumarakom, Kottayam, ($09^0$33'N; $76^0$25'E); 2) Chalakkudy River at Kanakkankadavu ($10^0$08'N; $76^0$07'E) and 3) Nethravathi River at Kankanadi, Mangalore ($12^0$52'N; $74^0$54'E) (Fig. 2). The riverine locations were chosen to cover geographically separate populations of *H. brachysoma.* West-flowing rivers from the Western Ghats are relatively shorter (mean riverine length 76 KM) and generally, many do not have tributaries. More over, the species is restricted to lower stretches of these 3 rivers (approximately within 30 kilometers distance). Hence, within a river, there was only a sampling site. Fishes were collected using cast nets and other conventional methods and their total length, total weight and sex were recorded. The details of fish samples collected in different periods are given in Table-1.

Table 1. Sample size of *H. brachysoma* and sampling period at three riverine locations

No.	River system	Collection Site	Sampling Date	No. of specimens			Total samples (N)
				Male	Female	Total	
1.	Meen-achil	Kumarakom, Kottayam	10.06.2001	10	6	16	70
			14.06.2001	9	16	25	
			21.07.2002	8	11	19	
			16.08.2002	7	3	10	
2.	Chala-kkudy	Kanakkan-kadavu, Chalakkudy	14.06.2001	16	19	35	70
			19.06.2003	7	8	15	
			17.07.2003	10	10	20	
3.	Nethr-avathi	Kankanadi, Mangalore	04.05.2001	12	14	26	70
			14.05.2002	18	26	44	

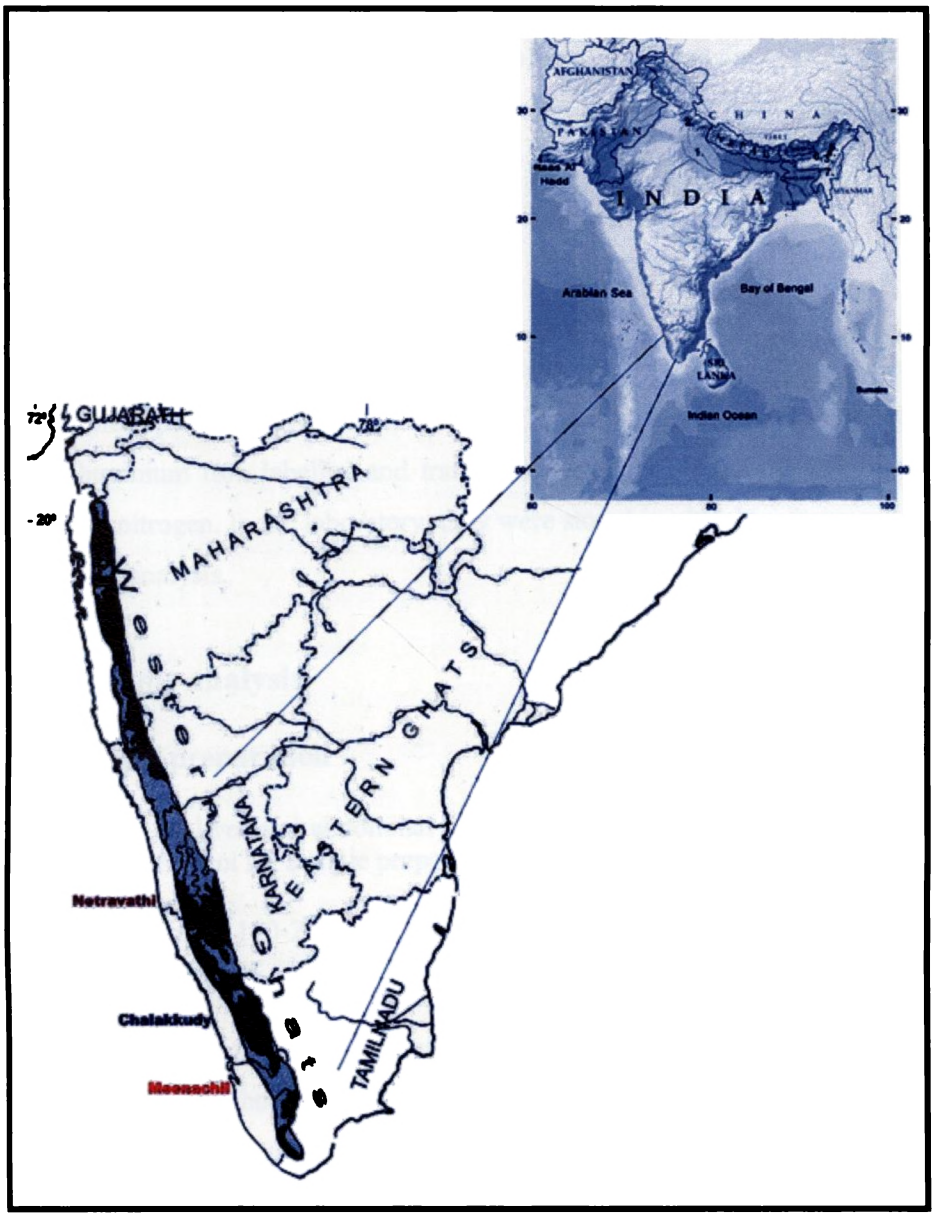

Fig. 2. Map showing the sampling sites of *H. brachysoma*

3.2. Collection of tissues

3.2.1. Collection of blood samples

Blood samples (0.25 ml) for DNA extraction were collected from the live fish immediately after capture by puncturing the caudal vein, using sterile syringes, rinsed with anticoagulant Heparin (Biological E. Limited, India.). Blood samples were immediately poured into sterile 1.5 ml microfuge tubes containing 1.25 ml of 95% ethyl alcohol. To avoid clotting of blood in ethyl alcohol, the tubes were thoroughly shaken; sealed using 'Parafilm'; transported to the laboratory and stored in refrigerator at 4^0C until further analysis.

3.2.2. Collection of liver and muscle

Liver and abdominal muscle tissues (one gram each) were dissected out from freshly killed specimens after the collection of blood samples at each site, wrapped in sterile aluminium foil, labelled and transported to the laboratory in cryocans filled with liquid nitrogen. In the laboratory, they were stored in ultra-low freezers at -85^0C, until further analysis.

3.3. Allozyme analysis

3.3.1. Sample preparation

Pieces of liver and abdominal muscle tissues were removed from the frozen samples. The protocol for sample preparation is given below.

- Approximately 100-250 mg tissue (liver or muscle) was taken in a labelled 1.5 ml centrifuge tube kept on ice.
- The tissues were homogenized approximately in 4 volumes of chilled extraction solution (given in box below), while keeping on ice.
- Centrifuged the homogenized samples at 12000 rpm at 4°C for 1 hour.
- 100-200 µl of the supernatant was pipetted out (from middle portion), in another cold vial avoiding the white layer at the meniscus and debris at the bottom.
- Centrifuged at 12000 rpm at 4°C for 30 minutes in 'Heraeus - Biofuge Stratos'
- The clear solution was taken from middle portion for allozyme analysis.

Extraction solution		
For liver (250 mg/ml)	50% sucrose	-2.0 ml
	EDTA (64 mg/100 ml)	-5.0 ml
	0.2 M Tris-Cl	-0.5 ml
	Double distilled water	-2.5 ml
	Total volume	-10.0 ml
For muscle (125 mg/ml)	10% sucrose solution.	

3.3.2. Selection of allozymes

Twenty-five enzymes were used for initial screening and of these, fourteen were found to give scorable activity and hence selected for detailed investigation on stock structure of *H. brachysoma*. The name of enzyme loci, enzyme commission numbers and quaternary structure are given in Table-2. The selected fourteen enzymes were Aspartate amino transferase (AAT, 2.6.1.1), Esterase (EST, 3.1.1.1), Glucose-6-phosphate dehydrogenase (G_6PDH, 1.1.1.49), Glucose phosphate isomerase (GPI, 5.3.1.9), Glucose dehydrogenase (GLDH, 1.1.1.47), α-Glycerophosphate (Glycerol 3-phosphate) dehydrogenase (αG_3PDH, 1.1.1.8), Lactate dehydrogenase (LDH, 1.1.1.27), Malate dehydrogenase (MDH, 1.1.1.37), Octonol dehydrogenase (ODH, 1.1.1.73), Phosphoglucomutase (PGM, 5.4.2.2), Superoxide dismutase (SOD, 1.15.1.1) and Xanthine dehydrogenase (XDH, 1.1.1.204). Among these, two enzymes were monomorphic, *viz.* Malic enzyme (ME, 1.1.1.40) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1.2.1.12) and rest 12 were polymorphic.

Enzymes that did not give scorable activity and hence were discarded included: Acid phosphatase (ACP), Adenylate kinase (AK), Alcohol dehydrogenase (ADH), Alkaline phosphate (ALP), Creatine kinase (CK), Fumerase (FUM), Glutamate dehydrogenase (GDH), Hexokinase (HK), Isocitrate dehydrogenase (ICDH), Phosphogluconate dehydrogenase (6PGDH) and Pyruvate kinase (PK).

Table 2. Name of enzymes with their enzyme commission (E.C.) number used in allozyme analysis in *Horabagrus brachysoma*

Enzymes	Abbreviation	E.C. number	Quaternary Structure
Acid phosphatase	ACP	3.1.3.2	Dimer
Adenylate kinase	AK	2.7.4.3	Monomer
Alcohol dehydrogenase	ADH	1.1.1.1	Dimer
Alkaline phosphatase	ALP	3.1.3.1	Mono/ Dimer
Aspartate amino transferase	AAT	2.6.1.1	Dimer
Creatine kinase	CK	2.7.3.2	Dimer
Esterase	EST	3.1.1. -	Monomer
Fumerase	FUM	4.2.1.2	Tetramer
Glutamate dehydrogenase	GDH	1.4.1.3	Hexamer
Glucose-6-phosphate dehydrogenase	G_6PDH	1.1.1.49	Dimer
Glucose phosphate isomerase	GPI	5.3.1.9	Dimer
Glucose dehydrogenase	GLDH	1.1.1.47	Dimer
α-Glycerophosphate dehydrogenase	αG_3PDH	1.1.1.8	Dimer
Glyceraldehyde-3-Phosphate dehydrogenase	GAPDH	1.2.1.12	Tetramer
Hexokinase	HK	2.7.1.1	Monomer
Isocitrate dehydrogenase	ICDH	1.1.1.42	Dimer
Lactate dehydrogenase	LDH	1.1.1.27	Tetramer
Malate dehydrogenase	MDH	1.1.1.37	Dimer
Malic enzyme	ME	1.1.1.40	Tetramer
Octonol dehydrogenase	ODH	1.1.1.73	Dimer
Phosphogluconate dehydrogenase	6PGDH	1.1.1.44	Dimer
Phosphoglucomutase	PGM	5.4.2.2	Monomer
Pyruvate kinase	PK	2.7.1.40	Tetramer
Superoxide dismutase	SOD	1.15.1.1	Dimer
Xanthine dehydrogenase	XDH	1.1.1.204	Dimer

3.3.3. Electrophoresis

Supernatant from tissues was analysed using Polyacrylamide gel electrophoresis (PAGE) and the concentration of the gel was 7.25%. Band patterns (zymogram) were detected by specific enzyme substrate staining procedures of Shaw and Prasad (1970) and Shaklee *et al.* (1990). Since the liver tissue produced sharp and reproducible band patterns without trailing, it was selected for further studies. Electrophoresis was carried out in a vertical gel apparatus (Hoefer - Pharmacia, LKB). The gel composition for PAGE is given below.

Stock solutions	Volume
Acrylamide (40%)	3.5 ml
Bis acrylamide (2.1%)	2.5 ml
Double distilled water	6.0 ml
Tank buffer (1X)	5.0 ml
Ammonium persulphate (0.28%)	3.0 ml
TEMED	20.0 µl

Two buffer systems, TBE (90 mM Tris-borate and 2 mM EDTA, pH 8.0) and TG (5 mM Tris-Cl and 0.038 M Glycine, pH 8.3) were tried for the present study. Stock solutions of acrylamide and bis-acrylamide, gel buffer, ammonium persulphate (APS) and TEMED (N,N,N',N'- Tetramethyl ethylene diamine) were prepared as given above. To increase the resolution of the bands in the gel mixture NAD or NADP was added in the gel and upper tank buffer, based on the nature of the enzyme (Gopalakrishnan *et al.*, 1997). The gel mixture was loaded and the combs were kept to make the wells in the gel. The 1X TBE or Tris-glycine buffer was poured in upper and lower chamber with or without NAD or NADP. In all the enzymes, except MEP, PGM and SOD, NAD was added in upper and lower chamber during electrophoresis. Approximately 6 µl of sample (clear supernatant) was loaded in each well at the cathodal end and it took nearly one to one and half-hours for the indicator dye to reach the anodal end of the gel. A cooling system was connected to the electrophoretic apparatus so as to minimize the heat produced during each electrophoretic run. After completion of each run, the gel was stained for a specific allozyme using standardized protocols.

3.3.4. Staining and Imaging

The staining recipe used for the allozyme detection was modified from that of Shaw and Prasad (1970), Shaklee *et al.* (1990) and Gopalakrishnan *et al.* (1997).

Zones of activity for each enzyme were revealed by incubating the gels in the dark at 37^{0}C in the presence of specific substrate and histochemical staining solution until sharp bands were visualized. The stock solutions used were also of the same concentration mentioned in the original recipe. In PGM and GPI, 2% agar overlay was done for better resolution and to prevent leaching out of the enzyme-stain complex from the gel. After staining, the gels were photographed (using Image Master ID elite) or scanned in a high-resolution scanner to fix the original colour of the gels. The details of the staining recipe for these fourteen enzymes are given below.

Aspartate Amino Transferase (AAT) 2.6.1.1, Dimer

α -ketoglutaric acid (20mg/ml)	=1ml
L-Aspartic Acid (50 mg/ml)	=1 ml
Pyriodoxal 5-phosphate (1mg/ml)	=0.5 ml
BB Salt (40 mg/ml)	=0.5 ml
Tris-Cl buffer (pH 8.0)	=2.5 ml
Dist water	=4.5 ml
Running buffer	TBE
Running time	65 min

Esterase (EST) 3.1.1.- , Monomer

α -naphthyl acetate (20mg in 0.5ml acetone + 0.5 ml H_2O)	=0.5 ml
β-Naphthyl acetate	=0.5 ml
Fast Blue RR (5 mg/ml)	=0.5 ml
Tris-Cl buffer (pH 8.0/7.0)	=2.5 ml
Dist water	=6.0 ml
Running buffer	TBE
Running time	50 min

Glucose Dehydrogenase (GLDH) 1.1.1.47, Dimer

D-glucose	=500mg
NAD (15 mg/ml)	=0.6 ml
NBT (8 mg/ml)	=0.4 ml
PMS (1.7 mg/ml)	=0.2 ml
Tris-Cl (pH 8.6)	=5.0 ml
Dist. Water	=3.8 ml
Running buffer	TBE
Running time	90 min

Glucose –6-PO_4 Dehydrogenase (G_6PDH) 1.1.1.49, Dimer

Glucose-6-PO_4(50mg/ml)	=0.6 ml
NADP (4 mg/ml)	=1.6 ml
NBT (8 mg/ml)	=0.4 ml
PMS (1.7 mg/ml)	=0.4 ml
Tris-Cl (pH 8.0)	=2.0 ml
$MgCl_2$ (20 mg/ml)	=0.4 ml
Dist H_2O	=4.6 ml
Running buffer	TBE
Running time	90 min

Glucose phosphate Isomerase (GPI) 5.3.1.9, Dimer

Fructose-6-PO_4 (20 mg/ml)	=1 ml
NADP (4 mg/ml)	=1.6 ml
Mg Cl_2 (20 mg/ml)	=0.5 ml
G_6PDH (1000 units/ml)	=20 µl
NBT (8 mg/ml)	=0.4 ml
PMS (1.7 mg/ml)	=0.2 ml
Tris-HCl (pH 8.0)	=2.5 ml
Dist H_2O	=3.8 ml
Agar overlay (2%).	
Running buffer	TBE
Running time	90 min

Glyceraldehyde-3-PO_4 Dehydrogenase (GAPDH) 1.2.1.12, Tetramer

Fructose-1, 6-diphosphate (Na_4 Salt) (20mg/ml)	=2.5 ml
Aldolase (1000 units/ml)	=220 µl
NAD (15 mg/ml)	=0.4 ml
NBT (8 mg/ml)	=0.4 ml
PMS (1.7 mg/ml)	=0.2 ml
Arsenate (Na Salt) (20mg/ml)	=1.5 ml
Tris-HCl (pH 8.0) buffer	=2.5 ml
Dist H_2O	=2.5 ml
Running buffer	TBE
Running time	110 min

αGlycerol-3-PO_4 Dehydorgenase (αG_3PDH) 1.1.1.8, Dimer

α-DL-glycerophosphate	=260mg
NAD (15 mg/ml)	=0.6 ml
NBT (8 mg/ml)	=0.4 ml
PMS (1.7 mg/ml)	=0.3 ml
0.2m Tris-Cl (pH 8.0) buffer	=3.5 ml
0.1M $MgCl_2$ (or 20 mg/ml)	=0.1 ml
Dist H_2O	=4.0 ml
Running buffer	TBE
Running time	90 min

Lactate Dehydrogenase (LDH) 1.1.1.27, Tetramer

Lithium lactate (40mg/ml)	=1.6 ml
NAD (15mg/ml)	=0.4 ml
NBT (8mg/ml)	=0.4 ml
PMS (1.7mg/ml)	=0.2 ml
Tris-HCl (pH 8.0)	=2.5 ml
Dist H_2O	=4.7 ml
Running buffer	TBE
Running time	90 min

Malate Dehydrogenase (MDH) 1.1.1.37, Dimer

Sodium malate (50 mg/ml) (Malic acid and Sod. Salt)	=2.0 ml
NAD (15 mg/ml)	=0.4 ml
NBT (8 mg/ml)	=0.4 ml
PMS (1.7 mg/ml)	=0.2 ml
0.2M Tris-Cl (pH 8.0)	=2.5 ml
Dist H_2O	=4.5 ml
Running buffer	TBE
Running time	90 min

Malic Enzyme (MEP) 1.1.1.37, Dimer

Sodium malate (50mg/ml)	=1.0 ml
NADP (4 mg/ml)	=1.6 ml
NBT (8 mg/ml)	=0.4 ml
PMS (1.7 mg/ml)	=0.2 ml
Tris-Cl (pH-8)	=2.5 ml
Dist H_2O	=3.5 ml
$MgCl_2$ (20mg/ml)	=0.5 ml
Oxaloacetic acid (to inhibit MDH)	=9 mg
Running buffer	TBE
Running time	90 min

Octanol Dehydrogenase (ODH)
1.1.1.73, Dimer

0.05m Tris-HCl (pH 8.5)	=5.0 ml
Octanol	=20 µl
NAD (15 mg/ml)	=0.4 ml
NBT (8 mg/ml)	=0.4 ml
PMS (1.7 mg/ml)	=0.4 ml
Dist. Water	=3.7 ml
Sod. Pyruvate	220mg/ 10 ml
Running buffer	TBE
Running time	70 min

Phosphoglucomutase (PGM)
5.4.2.2, Monomer

Glucose-1-PO_4(50mg/ml)	=1.0 ml
NADP (4 mg/ml)	=1.6 ml
$MgCl_2$ (20mg/ml)	=1.0 ml
G_6PDH (1000 units/ml)	=20 µl
NBT(8 mg/ml)	=0.4 ml
PMS (1.7 mg/ml)	=0.2 ml
Tris-HCl (pH 8.0)	=2.5 ml
Dist. Water	=6.0 ml
Agar overlay (2%)	
Running buffer	TBE
Running time	90min

Superoxide Dismutase (SOD)
1.15.1.1, Dimer

NBT (8 mg/ml)	=0.4 ml
PMS (1.7 mg/ml)	=0.4 ml
NAD (15 mg/ml)	=0.4 ml
Tris-HCl (pH 8.0)	=3.0 ml
Dist. Water	=6.0 ml

Keep in dark for 20 min sharply without PMS & NBT. Then expose to a bulb (not tube light) with NBT & PMS.

Running buffer	TG
Running time	80 min

Xanthine Dehydrogenase (XDH)
1.1.1.204 - Dimer

Hypoxanthine (100mg/ml)	=1.6 ml
NAD (15 mg/ml)	=0.4 ml
0.2m Tris-HCl (pH-7.5)	=7.5 ml
NBT (8 mg/ml)	=0.4 ml
PMS (1.7 mg/ml)	=0.2 ml
Dist H_2O	=0.4 ml
Pyruvate	150 mg
Running buffer	TBE
Running time	130 min

3.3.5. Scoring of alleles

Enzyme activity obtained on each gel was differentiated into specific zones. Nomenclature of loci and alleles followed that recommended by Shaklee *et al.* (1990). The slowest moving zone was marked as locus 1 and the faster one as locus 2. Zone possessing bands with different electrophoretic mobilities were counted as polymorphic (more than one allele) and the one without as monomorphic loci (single allele). The differences in the electrophoretic mobilities of bands in a polymorphic locus were actually measured to distinguish the multiple forms of the alleles at the locus. The banding pattern of heterozygotes in polymorphic loci, conformed to that expected as per the structure of the respective protein (Whitmore, 1990). When an

allozyme genotype had only two bands, the enzyme structure was described as monomeric heterozygote and when it formed three bands, it was considered as a heterozygous pattern of a dimeric enzyme. As a general practice, the most common band was given the electrophoretic mobility value 100. Alternate alleles were designated as per their mobility, in relation to the most common allele. Since, protein/allozyme bands are co-dominant allelic products (genotypes), a single banded genotype was counted as a homozygote formed of homozygous alleles. When genotypes were formed of more than two different alleles already considered, then the locus was counted as multiple allelic loci as for GLDH in this study with alleles 080, 089, 100 and 117 (Fig. 5). The number of different genotypes observed at each locus was counted in each sample.

3.3.6. Analysis of Data

3.3.6.1. Allele frequencies, polymorphic loci and heterozygosity

To analyze variation at allozyme loci, allele frequencies at each locus were calculated with GENETIX Software (version 4.0, Belkhir *et al.*, 1997). A locus was considered to be polymorphic when the frequency of the most common allele was equal to or less than 0.99 (Nei, 1987). The mean number of alleles per locus; observed and expected heterozygosities (Hob and Hex) and percentage of polymorphic loci for overall and each population were calculated with GENETIX. The allele frequencies of multiple collections of the same river in different years were tested for significant homogeneity and the genotype data from different collection sets for the same river that exhibited homogeneity were pooled. The combined data sets were used for further analysis of parameters of genetic variation and population structure of *H. brachysoma.*

3.3.6.2. Linkage disequilibrium

This parameter was tested using a contingency table test for genotype linkage disequilibrium between pairs of loci in a population, based upon the null hypothesis that genotypes at one locus are independent of genotypes at other loci. Calculations were performed using the GENEPOP Ver. 3.3d programme (Raymond and Rousset, 1998), which performs a significance test using Markov chain procedures.

3.3.6.3. Hardy-Weinberg Equilibrium

Exact *P*-tests for conformity to Hardy-Weinberg Equilibrium (probability and score test) were performed by the Markov Chain method using GENEPOP version 3.3d (Raymond and Rousset, 1998) with parameters, dememorization = 1000; batches = 10 and iterations = 100; and based upon a null hypothesis of random union of gametes. The significant criteria were adjusted for the number of simultaneous tests using sequential Bonferroni technique (Rice, 1989).

3.3.6.4. Estimates of population differentiation

The genetic differentiation between populations was investigated by: 1) Exact test to assess genotypic homogeneity between different pairs of populations over each locus and all loci combined using GENEPOP. This test was performed on genotype tables, assuming possible non - independence of alleles within genotypes will not affect the test validity (Raymond and Rousset, 1995a). A Markov Chain method (Guo and Thompson, 1992) was used to generate an unbiased estimate of the exact test. Although exact test of genotype and allele frequencies may be the most sensitive detector of population differentiation, it provides no estimate of the magnitude of the differences (Donnelly *et al.*, 1999); hence, to assess the population structure in a quantitative way, F_{ST} estimator was used. 2) F-statistics (F_{IS} and F_{ST}): The coefficient of genetic differentiation (F_{ST}) and the inbreeding coefficient (F_{IS}) were estimated using the estimator of Weir and Cockerham, (1984). Estimation of average F_{ST} and determination of whether the values were significantly different from zero; and calculation of pair-wise population F_{ST} values (θ) and their significance levels, were carried out using GENEPOP. This programme performs numerical resampling by bootstrapping (1000 times in the present study) and jack-knife procedures in order to estimate confidence intervals and the significance of values. F_{ST} values range from 0 to 1, the greater the value, the greater the differences among populations (Beaumont and Hoare, 2003). F_{IS} refers to the Hardy-Weinberg distribution (or otherwise) of genotypes of individuals within sub-population and is defined as the correlation between homologous alleles within individuals with reference to the local population. It is a measure of deviations from Hardy-Weinberg proportions within samples and is some times known as the fixation index. Positive values for a fixation index

demonstrate an excess of homozygotes (positive correlation between homologous allele) or conversely, a deficiency of heterozygotes, relative to the Hardy-Weinberg model. This could be due to inbreeding and this index is often labeled an inbreeding coefficient.

3.3.6.5. Gene flow

F_{ST} values can be used to estimate the amount of gene flow (Nm) using the corrected private - allele-based model of Barton and Slatkin (1986) in GENEPOP, between populations, since, $F_{ST} = 1 / (4Nm+1)$. Therefore, $Nm = (1-F_{ST})/4F_{ST}$, where N is the effective population size and 'm' is the proportion of migrants arriving into each of the population in each generation.

3.3.6.6. Genetic similarity and distance

Genetic similarity/identity and distance between pairs of populations of *H. brachysoma* were estimated using POPGENE Version 1.31 (Yeh *et al.,* 1999). Nei and Li's (1979) pair-wise genetic similarity (SI) among yellow catfish specimens were computed and converted by POPGENE into genetic distance (GD) according to Hillis and Moritz's (1990) formula, GD = 1- SI. The SI reflects the proportion of bands shared between the individuals and values range from 0 when no bands are shared between RAPD profiles of two populations to 1, when no difference are observed, *i.e.,* all bands are identical. The opposite holds true for 'GD' values.

3.3.6.7. Dendrogram

Phylogenetic relationships based on genetic distance values generated from allozyme data among three populations of *Horabagrus brachysoma* were made and a dendrogram plotted, using the unweighted pair group method and arithmetic averages (UPGMA, Sneath and Sokal, 1973) based on Nei (1978) modified from NEIGHBOR procedure of PHYLIP version 3.5c (Felsenstien, 1993) using POPGENE version 1.31(Yeh *et al.,* 1999). To test the confidence level of each branch dendrogram, the data were bootstrapped 1000 times using WinBoot (Yap and Nelson, 1996). Bootstrap values between 75 and 95 were considered significant and above 95 highly significant (Lehmann *et al.*, 2000).

3.4. Random amplified polymorphic DNA (RAPD)

3.4.1 Genomic DNA isolation

Total DNA was extracted from the blood samples following the procedures of Taggart *et al.* (1992) and Cenis *et al.* (1993) with minor modifications.

- 500 µl of blood sample (0.25 ml stored in 1 ml of 95% ethanol) from each specimen separately was taken in 50 ml autoclaved centrifuge tube. Ethanol was decanted by centrifugation at 10000 rpm for 10 min. at 4^0C.

- The blood sample was washed by mixing with 1.0 ml of high molar TE buffer (0.1M Tris and 0.04 M EDTA).

- The buffer was decanted by centrifugation at 10,000 rpm for 10 minutes at 4^0C, and repeated the above two steps once to get clear pellets of blood cells.

- To lyse the blood cells, 3 ml of incubation buffer (given in box) was added to each tube and incubated at 56^0C for 1 hour in a water bath.

Incubation Buffer	
Tris-Cl (pH 8.3)	-10 mM
EDTA (pH 8.0)	-1 mM
NaCl	-0.4 M (2.337 g/100 ml)
Proteinase K(20 mg / ml)	-10 µl /ml
SDS	-10%.

- The sample was taken out from water bath and 2.0 ml of lysis buffer (10 mM Tris-Cl (pH 8.3), 1 mM EDTA (pH 8.0) and 0.4 M (2.337 g / 100 ml) NaCl) was added in each tube.

- The DNA was purified by extraction with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and mixed very gently by repeatedly inverting the tube slowly, to avoid the denaturing of DNA, for 10 minutes (protocol for saturation of phenol is given in box below).

Saturation of Phenol with Tris-Cl (pH 8.0)
▪ If phenol is transparent, added 0.1 % (20 mg) 8-hydroxy-quinoline (to avoid the oxidation of phenol) to 200 ml of water saturated phenol ▪ Covered the flask containing phenol with aluminium foil to avoid light reaction. ▪ 200 ml of 0.5 M Tris-HCl was added. ▪ Stirred the solution using magnetic stirrer for 15 minutes ▪ Kept the solution for 30 minutes to allow the phenol to settle. ▪ The supernatant (Tris) was decanted. ▪ 200 ml of 0.1 M Tris-Cl was added. ▪ Repeated the above four steps once. ▪ 200 ml of 0.1 M Tris-Cl was added to phenol ▪ And stored at 4^0C

- The sample was then centrifuged at 12,000 rpm for 15 min at 4^0C and aqueous phase was transferred to a fresh autoclaved tube by using 1.0 ml cut tips. The organic phase containing the denatured proteins and other debris was discarded.

- Equal volume of the aqueous phase and chloroform: isoamyl alcohol (24:1v/v) mixture was added to the sample, mixed gently and centrifuged at 12,000 rpm for 15 min at 4^0C.

- The aqueous phase was transferred to a fresh autoclaved tube and organic phase containing the lipids and carbohydrates were discarded.

- Then $1/10^{th}$ volume of 3 M sodium acetate (pH 5.2) was added to the separated aqueous phase and the DNA was precipitated with 2.5 volume of ice-cold ethanol.

- The tube was then kept at 4^0C for overnight in a refrigerator to get the maximum pellet of DNA.

- The precipitated DNA was pelleted by centrifuging at 12,000 rpm for 10 minutes at 4^0C and ethanol was decanted and the DNA pellet was marked in tube.

- To wash the DNA pellet 3.0 ml of 70% ethanol was added, and mixed. The solution was centrifuged at 12,000 rpm for 10 minutes at 4^0C.

- Carefully discarded the ethanol and kept the tubes inverted to drain off remaining ethanol and then the DNA was vacuum dried and suspended in 100 μl TE buffer (10 mM: 1 mM, pH 8.0).

- RNA in the sample was degraded by incubating at 37^0C for 1 h after the addition of 5.0 μl of DNAase free RNAase (10 mg/ml-Bangalore Genei).

- The DNA samples were stored at -20^0C for further use.

The extracted DNA was checked through 0.7% agarose gel electrophoresis with ethidium bromide incorporated in 1X TBE buffer (90 mM Tris-borate and 2 mM EDTA, pH 8.0).

3.4.2. DNA Quantification

The quality and quantity of the extracted DNA was checked in UV spectrophotometer (Beckman, USA) by taking the optical density (OD) at 260 nm and 280 nm. The quality was checked by measuring the ratio of absorbance at 260 nm and 280 nm (260/280). The value between 1.7 - 1.8 indicates good quality DNA without protein contamination. DNA quantification was done according to the following calculation: sample showing 1 OD at 260 nm is equivalent to 50 µg of DNA/ml. The OD of each DNA sample at 260 nm was measured and quantified accordingly.

3.4.3. Screening of RAPD primers

Eighty decamer primers (20 from each series OPA, OPAA, OPAC and OPAH) (Operon Technologies, Alameda, USA) were used for screening *Horabagrus*

brachysoma samples. Thirty two primers (Table-3) out of 80 produced amplicons and they were selected for primary screening, however only 10 primers *viz.*, OPA-07, OPA-09, OPA-11, OPA-20, OPAC-14, OPAH-01, OPAH-02, OPAH-04, OPAH-08 and OPAH-09 were selected for population genetic analysis taking into consideration the repeatability, sharpness and intensity of bands. In Table-3, the sequences and molecular weights of the primers are given.

Table 3. Selected primers with concentration and molecular weight, used in RAPD analysis in *H. brachysoma* (the primers asterisked are selected for population analysis)

Sl. No:	Primer	Sequences (5'-3')	M.W (dalton)	Conc. (pmoles/µl)
1.	OPA 06	GGTCCCTGAC	2995	5.743
2.	OPA 07*	GAAACGGGTG	3108	4.627
3.	OPA 08	GTGACGTAGG	3099	4.894
4.	OPA 09*	GGGTAACGCC	3044	5.160
5.	OPA 11*	CAATCGCCGT	2979	5.533
6.	OPA 12	TCGGCGATAG	3059	5.090
7.	OPA 15	TTCCGAACCC	2939	5.785
8.	OPA 16	AGCCAGCGAA	3037	4.712
9.	OPA 19	CAAACGTCGG	3028	4.990
10.	OPA 20*	GTTGCGATCC	3018	5.656
11.	OPAA 07	CTACGCTCAC	2939	5.785
12.	OPAA 08	TCCGCAGTAG	3019	5.302
13.	OPAA 11	ACCCGACCTG	2964	5.616
14.	OPAA 12	GGACCTCTTG	3010	5.656
15.	OPAA 16	GGAACCCACA	2997	4.894
16.	OPAA 17	GAGCCCGACT	3004	5.379
17.	OPAC 05	GTTAGTGCGG	3090	5.192
18.	OPAC 06	CCAGAACGGA	3037	4.710
19.	OPAC 09	AGAGCGTACC	3028	4.988
20.	OPAC 14*	GTCGGTTGTC	3041	5.783
21.	OPAC 15	TGCCGTGAGA	3059	5.088
22.	OPAC 18	TTGGGGGAGA	3139	4.710
23.	OPAH 01*	TCCGCAACCA	2948	5.413
24.	OPAH 02*	CACTTCCGCT	2930	6.207
25.	OPAH 03	GGTTACTGCC	3010	5.654
26.	OPAH 04*	CTCCCCAGAC	2924	5.874
27.	OPAH 06	GTAAGCCCCT	2979	5.531
28.	OPAH 08*	TTCCCGTGCC	2946	6.473
29.	OPAH 09*	AGAACCGAGG	3077	4.542
30.	OPAH 10	GGGATGACCA	3068	4.799
31.	OPAH 11	TCCGCTGAGA	3019	5.30
32.	OPAH 19	GGCAGTTCTC	3010	5.654

3.4.4. PCR amplification

RAPD-PCR reactions were carried out in a PTC 200 gradient thermal cycler (M.J. research, Inc., Watertown, Massachusetts, USA) employing the RAPD primers described in Table-3. PCR amplifications were performed in 25µl reactions containing 1x reaction buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) with 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 6-8 pmoles of primer (random primers), 200 mM dNTPs, 2 U *Taq* DNA polymerase (Genei, Bangalore, India) and 25 ng of template DNA. To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. The reaction mixture was pre-heated at 95°C for 3 minutes followed by 40 cycles (94°C for 3 minutes, 40°C for 1.30 minutes and 72°C for 2 minutes). The reaction was then subjected to a final extension at 72°C for 10 minutes. The composition of PCR reaction mixture is given in box below.

PCR reaction Mixture	Vol. per reaction
Double distilled water	17.3µl
Assay buffer (10x)	2.5 µl
dNTPs	2.0 µl
Primer (Operon Technologies)	1.5µl
Taq polymerase (Genei, Bangalore)	0.7µl
Template DNA	1.0 µl
Total volume	25.0 µl

3.4.5. Agarose electrophoresis and visualization of bands

The resulting products were electrophoretically analyzed through 1.5% agarose gels stained with ethidium bromide (5µg/ml) in 1x TBE buffer (pH 8.0). The gels were visualized under UV transilluminator and documented using Image Master VDS (Pharmacia Biotech, USA).

RAPD-PCR technique can often produce non-reproducible amplification product (Callejas & Ochando, 2001). Reactions were therefore performed following a strict protocol with standardized conditions. Also, all amplification reactions were carried out at-least thrice in order to make sure

consistency and repeatability of fingerprints generated using selected RAPD primers.

3.4.6. Analysis of Data

3.4.6.1. Scoring of bands

Images of gels were used to analyze banding patterns. A binary matrix was produced whereby the presence or absence of each DNA fragment for each sample was recorded 1 or 0, respectively. Faint or poorly amplified fragments were excluded from the analysis as were fragments with very high (above 3000 bp) or low (below 150 bp) molecular weight. The analysis was based on a few assumptions. First, all RAPD fragments scored represented 2-allele system, *i.e.,* presence (dominant) and absence (recessive) of bands. Second, fragments that migrated at the same position, had the same molecular weight, and stained with the same intensity were homologous bands from the same allele, and the alleles from different loci did not co-migrate. A third assumption was that the populations conformed to Hardy-Weinberg equilibrium, $p^2 + 2pq + q^2 = 1$, with frequencies p (dominant, band present) and q (recessive, band absent) (Clark and Lanigan, 1993; Lynch and Milligan, 1994). From the binary matrix, the total number of RAPD fragments and polymorphic fragments were calculated for each primer and for all primers. The molecular weights of the bands were calculated by using Image Master 1D Elite software (Pharmacia Biotech, USA) in relation to the molecular marker λDNA with *Eco*RI / *Hind*III double digest applied along with the samples (Fig.19).

3.4.6.2. Allele frequencies and polymorphic loci

Genetic variability in three populations of *Horabagrus brachysoma* was estimated from the gene (allele) frequencies, percentage of polymorphic loci (%P). The %P values were calculated using the criterion for polymorphism, of which the frequency of the most common allele was ≤0.95. RAPD allele frequencies were calculated taking into account the above assumptions using POPGENE version 1.31 (Yeh *et al.,* 1999).

3.4.6.3. Average gene diversity (H)

Average gene diversity index also known as average heterozygosity (H) (Nei, 1987; Khoo *et al.*, 2002) is a measurement of genetic variation for randomly mating populations and is analogous to Wright's (1951) F_{ST} statistics (fixation index). It was calculated using the POPGENE version 1.31 (Yeh *et al.*, 1999). H is defined as the mean of heterozygosities (h) for all loci.

3.4.6.4. Genetic differentiation and geneflow

The value of coefficient of genetic differentiation (G_{ST}) and effective migration rate or gene flow were also calculated using POPGENE version 1.31 (Yeh *et al.*, 1999).

3.4.6.5. Genetic similarity and distance

Genetic similarity/identity and distance between pairs of populations of *H. brachysoma* were estimated using POPGENE Version 1.31 (Yeh *et al.*, 1999). Nei and Li's (1979) pair-wise genetic similarity (SI) among yellow catfish specimens were computed and converted by POPGENE into genetic distance (GD) according to Hillis and Moritz's (1990) formula, GD = 1- SI. The SI reflects the proportion of bands shared between the individuals and values range from 0 when no bands are shared between RAPD profiles of two populations to 1, when no difference observed, *i.e.*, all bands are identical. The opposite holds true for 'GD' values.

3.4.6.6. Dendrogram

Cluster analysis was performed and dendrogram plotted based on RAPD data among three populations of *Horabagrus brachysoma,* following unweighted pair group method using arithmetic averages (UPGMA; Nei, 1978) modified from NEIGHBOR procedure of Phylip version 3.5c (Felsenstien, 1993) using POPGENE Version 1.31 (Yeh *et al.* 1999). To test the confidence level of each branch of UPGMA based dendrogram, the binary data matrix was bootstrapped 1000 times, using WinBoot (Yap and Nelson, 1996). Bootstrap values between 75 and 95 were considered significant and above 95 highly significant (Lehmann *et al.*, 2000).

3.5. Microsatellite analysis

3.5.1. Identification of markers

3.5.1.1 Collection of microsatellite primer sequences

Available microsatellite information for closely related species (up to order or family level) was collected from the Genebank through Internet (National Centre for Biotechnology Information – NCBI website). For cross-species amplification of microsatellite loci, a total of 25 microsatellite primers from *Pangasius hypophthalmus* (4)(Volchaert *et al.*, 1999); *Clarias macrocephalus* (4) (Na-Nakorn *et al.*, 1999); *Clarias gariepinus* (7) Galbusera *et al.*, 1996 and *Scleropages formosus* (10) Yue *et al.*, 2000) were used (Table-4).

Table 4. Microsatellite primers of related species tested for cross-species amplification in *Horabagrus brachysoma*

Sl. No.	Donor species	No. of primer pairs tested	Loci/ Primer	Gene bank Accession No.	References
1	*Pangasius hypophthalmus*	4	Phy01 Phy03 Phy05 Phy07	AJ131380 AJ131381 AJ131382 AJ131383	Volchaert *et al*, (1999).
2	*Clarias macrocephalus*	4	Cma1 Cma2 Cma3 Cma4	-----	Na-Nakorn *et al.*, (1999)
3	*Clarias gariepinus*	7	Cga01 Cga02 Cga03 Cga05 Cga06 Cga09 Cga10	-----	Galbusera *et al.*, 1996.
4	*Scleropages formosus*	10	D11 D13 D14 D16 D33 D35 D37 D38 D42 D72	AF219953 AF219954 AF219955 AF219957 AF219961 AF219962 AF219963 AF219964 AF219965 AF219966	Yue *et al*, (2000)
	Total tested	25			

3.5.1.2. Designing of primers for microsatellite sequences

The primers for microsatellite sequences were designed based on their melting temperature, secondary structure and sequence homology between the forward and reverse primers using the following programs: PRIMER3 (Rozen and Skaletsky, 1998) and DNASIS and custom synthesized for use.

Table 5. Sequences, concentration and annealing temperature of selected microsatellite primers in *H. brachysoma*

Sl No:	Primers		Sequence 5'-3'	Conc. (nmol)	Ta for each primer
1	Phy 01	F	CGAACACGCCACAGAGAGTA	49.5	57°C
		R	CCACACCCACAACACCATAA	51.4	55°C
2	Phy 05	F	CCAGCAACCCACATAATTGA	43.2	53°C
		R	CAGCTCAGGGCCAAAAGTAG	45.1	57°C
3	Phy 07	F	AGTCACTTCAGCACCTGCCT	38.4	57°C
		R	ATCTCTGTGATGGTGAGCCA	53.9	55°C
4	Cma 3	F	TTCGGATTGTTTCTGTGG	53.1	47°C
		R	ACACTCTTTACACTGATT	50.2	43°C
5	Cma 4	F	TTTCGCCACGCAGGTTT	46.9	47°C
		R	TGGATTTTGACTGTGTATT	50.7	45°C
6	Cga 06	F	CAGCTCGTGTTTAATTTGGC	79.6	53°C
		R	TTGTACGAGAACCGTGCCAGG	52.0	61°C
7	D-33	F	CACATGCATGGAATTATGGC	47.7	53°C
		R	GAGCCAGAAGCAGGACTGAC	42.0	59°C
8	D-38	F	AATGCTGATGGACCTGCTCT	56.3	55°C
		R	CAAACAGGGAACCCACAGAT	53.3	55°C

3.5.1.3. PCR amplification

PCR reactions were carried out in a PTC 200 gradient thermal cycler (M.J. Research, Inc., Watertown, Massachusetts, USA) employing the microsatellite primers (Table-5). Amplifications were performed in 25 µl reaction mixture containing 1x reaction buffer (10 mM Tris, 50 mM KCl, 0.01% gelatin, pH 9.0) with 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 5 pmoles of each primer, 200 mM dNTPs, 2 U *Taq* DNA polymerase (Genei, Bangalore, India) and 25-50 ng of template DNA. The volume of reaction mixture is given in box below.

PCR reaction mixture	Vol. per reaction
Double distilled water	18.3 µl
Assay buffer (10x)	2.5 µl
dNTPs	2.0 µl
Primer (forward and reverse)	0.5µl
Taq polymerase (Genei, Bangalore)	0.7µl
Template DNA	1.0 µl
Total volume	25.0 µl

To check DNA contamination, a negative control was set up omitting the DNA from the reaction mixture. The reaction mixture was pre- heated at 94°C for 5 minutes followed by 25 cycles (94°C for 30 seconds, annealing temperature depending upon the T_m value of primer (usually 50°C - 60°C) and 72°C for 1 minute).

The annealing temperature (T_a) of a microsatellite primer was calculated using the following formula, $T_m = \{2 (A+T) + 4 (G+C)\}$, where T_m = melting temperature of the primer; A, T, G and C are the number of bases in the primer. The T_m values of both forward and reverse primers were calculated separately and the annealing temperature (T_a) for a primer combination was fixed 3-5°C below the lowest T_m value obtained for the forward/reverse primer in that combination (Table-5). The reaction was then subjected to a final extension at 72°C for 2 minutes. The amplified product was checked in 10% polyacrylamide gel electrophoresis (PAGE).

3.5.1.4. Polyacrylamide gel electrophoresis (PAGE)

The PCR products were analyzed electrophoretically using a 10% non-denaturing polyacrylamide (19:1 acrylamide and bisacrylamide) gel. The molten agarose (1%) was poured between glass and alumina plate with glass syringe and a needle for approximately 1 cm height at the bottom for sealing the unit. After solidification of agarose, the polyacrylamide (10%) was poured in the order given below and comb was inserted in between the plates to make wells in the gel.

Acrylamide (19:1)	: 5 ml
Double distilled water	: 2.0 ml
5 x TBE	: 2.0 ml
10% (Ammonium persulphate)	: 70 µl
TEMED	: 3.5µl

After the gel had polymerized, the comb was removed without distorting the shapes of the wells. The IX TBE buffer was poured in upper and lower chambers. The PCR amplified samples (8 µl) were loaded with 2 µl of bromophenol blue (BPB) into the wells using micropipette; and run with 1xTBE buffer (pH 8.0) for 5 hours at constant voltage of 10 V/cm, at 4 ^{0}C in a cold chamber.

3.5.1.5. Visualization of microsatellite products

The amplified microsatellite loci were visualized using silver staining of the polyacrylamide gel. The gels were fixed in 50 ml of fixing solution (diluted five times with 30.4 ml double distilled water and 9.6 ml ethanol) for 30 min. and silver-impregnated (with 1X staining solution) for another 30 minutes. This was followed by washing the gels in double distilled water for 1 to 2 minutes, after removing the staining solution. The gels were then kept in the 1X developing solution in darkness for 10 minutes. When the bands were dark enough, the developing solution was poured out and the stopping and preserving solution (1X) was immediately added. The composition of silver staining solutions is given in box below.

Non-amplified (null) alleles are a common feature of microsatellite genotyping and can bias estimates of allele and genotype frequencies, there by hindering population genetic analysis. Whenever microsatellite product was not visualized in gels with any of the samples, the same DNA extract was again subjected to PCR amplification and electrophoresis to rule out the possibility of null alleles.

Item	Composition
Fixing solution (5X)	Benzene sulphonic acid; 3.0% w/v in 24% v/v ethanol
Staining solution, 5X	Silver nitrate; 1.0% w/v Benzene sulphonic acid; 0.35% w/v.
Developing solution, 5 X Sodium carbonate solution, 5X Formaldehyde; 37% Sodium thiosulphate; 2%	 Sodium carbonate, 12.5% w/v. Formaldehyde; 37% w/v in water Sodium thiosulphate; 2% w/v in water
Stopping and Preserving solution, 5X	Acetic acid, 5% v/v Sodium acetate, 25% w/v Glycerol; 50% v/v

3.5.1.6. Calculation of the molecular weights of the bands

Molecular weights of the bands were calculated in reference to the molecular weight markers with the software Image Master ID Elite. The alleles were designated according to PCR product size relative to molecular marker (*pBR*322 DNA/*Msp*I digest, Fig. 31).

3.5.1.7. Confirmation of microsatellite by cloning and sequencing

The microsatellites loci were confirmed by sequencing the loci after cloning them in TOPO vector (Invitrogen, Carlsbad, USA) (Fig. 41).

3.5.1.7a. PCR Amplification of target sequence

The band of the target sequence with particular primer (forward and reverse) was amplified using PCR protocol as given in section 3.5.1.3. The samples were electrophoresed in PAGE (section 3.5.1.4) to check the concentration of DNA.

3.5.1.7b. Elution of amplified products from agarose gel

The PCR product of microsatellite loci was eluted from the agarose gel by the following method:

i) The samples (20 µl) were quick spinned with 3 µl bromophenol blue dye.

ii) They were run in 2.0% agarose gels and the DNA bands were cut out from the lane after viewing the gel over long wave length UV light quickly so as to avoid nicks.

iii) The gel slices were taken in a 1.5 ml micro-centrifuge tube and 1.0 ml Tris-saturated phenol was added.

iv) The sample was kept at -80^{0}C for over night. The frozen samples were centrifuged at 10000 rpm for 20 min at 4^0 C.

v) The supernatant was transferred to a fresh tube and DNA precipitated by adding 2.5 vol. of ethanol and 1/10 times 3 M Na-Acetate (pH 5.2).

vi) The pooled sample was kept in –20 °C for over night and centrifuged at 10,000 rpm at 4 °C for 20 min.

vii) Ethanol was decanted; the DNA pellet was washed with 0.5ml of 70% ethanol and centrifuged at 10,000 rpm at 4°C for 20 min.

viii) Discarded the ethanol and the DNA was vacuum dried and suspended in 15 µl double distilled water.

ix) For checking the concentration, DNA samples were run (4 µl) in 2.0% agarose gel.

3.5.1.7c. Construction of recombinant plasmid

TOPO (Invitrogen, Carlsbad, USA) vector (Fig. 41) was used for constructing the recombinant DNA. *Taq* polymerase has a nontemplate- dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3'phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994) (Fig. 42).

3.5.1.7d. Competent cell preparation

Competent cell preparation was done as follows by using *E.coli* strain *DH5α:*

i. From a glycerol stock, the *E. coli* strain was streak plated to LB agar media.

ii. Single colony from the plate was picked and cultured in 3 ml LB overnight at 37^0C in an environmental shaker (New Brunswick Scientific, USA).

iii. Next day, 2% of the overnight grown cultures (100 µl) were inoculated to 5 ml LB and grown for 3-4 h (till the OD reaches 0.3 – 0.5).

iv. The cultures were then kept in crushed ice and distributed 1 ml each to 1.5 ml micro-centrifuge tubes.

v. The cells were harvested by spinning at 5000 rpm for 3 min at 4^0C.

vi. After discarding the supernatant, the tubes were kept in ice and 200 µl 0.1 M freshly prepared $CaCl_2$ was added with a pre-cooled pipette tip. The cells were kept suspended in 0.1 M $CaCl_2$ for 20 min on ice.

vii. The tubes were then spun at 5000 rpm for 3min at 4^0C and the supernatant was discarded.

viii. The cells were re-suspended in 200 µl 0.1 M ice-cold $CaCl_2$ and either quickly frozen to -70^0C for storage or kept in ice for immediate use.

3.5.1.7e. Transformation of recombinant plasmid

The *E.coli* strain *DH5α* was used for the transformation purpose. The transformation was done as follows.

a. For transformation, 4 µl of the ligated mix was added to the competent cells and incubated in ice for 30 min.

b. Then a heat shock was given at 42^0C for 1 min.

c. After the heat shock, the tube was immediately transferred to ice and allowed to chill for 1-2 min.

d. Then 800µl LB medium was added and the culture was incubated at 37^0C for 90 min with shaking.

e. The transformed competent cells (100 µl) were spread over an LB plate (90 mm) containing 50 µg/ml ampicillin coated with 40 µl X-gal (20 mg/ml) and 4 µl IPTG (200 mg/ml).

f. The plates were incubated for 12-16 h at 37^0C.

g. Transformants containing the insert were selected by blue/white screening.

3.5.1.7f. Selection of recombinants

The transformed cells were cultured in LB media plate (90 mm) containing 50 µg/ml ampicillin coated with 40 µl X-gal (20 mg/ml) and 4 µl IPTG (200 mg/ml). The plates were incubated for 12-16 hours at 37^{0}C. The transformants containing inserts were selected by blue/white screening (Sambrook *et al,* 1989).

3.5.1.7g. Confirmation of cloning

a) Through PCR

Both blue and white colonies were cultured overnight in 3 ml LB containing appropriate antibiotic and were pelleted down quickly by spinning down at maximum speed for 30 sec in a table-top micro centrifuge. The pellet was suspended in 50 µl sterile double distilled water and boiled for 10 min. The suspension was spun down and the supernatant was used as the template for PCR reactions. PCR reactions were performed with 2 µl of the template plasmid DNA. The PCR products from blue and white colonies were analysed in 1.5% agarose gels.

b) By comparing the plasmid size

Plasmid extraction from recombinant bacteria and non-recombinant bacteria were done according to the alkaline lysis procedure of Birnboim and Doly (1979).

- An overnight grown 3 ml culture was inoculated in to 100 ml of LB broth containing the respective antibiotic and kept under constant shaking for 12-16 h at 37^{0}C.

- The cells were harvested by centrifugation at 6000 rpm for 5 min at 4^{0}C and the supernatant was drained completely.

- The bacterial pellet was washed with 10ml ice-cold STE buffer (composition of buffer given in the box).

- The washed pellet was re-suspended in 3 ml of solution I.

- Cells were lysed by adding freshly prepared solution II (6 ml), mixed thoroughly and kept at room temperature for 5-10 min.

- Finally, 4.5 ml of ice-cold solution III was added to the lysate mixed thoroughly and kept in ice for 10 min.

STE buffer.
0.1 M NaCl, 10 mM Tris-Cl (pH-8), 1 mM EDTA (pH8.0)

Solution I
50 mM Glucose, 25 mM Tris-Cl (pH-8), 10 mM EDTA (pH 8.0)

Solution II
0.2 N NaOH, 1% SDS

Solution III
5 M Potassium acetate-60 ml, Glacial acetic acid-11.5ml
H_2O 28.5ml

- The precipitated mix which contains chromosomal DNA and high mol. wt. RNA was removed by centrifuging at 6000 rpm for 15 min at 4^0C.

- The supernatant was treated with 5 µl of RNAse (10 mg/ml) and incubated at 37^0 C for 1h.

- The supernatant was then washed twice with an equal volume of chloroform: isoamyl alcohol and centrifuged at 10,000 rpm for 10 min.

- Plasmid DNA from the supernatant was precipitated by the addition of 0.6 vol. of isopropanol and incubated at room temperature for 10 min.

- The plasmid DNA was recovered by centrifugation at 10,000 rpm for 10 min.

- Pellet was washed in 70% ethanol, air-dried and dissolved in 100 µl of TE buffer and stored at -20^0C.

- The plasmids isolated from recombinant colonies and non recombinant colonies were compared in 0.8% agarose gels.

3.6.1.7h. Sequencing of microsatellite loci

The recombinant plasmids were isolated in large scale by alkaline lysis method (section 3.5.1.7g) and were further purified through PEG precipitation for sequencing purpose. To 32 µl of plasmid DNA, 8 µl of 4 M NaCl and 40 µl of 13% PEG_{8000} were added. After through mixing, the sample was incubated on ice for 20 min. and the precipitated plasmid DNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4^0C. Then the supernatant was discarded and the pellet was rinsed with 70% ethanol. Pellet was air-dried and re-suspended in 20µl of sterile double distilled H_2O and stored at -20^0C.

The sequencing was done using forward and reverse sequencing primers with the automated DNA sequencer AB1377 according to manufacturers instructions at the Department of Biotechnology facility, Delhi University, North campus, New Delhi.

3.6.2. Population structure analysis

3.6.2.1. PCR amplification

PCR reactions were carried out by employing eight selected microsatellite primers. Amplifications were performed as given in section 3.5.1.3.

3.6.2.2. Scoring of alleles

Gels having a zymogram pattern following the electrophoresis and silver staining procedures (described on sections 3.6.1.4. and 3.6.1.5) were gel documented using Image Master VDS gel documentation system. The slowest moving zone was marked as locus 1 and the faster one as locus 2. The zone having bands with different electrophoretic mobilities was counted as polymorphic (more than one allele) and the one without as monomorphic loci (single allele). Since, microsatellite bands are co-dominant allelic products (genotypes), a single banded genotype was counted as a homozygote formed of homozygous alleles where as a two or more banded genotype was as heterozygote, formed of two heterozygous alleles at the locus. The number of

different genotypes observed at each locus was counted in each sample. The molecular weight of the bands was calculated by using Image Master 1D Elite software (Pharmacia Biotech, USA) in relation to the molecular marker *pBR*322 with *Msp*I digest (Fig. 31).

3.6.2.3. Analysis of Data

In the analysis of microsatellites, parameters tested were as in the case of allozymes and the softwares used were also same (pages 46 to 48). The parameters estimated include number of alleles, allelic frequencies, percentage of polymorphic loci, observed and expected heterozygosity, linkage disequilibrium, conformity of allele frequencies to that expected under Hardy- Weinberg equilibrium and estimates of population differentiation including F-statistics and gene flow, Genetic similarity and distance and plotting dendrogram using GENEPOP version 3.1 (Raymond and Russet, 1998), GENETIX version 4.0 (Belkhir *et al.*, 1997) and POPGENE version 1.31 (Yeh *et al.*, 1999). Occurrence of null alleles results in false homozygotes leading to genotyping errors and heterozygotes deficiency that can cause deviations from Hardy-Weinberg Equilibrium. This can mimic the true causative factors of Hardy-Weinberg disequilibrium (inbreeding, assortative mating or Wahlund effect) and potentially bias population genetic analysis. The expected frequency of null alleles was calculated according to Van Oosterhout *et al.* (2004, 2006) using MICRO-CHECKER (available from http://www.microchecker.hull.ac.uk/.) and all the genotypes of the loci with known inbreeding coefficient or fixation indices (F_{IS}) were tested for null alleles and further analyzed for population differentiation.

4

RESULTS

4.1. Allozyme analysis

4.1.1. Selection of allozymes

The allozyme analysis was conducted to detect 25 enzymes, but only 14 showed their presence with scorable activity (Enzyme commission numbers and abbreviations are given in Table-2. Out of these fourteen enzymes, 12 enzymes were polymorphic and two enzymes were monomorphic. The polymorphic enzymes were Aspartate amino transferase (AAT), Esterase (EST), Glucose dehydrogenase (GLDH), Glucose phosphate isomerase (GPI), Glucose-6-phosphate dehydrogenase (G_6PDH), αGlycerol-3-phosphate dehydrogenase (αG_3PDH), Lactate dehydrogenase (LDH), Malate dehydrogenase (MDH), Octonol dehydrogenase (ODH), Phosphogluco mutase (PGM), Superoxide dismutase (SOD) and Xanthine dehydrogenase (XDH). The monomorphic enzymes were Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Malic enzyme (MEP) (Table-6).

The fourteen enzymes yielded 25 scorable loci in all populations. EST has maximum number of loci *i.e.*, 5 (*EST-1*, EST-2*, EST-3*, EST-4** and *EST-5**). Three loci were present in ODH (*ODH-1*, ODH-2** and *ODH-3**) and two loci each were present in AAT (*AAT-1** and *AAT-2**), LDH (*LDH-1** and *LDH-2**), GAPDH (*GAPDH-1** and *GAPDH-2**), GPI *(GPI-1** and *GPI-2**) and XDH (*XDH-1** and *XDH-2**). All other enzymes (GLDH, G_6PDH, αG_3PDH, MDH, MEP, PGM and SOD) had only a single locus. A detailed description of the polymorphic and monomorphic enzymes are given in Table-6 and the distribution of genotypes are given in Table-7.

Scorable activity of the following enzymes could not be detected in *Horabagrus brachysoma:* Acid phosphatase (ACP), Adenylate kinase (AK), Alcohol dehydrogenase (ADH), Alkaline phosphate (ALP), Creatine kinase (CK), Fumarase

(FUM), Glutamate dehydrogenase (GDH), Hexokinase (HK), Isocitrate dehydrogenase (ICDH), Phosphogluconate dehydrogenase (6PGDH) and Pyruvate kinase (PK).

Table 6. The names of enzyme loci, enzyme commission (E.C.) number and observed alleles for allozyme analysis in *Horabagrus brachysoma*. The enzymes mark 'ns' did not yield any scorable activity

Enzymes	No. of loci	Locus	Alleles	Monomorphic /Polymorphic
Acid phosphatase	ns	*ACP**	*ns*	ns
Adenylate kinase	ns	*AK**	*ns*	ns
Alcohol dehydrogenase	ns	*ADH**	*ns*	ns
Alkaline phosphate	ns	*ALP**	*ns*	ns
Aspartate amino transferase	2	*AAT-1**	*100*	Monomorphic
		*AAT-2**	*100,117,126*	Polymorphic
Creatine kinase	ns	*CK**	*ns*	ns
Esterase	5	*EST-1**	*083, 100*	Polymorphic
		*EST-2**	*100,106*	Polymorphic
		*EST-3**	*095,100*	Polymorphic
		*EST-4**	*100*	Monomorphic
		*EST-5**	*100*	Monomorphic
Fumarase	ns	*FUM**	*ns*	ns
Glutamate dehydrogenase	ns	*GDH**	*ns*	ns
Glucose dehydrogenase	1	*GLDH**	*080,089,100,117*	Polymorphic
Glucose phosphate isomerase	2	*GPI-1**	*100*	Monomorphic
		*GPI-2**	*096,100*	Polymorphic
Glucose-6-phosphate dehydrogenase	1	G_6PDH*	*086,100*	Polymorphic
α-Glycerophosphate dehydrogenase	1	αG_3PDH*	*088, 100*	Polymorphic
Glyceraldehyde-3-Phosphate dehydrogenase	2	*GAPDH-1**	*100*	Monomorphic
		*GAPDH-2**	*100*	Monomorphic
Hexokinase	ns	*HK**	*ns*	ns
Isocitrate dehydrogenase	ns	*ICDH**	*ns*	ns
Lactate dehydrogenase	2	*LDH-1**	*100*	Monomorphic
		*LDH-2**	*100,112,134*	Polymorphic
Malate dehydrogenase	1	*MDH**	*086,100*	Polymorphic
Malic enzyme	1	*ME**	*100*	Monomorphic
Octonol dehydrogenase	3	*ODH-1**	*100*	Monomorphic
		*ODH-2**	*091,100*	Polymorphic
		*ODH-3**	*100*	Monomorphic
Phosphogluconate dehydrogenase	ns	*6PGDH**	*ns*	ns
Phosphogluco mutase	1	*PGM**	*093,100*	Polymorphic
Pyruvate kinase	ns	*PK**	*ns*	ns
Superoxide dismutase	1	*SOD**	*093,100*	Polymorphic
Xanthine dehydrogenase	2	*XDH-1**	*093, 100*	Polymorphic
		*XDH-2**	*100*	Monomorphic

4.1.1.1. Polymorphic Enzymes

4.1.1.1a. Aspartate Amino Transferase (AAT. 2.6.1.1)

Aspartate Amino Transferase is dimeric in vertebrates. The banding pattern of Aspartate amino transferase patterns in of *H. brachysoma* are shown in fig. 3. The banding patterns showed two different zones (a fast moving zone and slow moving zone) of enzyme activity, which were presumed to be under the control of two independent loci. They were designated as *AAT-1** and *AAT-2** according to their order of increasing mobility differences. The first locus (slow moving) was monomeric and had only a single allele. The second locus (fast moving) was polymorphic and had three alleles *A, B* and *C* and exhibited the typical 3 banded dimeric heterozygous pattern.

Meenachil and Chalakkudy: In these two populations, *AAT-2** had two alleles *A* and *B* (Rf value*100* and *117* respectively) (overall three alleles were present in this locus) they exhibited three types of genotypes *AAT-2*AA, AAT-2*BB* and *AAT-2*AB*. The third allele *C* was absent in these populations.

Nethravathi: In this population, *AAT-2** exhibited three alleles *viz, A, B* and *C* (Rf values*100, 117* and *126* respectively). They exhibited three types of genotypes *AAT-2*AA, AAT-2*BB* and *AAT-2*CC*. The heterozygous condition *AAT-2*AB* could not be detected in all the 70 samples of this population.

4.1.1.1b Esterase (EST. 3.1.1.-)

Esterase enzymes are mostly monomeric in structure except the dimeric Esterase-D. The banding pattern of esterase enzyme system of *H. brachysoma* is shown in Fig-4. It showed five different zones of enzyme activity, which were presumed to be under the control of five independent loci. They were designated as *EST-1*, EST-2*, EST-3*, EST-4** and *EST-5** according to their order of increasing mobility differences. *EST-1*, EST-2** and *EST-3** had more than one allele (*EST-1** had three alleles whereas, *EST-2** and *EST-3** had two alleles each) and heterozygous

condition was also recorded in some individuals. *EST-4** and *EST-5** exhibited only one allele in all the populations.

Meenachil: In this population, *EST-1** locus exhibited one allele (Rf value *100*) hence this locus was monomorphic in this population. *EST-2** locus had two alleles, *A* and *B* (Rf values *100* and *106* respectively), and exhibited three types of genotypes, *viz, EST-2*AA, EST-2*BB* and *EST-2*AB. EST-3** locus was monomorphic in this population.

Chalakkudy: *EST-1** locus exhibited by only one allele (Rf value *100*) in samples from Chalakkudy River; hence this locus was monomorphic in this population. *EST-2** locus had two alleles, *A* and *B* (Rf values *100* and *106* respectively) and exhibited three types of genotypes, *viz, EST-2*AA, EST-2*BB* and *EST-2*AB. EST-3** was polymorphic in this population, and it represented by two alleles *A* and *B* (Rf values *100* and *095* respectively) giving two genotypes, *EST-3*AA* and *EST-3*AB.*

Nethravathi: In this population, the first locus of Esterase, *EST-1** was polymorphic and had two alleles *viz, A* and *B* (Rf values *100* and *083* respectively). The second allele *B* (*083*) was very common in this population and it was represented in 57 out of 70 individuals (81.4%). These three alleles exhibited three genotypes *viz, EST-1*AA, EST-1*BB* and *EST-1*AB. EST-2** locus had two alleles, *A* and *B*, and exhibited three types genotypes, *viz EST-2*AA, EST-2*BB* and *EST-2*AB. EST-3** locus was monomorphic in this population having only one allele *A* (*100*).

4.1.1.1c Glucose dehydrogenase (GLDH-1.1.1.47)

The quaternary structure of this enzyme varied in different groups; monomeric or dimeric (Richardson et al., 1986). The pattern was found to be dimeric in *H. brachysoma* as evidenced by the typical 3 banded pattern heterozygotes. This enzyme was found to be under the control of highly polymorphic single locus (Fig. 5) in the species. This polymorphic locus contained four alleles *viz, A, B, C* and *D*, but these 4 alleles were not present in all three stocks. These 4 alleles showed 6 different

genotypes *viz,* most common homozygote *(GLDH*AA), GLDH*BB, GLDH*CC, GLDH*DD,* two heterozygotes *GLDH*AB* and *GLDH*AD.*

Meenachil: This population contained two types alleles *A* and *B* (Rf value*100* and *089* respectively), and these two alleles represented three types of genotypes *GLDH*AA, GLDH*BB* and *GLDH*AB.* In this population, the alleles *C* and *D* were absent.

Chalakkudy: In Chalakkudy population, the locus contained three types of alleles *A, B* and *C* (Rf values*100*, *089* and *117* respectively). The third allele *C* was seen only in this population. These three alleles represented four types of genotypes *GLDH*AA, GLDH*BB GLDH*AB* and *GLDH*CC.* The *GLDH*AA* genotype was exhibited in only two individuals out of 70 (2.85%) and *GLDH*CC* homozygote was present only in 4 individuals out of 70 (5.71%) in this population.

Nethravathi: In the samples caught from this river, the *GLDH** locus gave two alleles *viz., A* (Rf value *100*) and the private allele *D* (Rf value *117*). The alleles *B* and *C* were absent in this population. The two alleles represented three types of genotypes *GLDH*AA, GLDH*DD* and *GLDH*AD.*

4.1.1.1d Glucose Phosphate Isomerase (GPI-5.3.1.9)

The spacing of the bands of this dimeric enzyme had suggested that there were two loci in *H. brachysoma* (Fig.9). Agar overlay (2%) was used to prevent leaching out of end-products from the gel during staining. The two loci were *GPI-1** (slow homozygote) and *GPI-2** (fast heterozygote). *GPI-1** was monomorphic and the second locus, *GPI-2*,* exhibited two alleles *A* and *B* and produced 3 genotypes *viz, GPI-1*AA, GPI-1*BB* and *GPI-1*AB.*

Meenachil, Chalakkudy and Nethravathi: The samples from three river systems exhibited 2 alleles in second locus, *GPI-2*, viz, A* and *B* (Rf values *100* and *096* respectively) and three genotypes *viz, GPI-1*AA, GPI-1*BB* and *GPI-1*AB,* but their proportion varied in samples from 3 river systems (Table-7).

4.1.1.1e Glucose-6-Phosphate Dehydrogenase (G_6PDH. 1.1.1.49)

Glucose-6-phosphate dehydrogenase is a dimeric and has been one of the most thoroughly studied allozymes (Fig. 6). It has been of particular interest to geneticists because; it is controlled by a gene located on the X-chromosome in man (Richardson *et al.*, 1986) and some other group of animals. G_6PDH is found in various tissues but the maximum activity is observed in liver (Richardson *et al.*, 1986).

In *H. brachysoma*, the pattern of G_6PDH did not exhibit sex-linked inheritance. Both male and female specimens from all three rivers exhibited both homozygotes (*AA* and *BB*) and heterozygotes (*AB*). A sex-wise breakup of G_6PDH genotypes is given in Table-8. During the present investigation, liver extracts showed the presence of a single polymorphic locus of G_6PDH. *G_6PDH** locus had two alleles *A* and *B* (Rf values *100* and *086*) and it showed three types of genotypes *viz.*, fast homozygotes (*G_6PDH* AA*), heterozygotes (*G_6PDH* AB*) and slow homozygotes (*G_6PDH* BB*).

Meenachil, Chalakkudy and Nethravathi: In Meenachil population, the allele *B* (*086*) was most common, in Chalakkudy population, the first allele, *A* (*100*) was most common. In Nethravathi population, *A* and *B* alleles were equally represented.

Table 8. Distribution of dimeric *G_6PDH* genotypes in male and female *H. brachysoma* from different river systems

Locus	Genotypes (Alleles & Rf value)	No. of individuals					
		Meenachil		Chalakkudy		Nethravathi	
		Male	Female	Male	Female	Male	Female
G_6PDH	*AA* (100/100)	01	08	20	25	17	13
	BB (086/086)	22	16	09	05	09	21
	AB (086/100)	11	12	07	04	04	06

4.1.1.1f Glycerol-3-Phosphate Dehydrogenase (αG_3PDH-1.1.1.8)

Glycerol-3-phosphate dehydrogenase or α-glycerophosphate dehydrogense was tested in all the stocks, a single locus was found to be responsible for the enzyme activity (Fig. 7). The occurrence of the three different genotypes (*G_3PDH*AA*,

*G_3PDH*AB* and *G_3PDH*BB)* and their band positions suggested that the locus had two alleles, *A* and *B*.

Meenachil, Chalakkudy and Nethravathi: In Meenachil population, *A* (*100*) and *B* (*088*) alleles were equally represented. In Chalakkudy, the second allele *B* was most common. In Nethravathi population, the first allele *A* was most common. No private alleles could be detected in any fish from any of the rivers.

4.1.1.1g. Lactate Dehydrogenase (LDH-1.1.1.27)

Lactate dehydrogenase is tetrameric in structure in vertebrates. The banding patterns of LDH enzyme system of *H. brachysoma* are shown in Fig. 8. The banding patterns showed two different zones (a fast moving zone and slow moving zone) of enzyme activity, which were presumed to be under the control of two independent loci. They were designated as *LDH-1** and *LDH-2** according to their order of increasing mobility differences. The first locus (slow moving) was feebly staining and monomorphic and this locus exhibited only one genotype *LDH-1*AA*. The second locus (fast moving) had three types of alleles *A, B* and *C*. The *LDH-2*AC* heterozygous individuals had 5 bands indicating the tetrameric pattern of the enzyme.

Meenachil and Nethravathi: In Meenachil and Nethravathi populations, the second locus, *LDH-2** (fast moving), had two types of alleles *A* and *B* (Rf values*100* and *112* respectively). The third allele, *C* was absent in this population. The two genotypes, *LDH-2*AA* and *LDH-2*BB,* were recorded but no heterozygous condition was exhibited in this population.

Chalakkudy: In this population, the second locus, *LDH-2** had three types of alleles *A, B* and *C* (Rf values *100*, *112* and *134* respectively). The third allele *C* was a private allele of this population. The three genotypes, *LDH-2*AA, LDH-2*BB* and 5 banded heterozygous *LDH-2*AC*, were detected in this population. However, *LDH-2*AB* and *LDH-2*CC* pattern could not be observed.

4.1.1.1h. Malate Dehydrogenase (MDH-1.1.1.37)

Malate dehydrogenase is a dimeric allozyme. During the present investigation, liver extracts showed the presence of a single locus of MDH. The *MDH** locus stained intensely exhibiting polymorphic pattern (fig. 10). *MDH** locus had two alleles *A* and *B* in *H. brachysoma* hence, it showed three types of genotypes *viz.*, fast homozygotes (*MDH* AA*), heterozygotes (*MDH* AB*) and slow homozygotes (MDH* *BB*).

Meenachil, Chalakkudy and Nethravathi: The *MDH** locus exhibited 2 alleles (*A* and *B*; Rf values *100* and *086*) in samples from all the 3 rivers and in all the three populations showed similar pattern of MDH enzyme, and no private alleles were identified.

4.1.1.1i. Octanol Dehydrogenase (ODH-1.1. 1.73)

Three loci were found to be responsible for this dimeric enzyme activity (fig. 13). These three loci were represented by slow *ODH-1**, medium *ODH-2** and fast *ODH-3**. The loci *ODH-1** and *ODH-3** were monomorphic. The locus *ODH-2** was polymorphic with two types of alleles *A* and *B* and exhibited three types of genotypes *viz, ODH-2*AA* and *ODH-2*BB* and *ODH-2*AB*.

Meenachil, Chalakkudy and Nethravathi: In all the three populations, polymorphic *ODH-2** showed similar pattern with 2 alleles. In Meenachil and Chalakkudy, the allele *A* (*100*) was most common in the second locus (*ODH-2**). In Nethravathi, the second allele *B* (*091*) was most common in this locus.

4.1.1.1j. Phosphoglucomutase (PGM-5.4.2.2)

Phosphoglucomutase is monomeric in vertebrates and one zone (locus) of enzyme activity was recorded in all the stocks of *H. brachysoma* (Fig. 11). Agar overlay was used in the staining protocol. The single locus was polymorphic in nature with two alleles, *A* and *B*. Three types of individuals were seen, two types of homozygotes (*PGM*AA* and *PGM*BB)* and a heterozygote (*PGM*AB*).

Meenachil, Chalakkudy and Nethravathi: The *PGM** locus exhibited 2 alleles (*A* and *B*; Rf values *100* and *093*) in samples from all the 3 rivers and the proportion of the genotypes was also similar.

4.1.1.1k. Superoxide Dismutase (SOD-1.15.1.1)

Superoxide dismutase enzyme is a cuprozoic protein with a molecular weight of 32,000 and is a dimer, composed of two identical sub- units (McCord and Fridovich, 1969). A single zone of enzyme activity is shown in fig. 12. The *SOD** locus had two types of alleles, *A* (fast) and *B* (slow) and was polymorphic. The genotypes of *SOD** were represented by two homozygotes, *SOD*AA* and *SOD*BB* and a 3 banded heterozygote *SOD*AB*.

Meenachil, Chalakkudy and Nethravathi: The *SOD** locus exhibited 2 alleles (*A* and *B*; Rf values *100* and *093*) in samples from all the 3 rivers. In all the three populations, SOD showed similar pattern in structure. In Meenachil and Chalakkudy, the allele *A* (*100*) was most common while in Nethravathi, the second allele *B* (*093*) was most common.

4.1.1.1l. Xanthine Dehydrogenase (XDH-1.1.1.204)

Fig. 14 shows banding pattern of the dimeric xanthine dehydrogenase in *H.brachysoma*. From the banding pattern, it was inferred that XDH is controlled by the two presumptive loci. They were designated as *XDH-1** and *XDH-2**. *XDH-1** was polymorphic in nature and showed intensively staining bands. This locus was represented by three types of alleles *A*, *B* and *C*. These three alleles exhibited four types of genotypes; three homozygotes and a heterozygote *viz, XDH-1*AA, XDH-1*BB, XDH-1*CC* and *XDH-1*AB*. The locus *XDH-2** had only one allele in all the populations, so this locus was treated as monomorphic.

Meenachil, Chalakkudy and Nethravathi: In Meenachil and Chalakkudy, *XDH-1** was represented by only two alleles *A* (*100*) and *B* (*091*). This locus exhibited three types of genotypes in these population, *XDH-1*AA, XDH-1*BB* and the heterozygote

*XDH-1*AB*. The third allele, *C* (*114*) was the private allele of Nethravathi population, hence the samples represented one more genotype, *XDH-1*CC*. In Meenachil and Nethravathi, the allele *A* (*100*) was most common. In Chalakkudy, the second allele *B* (*091*) was most common.

4.1.1.2. Monomorphic enzymes

4.1.1.2a Glyceraldehyde-3-phosphate dehydrogenase (GAPDH. 1.2.1.12)

Glyceraldehyde-3-phosphate dehydrogenase (monomeric) was tested in all the stocks (Meenachil, Chalakkudy and Nethravathi). Two loci were found to be responsible for the enzyme activity (Fig. 15) *GAPDH-1** and *GAPDH-2**. Both the loci were monomorphic in all the populations.

4.1.1.2b Malic Enzyme (MEP-1.1.1.40)

Malic enzyme is tetrameric in structure. MEP was tested in all the stocks (Meenachil, Chalakkudy and Nethravathi) and an intensely staining single locus was found to be responsible for the enzyme activity (fig. 16). This locus was monomorphic in all the populations.

Table 8. The distribution of allozyme genotypes and their Rf values in *H. brachysoma* from 3 rivers systems

Sl. No:	Enzymes	Locus	Genotypes (Alleles & Rf value)	No. of individuals		
				Mee-nachil	Chala-kkudy	Nethra-vathi
1	AAT	*AAT-1**	*AA (100/100)*	70	70	70
		*AAT-2**	*AA (100/100)*	49	27	23
			BB (117/117)	12	27	41
			AB (100/117)	09	16	0
			CC (126/126)	0	0	06
2	EST	*EST-1**	*AA (100/100)*	70	70	08
			BB (083/083)	0	0	57
			AB (083/100)	0	0	05
		*EST-2**	*AA (100/100)*	45	56	60
			BB (106/106)	14	07	02
			AB (100/106)	11	07	08
		*EST-3**	*AA (100/100)*	70	44	70
			AB (095/100)	0	26	0
		*EST-4**	*AA (100/100)*	70	70	70
		*EST-5**	*AA (100/100)*	70	70	70

Table 7 continued

3	GLDH	*GLDH**	*AA (100/100)*	24	02	18
			BB (089/089)	28	37	0
			AB (089/100)	18	27	0
			CC (080/080)	0	04	0
			DD (117/117)	0	0	32
			AD (100/117)	0	0	20
4	G_6PDH	*G_6PDH**	*AA (100/100)*	09	45	30
			BB (086/086)	38	14	30
			AB (086/100)	23	11	10
5	GPI	*GPI-1**	*AA (100/100)*	70	70	70
		*GPI-2**	*AA (100/100)*	56	46	54
			BB (096/096)	10	12	14
			AB (096/100)	04	12	02
6	αG_3PDH	*αG_3PDH**	*AA (100/100)*	21	19	45
			BB (088/088)	21	32	11
			AB (100/088)	28	19	14
7	GAPDH	*GAPDH –1**	*AA (100/100)*	70	70	70
		*GAPDH –2**	*AA (100/100)*	70	70	70
8	LDH	*LDH-1**	*AA (100/100)*	70	70	70
		*LDH-2**	*AA (100/100)*	58	43	66
			BB (112/112)	12	20	04
			AB (100/112)	0	0	0
			CC (134/134)	0	0	0
			AC (100/134)	0	07	0
9	MDH	*MDH**	*AA (100/100)*	39	37	52
			BB (086/086)	19	23	15
			AB (091/100)	12	10	03
10	ME	*ME**	*AA (100/100)*	70	70	70
11	ODH	*ODH-1**	*AA (100/100)*	70	70	70
		*ODH-2**	*AA (100/100)*	48	36	12
			BB (091/091)	06	18	52
			AB (091/100)	16	16	06
		*ODH-3**	*AA (100/100)*	70	70	70
12	PGM	*PGM**	*AA (100/100)*	38	46	39
			BB (093/093)	12	10	08
			AB (093/100)	20	14	23
13	SOD	*SOD**	*AA (100/100)*	48	26	09
			BB (093/093)	05	33	41
			AB (093/100)	17	11	20
14	XDH	*XDH-1**	*AA (100/100)*	36	24	28
			BB (091/091)	23	28	19
			AB (091/100)	11	18	14
			CC (114/114)	0	0	09
		*XDH-2**	*AA (100/100)*	70	70	70

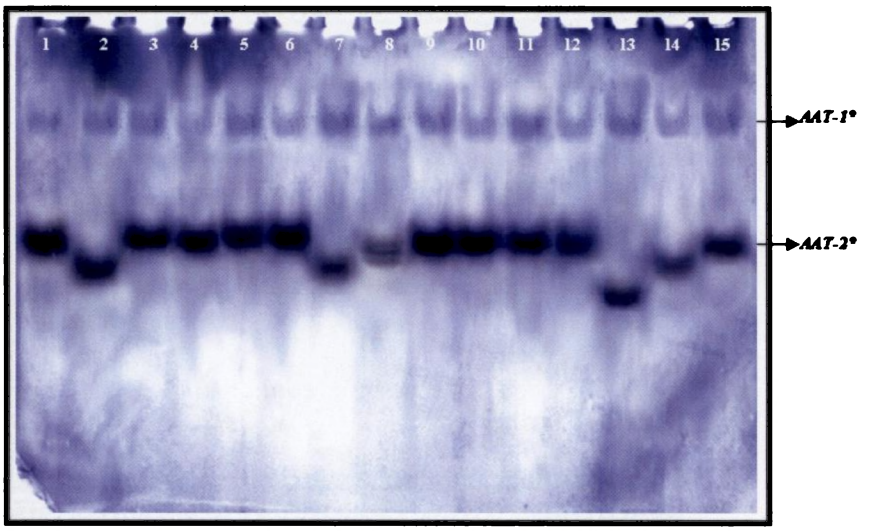

Fig. 3. Aspartate amino transferase (AAT) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

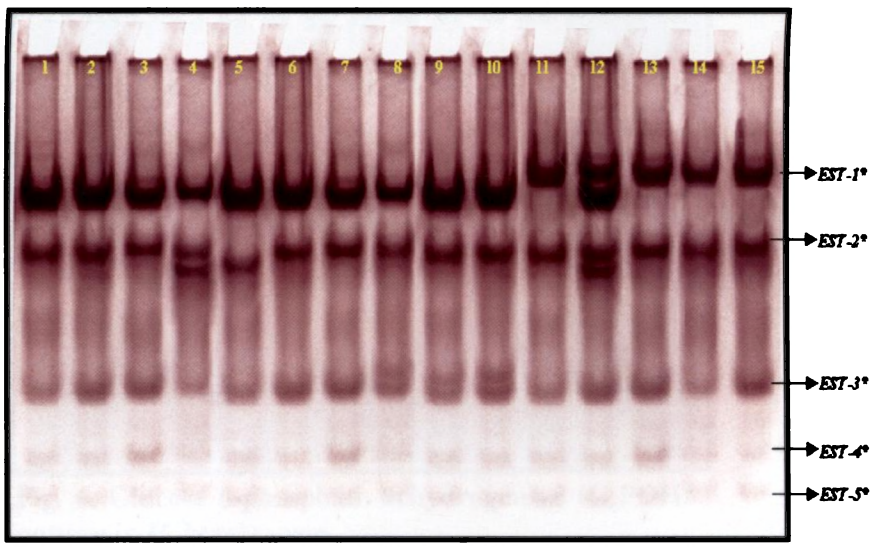

Fig. 4. Esterase (EST) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

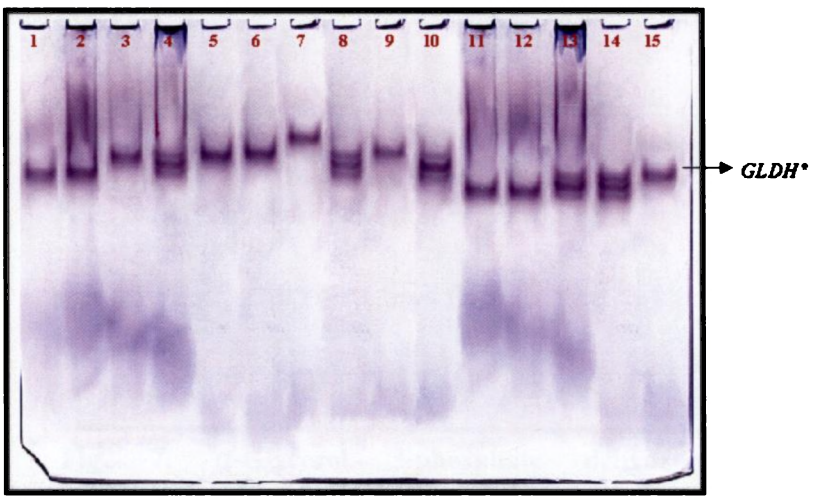

Fig. 5. Glucose dehydrogenase (GLDH) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

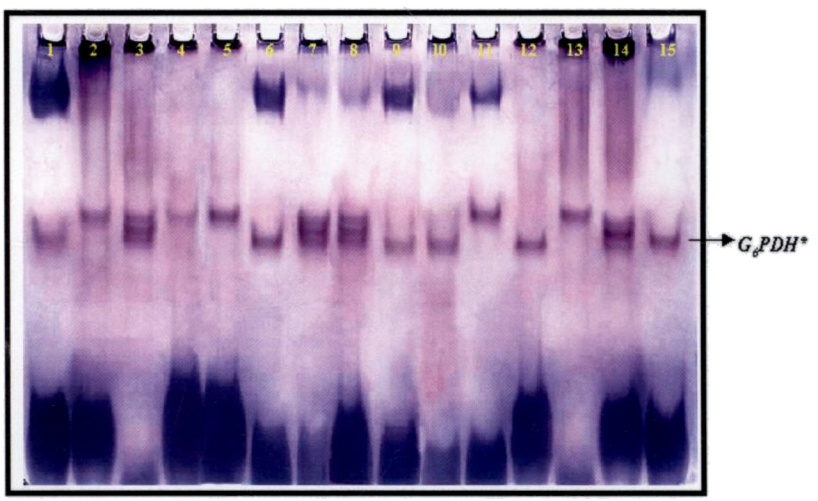

Fig. 6. Glucose 6-phosphate dehydrogenase (G_6PDH) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

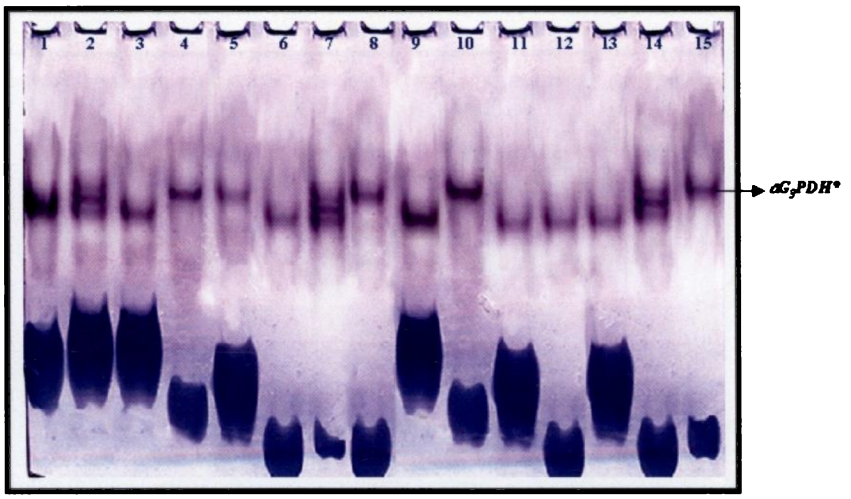

Fig. 7. α-Glycerol 3-phosphate dehydrogenase (αG_3PDH) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

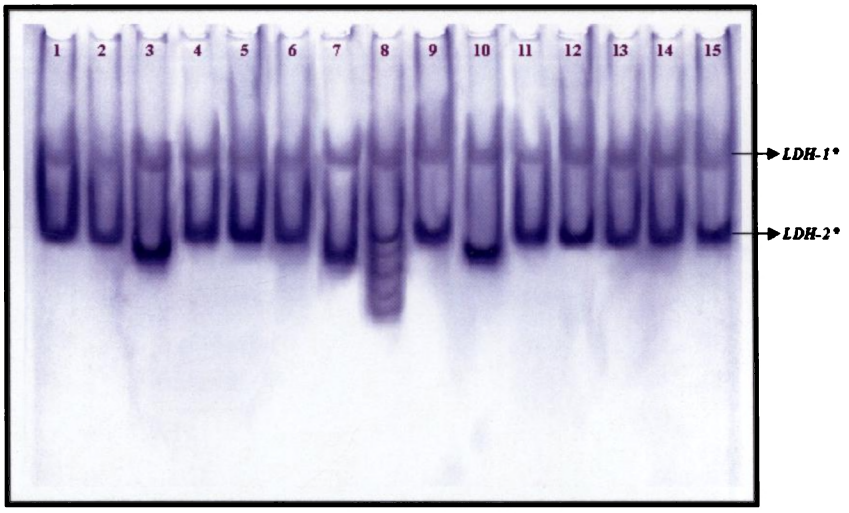

Fig. 8. Lactate dehydrogenase (LDH) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

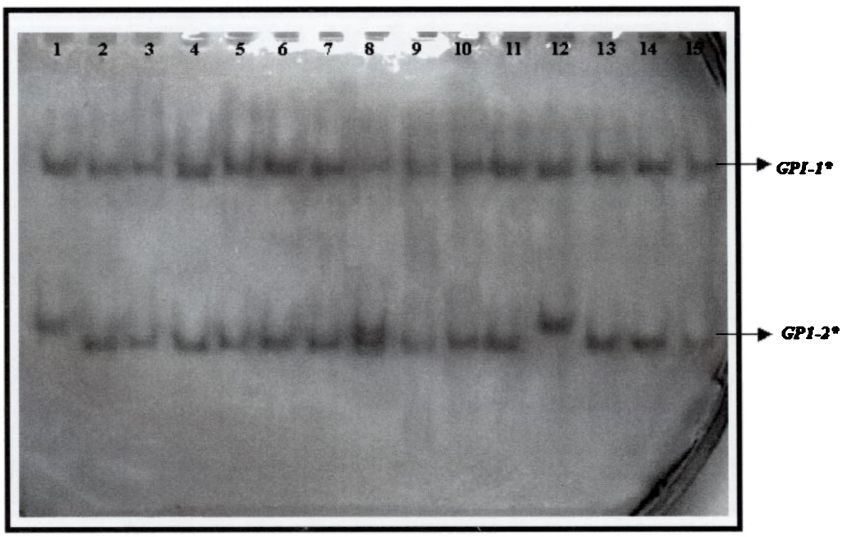

Fig. 9. Glucose phosphate isomerase (GPI) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

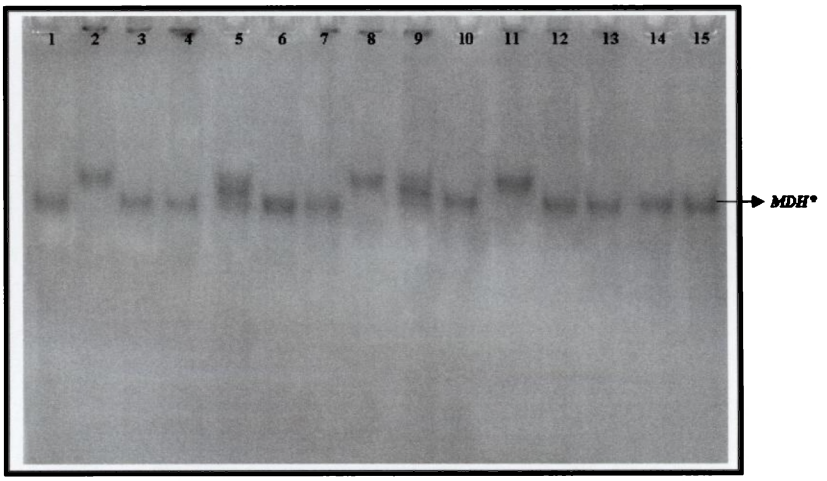

Fig.10. Malate dehydrogenase (MDH) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

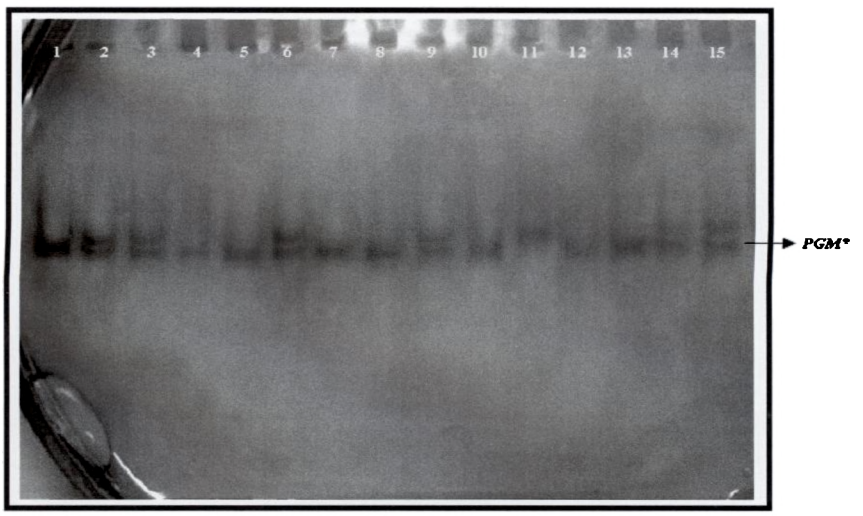

Fig. 11. Phosphoglucomutase (PGM) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

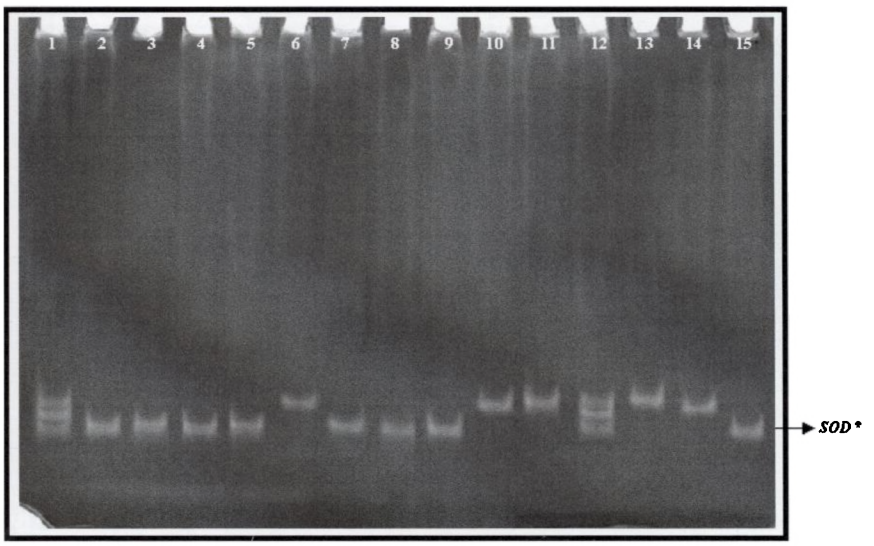

Fig. 12. Superoxide dismutase (SOD) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

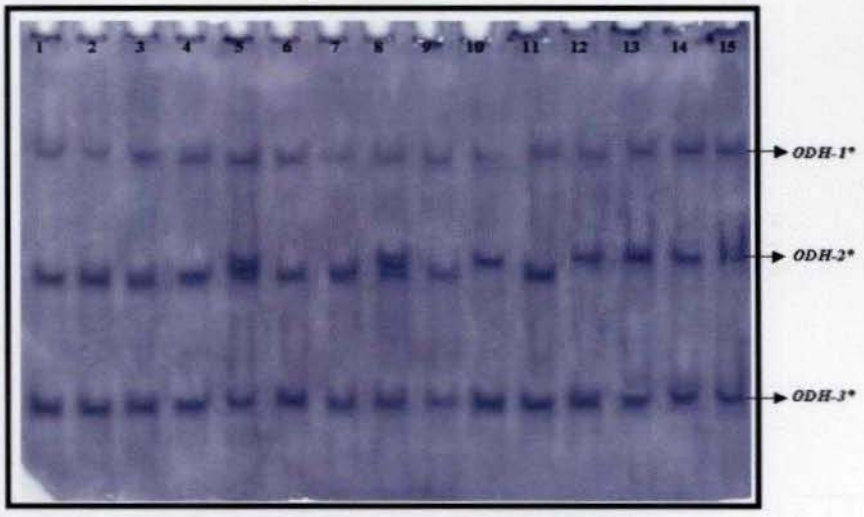

Fig. 13. Octanol dehydrogenase (ODH) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

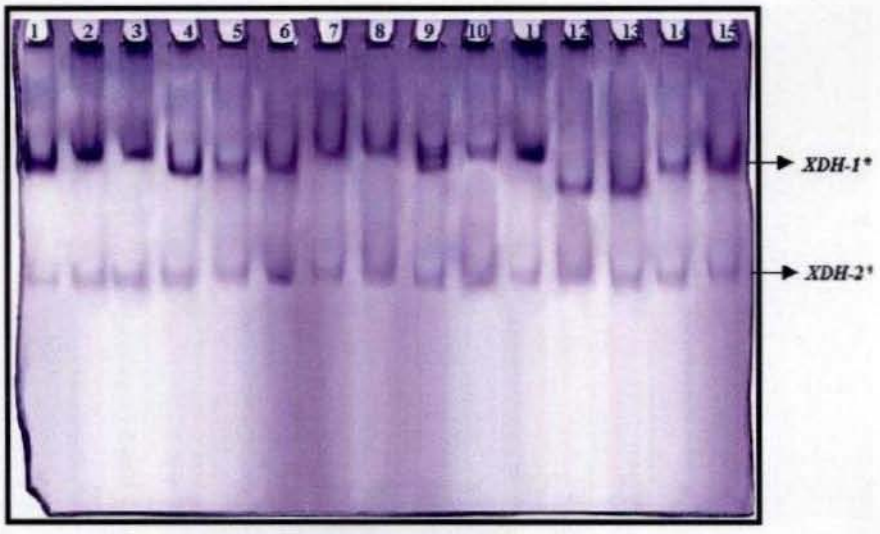

Fig. 14. Xanthine dehydrogenase (XDH) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

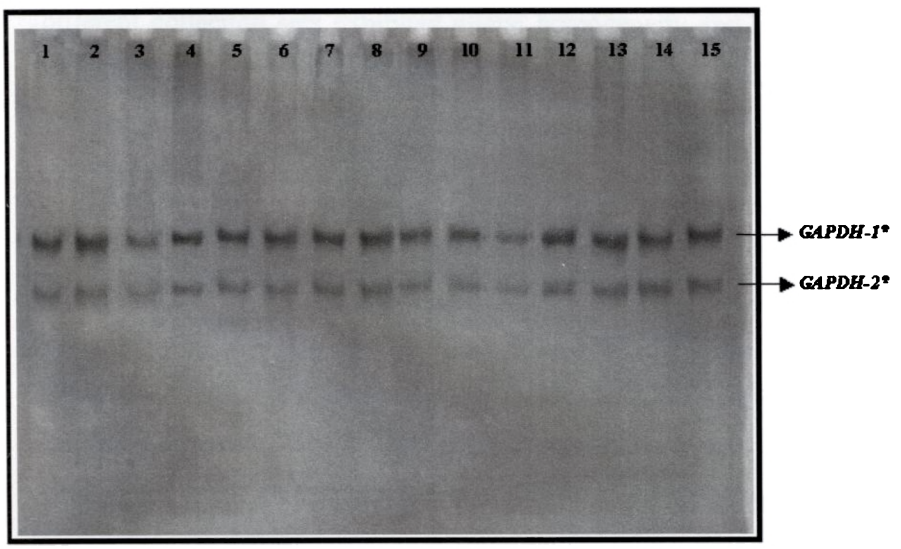

Fig. 15. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

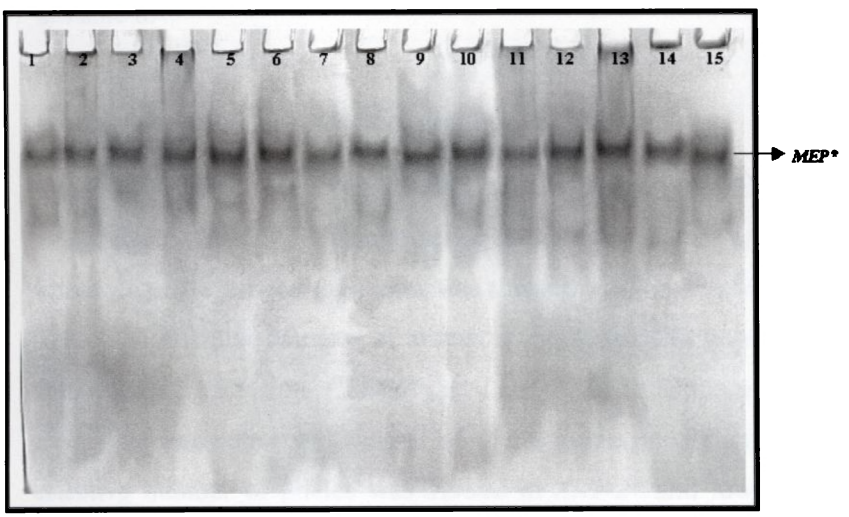

Fig. 16. Malic enzyme (MEP) pattern in *H. brachysoma*.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

4.1.2. Genetic Variability

The allele frequencies of multiple collections of the same river (three years-details in Table-1) were tested for significant homogeneity. The genotype data from different collection sets exhibited allelic homogeneity and hence they were pooled. This yielded three combined data sets *viz.*, Nethravathi, Chalakkudy and Meenachil; and they were used for analysis of parameters of genetic variation and population structure of *H. brachysoma.*

4.1.2.1. Number and percentage of polymorphic loci

Altogether, 14 polymorphic loci were obtained with 12 polymorphic allozymes across 3 populations (Table 9). However, in Meenachil population only 12 loci were variable. The two loci *EST-1**, and *EST-3** were monomorphic unlike in Chalakkudy and Nethravathi. The percentage of polymorphic loci was 48 in Meenachil. In Chalakkudy population, only 13 loci were polymorphic. Here too, *EST-1** was monomorphic. The percentage of polymorphic loci was 52. In samples from Nethravathi, 13 loci exhibited polymorphic pattern except *EST-3**. The percentage of polymorphic loci was 52. The overall percentage of polymorphic loci considering all the populations was 56. Polymorphic and monomorphic loci are given in Table 6.

4.1.2.2. Observed and effective number of alleles

The mean observed number of alleles (na) in Meenachil population was 1.857. The highest effective number of alleles (ne), (2) was exhibited by *G_3PDH** and lowest effective number of alleles was showed by *GPI-1** and *LDH-2** (1.3968 each). The mean effective number of alleles in the Meenachil population was 1.5967. In Chalakkudy population, *GLDH** and *LDH-2** had maximum number of alleles, *i.e.,* three. The mean observed number of alleles in this population was 2.071. The highest effective number of alleles was 1.9802 in *AAT-2** and lowest effective number of alleles was showed by *EST-2** (1.3423). The mean effective number of alleles in the Chalakkudy population was 1.7097. In Nethravathi population *AAT-2** and *XDH-1** had three alleles each and all other polymorphic loci had two alleles each except *EST-*

*3** that was monomorphic in this population. The mean observed number of alleles was 2.071. In Nethravathi population, the highest effective number of alleles was 2.0864 in *AAT-2** and lowest effective number of alleles was showed by *LDH-2** (1.1208). The mean effective number of alleles in the Nethravathi population was 1.6152 (Table 9).

The observed number of alleles ranged from 1 to 4 per locus in 25 loci of 14 allozymes among all the three populations studied. The highest numbers of alleles were found in glucose dehydrogenase (*GLDH**), which had four alleles. *AAT-2**, *EST-1**, *LDH-2**, *XDH-1** had three alleles each. Two alleles were present in the loci like *EST-1**, *EST-2**, *EST-3**, *G3PDH**, *G6PDH**, *GPI-2**, *MDH**, *ODH-2**, *PGM** and *SOD**. All the other loci *(AAT-1**, *EST-4**, *EST-5**, *GAPDH-1**, *GAPDH-2**, *GPI-1**, *LDH-1**, *MEP**, *ODH-1**, *ODH-3** and *XDH-2**) had one allele each. The mean observed number of alleles was 2.357. The observed number of alleles for each locus for each population is given in Table-9. The effective numbers of alleles (overall populations) ranged from 1.1314 (in *EST-3*)* to 2.8759 (in *GLDH*). The mean effective number of alleles in all the population was 1.8293 (Table-9).

Table 9. Observed (Na) and Effective (Ne) number of alleles for each population and overall populations

Locus	Meenachil		Chalakkudy		Nethravathi		Overall	
	na	ne	na	ne	na	ne	na	ne
*AAT-2**	2	1.5817	2	1.9802	3	2.0864	3	1.9868
*EST-1**	1	1.0000	1	1.0000	2	1.3604	2	1.6780
*EST-2**	2	1.6721	2	1.3423	2	1.1859	2	1.3968
*EST-3**	1	1.0000	2	1.4336	1	1.0000	2	1.1314
*G_3PDH**	2	2.0000	2	1.9333	2	1.6182	2	1.9802
*G_6PDH**	2	1.7070	2	1.6721	2	1.9963	2	1.9989
*GLDH**	2	1.9898	3	1.7459	2	1.9333	4	2.8759
*GPI-2**	2	1.3968	2	1.6182	2	1.5077	2	1.5077
*LDH-2**	2	1.3968	3	1.9033	2	1.1208	3	1.4517
*MDH**	2	1.8491	2	1.9231	2	1.5632	2	1.7948
*ODH-2**	2	1.6182	2	1.8760	2	1.5077	2	1.9935
*PGM**	2	1.7575	2	1.5817	2	1.6721	2	1.6721
*SOD**	2	1.4521	2	1.9518	2	1.6543	2	2.0000
*XDH-1**	2	1.9333	2	1.9742	3	2.4067	3	2.1430
Total	26	---	29	---	29	---	33	---
Mean	1.857	1.5967	2.071	1.7097	2.071	1.6152	2.357	1.8293
S.D.	0.363	0.3224	0.474	0.2915	0.475	0.3920	0.633	0.4202

4.1.2.3. Frequencies of alleles

The allelic frequencies of 14 polymorphic loci of 12 allozymes are given in Table 10. In Meenachil population, the allelic frequencies ranged from 0.1714 (in *GPI-2** and *LDH-2**) to 0.8286 (in *GPI-2** and *LDH-2**). The 2 monomorphic loci (in *EST-1** and *EST-3**) in this population gave a frequency of 1.000. In Chalakkudy population, the allelic frequencies ranged from 0.0500 (in *LDH-2**) to 0.8500 (in *EST-2**). *EST-1**, a monomorphic locus, gave a frequency of 1.000 in this population. In Nethravathi population, the allelic frequencies ranged from 0.0571 (in *LDH-2**) to 0.9429 (in *LDH-2**). *EST-3**, a monomorphic locus, gave a frequency of 1.000 in this population. The overall allele frequency value ranged from 0.0167 (in *LDH-2**) to 0.9381 (in *EST-3**).

Table 10. The allele frequencies of each locus, each population and overall populations (* private alleles)

Locus	Alleles	Meenachil	Chalakkudy	Nethravathi	Overall
*AAT-2**	*100*	0.7571	0.4500	0.6214	0.6095
	117	0.2429	0.5500	0.2929	0.3619
	126	------	----	0.0857*	0.0286
*EST-1**	*083*	----	------	0.8429*	0.2810
	100	1.0000	1.0000	0.1571	0.7190
*EST-2**	*100*	0.7214	0.8500	0.9143	0.8286
	106	0.2786	0.1500	0.0857	0.1714
*EST-3**	*095*	----	0.1857*	----	0.0619
	100	1.0000	0.8143	1.0000	0.9381
*G_3PDH**	*088*	0.5000	0.5929	0.2571	0.4500
	100	0.5000	0.4071	0.7429	0.5500
*G_6PDH**	*086*	0.7071	0.2786	0.4786	0.4881
	100	0.2929	0.7214	0.5214	0.5119
*GLDH**	*080*	----	0.0571*	-----	0.0190
	089	0.5357	0.7214	-----	0.4190
	100	0.4643	0.2214	0.4071	0.3643
	117	----	----	0.5929*	0.1976
*GPI-2**	*096*	0.1714	0.2571	0.2143	0.2143
	100	0.8286	0.7429	0.7857	0.7857
*LDH-2**	*100*	0.8286	0.6643	0.9429	0.8119
	112	0.1714	0.2857	0.0571	0.1714
	134	-----	0.0500*	----	0.0167
*MDH**	*086*	0.3571	0.4000	0.2357	0.3310
	100	0.6429	0.6000	0.7643	0.6690
*ODH-2**	*091*	0.2571	0.3714	0.7857	0.4714
	100	0.7429	0.6286	0.2143	0.5286
*PGM**	*093*	0.3143	0.2429	0.2786	0.2786
	100	0.6857	0.7571	0.7214	0.7214

SOD*	*093*	0.1929	0.5786	0.7286	0.5000
	100	0.8071	0.4214	0.2714	0.5000
XDH-1*	*091*	0.4071	0.5571	0.3714	0.4452
	100	0.5929	0.4429	0.5143	0.5167
	114	----	----	0.1143*	0.0381

4.1.2.4. Observed and expected heterozygosities

In Meenachil population, the range of observed heterozygosity was from 0.0571 (*GPI-2**) to 0.4 (*G_3PDH**). The mean observed heterozygosity was 0.1724 (±: 0.1278). The expected heterozygosity for this population ranged from 0.2861 (*GPI-2** and *LDH-2**) to 0.5036 (*G_3PDH**), with a mean of 0.3465 (± 0.1636) (Table-12). In Chalakkudy population, the mean of observed heterozygosity was 0.1908 (± 1055). It ranged from 0.1286 (*AAT-2**) to 0.3857 (*GLDH**). The expected heterozygosity for this population ranged from 0.2568 (*EST-2**) to 0.4986 (*AAT-2**), with a mean of 0.3969 (± 0.1371) (Table-12). In Nethravathi population, the observed heterozygosity ranged from 0.0286 (*GPI-2**) to 0.5827 (*AAT-2**). The mean observed heterozygosity was 0.1704 (± 0.1624). The expected heterozygosity for this population ranged from 0.1085 (*LDH-2**) to 0.5887 (*XDH-1**) with a mean of 0.3475 (± 0.1666) (Table-12).

The mean observed heterozygosity for overall populations was 0.1779 with a standard deviation of 0.0935. The observed heterozygosity ranged from 0.0286 (*EST-1**) to 0.3095 (*GLDH**). The expected heterozygosity for all populations ranged from 0.1164 (*EST-3**) to 0.6538 (*GLDH**) with a mean of 0.4276 (Table-12).

4.1.3. Stock-specific markers (private alleles)

Some alleles were present in a particular population only in *H. brachysoma*; these private alleles can be used as stock specific markers. In allozyme analysis, seven private alleles were recorded, one each in *AAT-2**, *EST-1**, *EST-3**, *LDH-2** and *XDH-1** and two alleles in *GLDH**. Out these seven private alleles, Chalakkudy population represented 3 and the rest represented by Nethravathi population. No private allele was obtained in Meenachil population. The private alleles and their frequencies are given in Table-11.

Table 11. Private alleles (Rf value) and their frequencies

Locus	Private Alleles (Rf value)	Frequency		
		Meenachil	Chalakkudy	Nethravathi
*AAT-2**	*126*	----	----	0.0857
*EST-1**	*083*	----	----	0.8429
*EST-3**	*095*	----	0.1857	----
*GLDH**	*080*	----	0.0571	----
	117	----	----	0.5929
*LDH-2**	*134*	----	0.0500	----
*XDH-1**	*114*	----	----	0.1143

4.1.4. Hardy-Weinberg expectations

The probability test provided the evidence that the observed allele frequencies in most of the loci significantly deviated ($P<0.05$) from that expected under Hardy-Weinberg equilibrium in all the three populations except in *G_3PDH**, *G_6PDH** and *SOD** in Meenachil population, *EST-3** in Chalakkudy population and *EST-2** and *PGM** in Nethravathi population, after the sequential bonferroni correction was made to the probability levels (Table-12). Wright's fixation index (F_{IS}) is a measure of heterozygote deficiency or excess and their significant values for each locus in each population and overall population are given in Table-12 and 13. The F_{IS} values for each locus ranged from –0.2281 for *EST-3** to 0.8846 for *LDH-2** and with an average of 0.5072. In most of the loci, the value of F_{IS} significantly deviated from zero, indicating deficiency of heterozygotes, except in *EST-3**.

4.1.5. Linkage disequilibrium

There was no significant association indicative of linkage disequilibrium between any pair-wise combinations of alleles across loci at any populations level ($P>0.05$; 165 pair-wise comparisons, comprising 55 pair-wise comparisons for 3 populations). It was therefore assumed that allelic variation at allozyme loci could be considered independent.

4.1.6. Genetic differentiation and gene flow

The co-efficient of genetic differentiation (F_{ST}) estimated through the estimator of Weir and Cockerham (1984) ranged from 0.0073 for *GPI-2** to 0.7815 for *EST-1**, with a mean of 0.1537, indicating that 15.37% of the total genetic variation exists among populations (Table-13). The estimate of pair-wise F_{ST} differed significantly ($P < 0.0001$) from zero for all pairs of riverine locations (Table-15). The loci exhibiting significant heterogeneity in genotype proportions between different population pairs and for overall population are depicted in Table-14a and b. Out of the possible 42 tests, 27 pairs had significant genotypic heterogeneity at least at one locus. The value of Nm (the average number of migrants per generation) derived from F_{ST} based on the overall estimate of gene flow between populations was 1.3760 in the study (Table-13).

Table 12. Summary of genetic variation and heterozygosity statistics of the fourteen allozyme loci in *H. brachysoma*

Locus	Populations (n=70 each)		
	Meenachil	Chalakkudy	Nethravathi
AAT-2*			
H obs.	0.1143	0.1286	0.5857
H exp	0.3704	0.4986	0.5245
F_{IS}	0.693	0.743	-0.118
P_{HW}	0.0000**	0.0000**	0.0000**
Pscore	0.0000**	0.0000**	0.0026*
EST-1*			
H obs.	0.0000	0.0000	0.0857
H exp	0.0000	0.0000	0.2668
F_{IS}	--	--	0.680
P_{HW}	--	--	0.0000**
Pscore	--	--	0.0000**
EST-2*			
H obs	0.1571	0.1000	0.1143
H exp	0.4048	0.2568	0.1579
F_{IS}	0.614	0.612	0.277
P_{HW}	0.0000**	0.0000**	0.0676
Pscore	0.0000**	0.0000**	0.0676
EST-3*			
H obs	0.0000	0.3714	0.0000
H exp	0.0000	0.3046	0.0000
F_{IS}	--	-0.221	--
P_{HW}	--	0.1052	--
Pscore	--	0.7451	--

Table 12 continued			
G_3PDH*			
H obs.	0.4000	0.2714	0.2000
H exp	0.5036	0.4862	0.3848
F_{IS}	0.207	0.444	0.482
P_{HW}	0.0977	0.0004*	0.0001**
Pscore	0.0665	0.0002*	0.0001**
G_6PDH*			
H obs	0.3286	0.1571	0.1286
H exp	0.4172	0.4048	0.5027
F_{IS}	0.214	0.614	0.746
P_{HW}	0.0872	0.0000**	0.0000**
Pscore	0.0673	0.0000**	0.0000**
GLDH*			
H obs	0.2429	0.3857	0.3000
H exp	0.5010	0.4303	0.4862
F_{IS}	0.517	0.104	0.385
P_{HW}	0.0000**	0.0000**	0.0025*
Pscore	0.0000**	0.0000**	0.0013*
GPI-2*			
H obs	0.0571	0.1714	0.0286
H exp	0.2861	0.3848	0.3392
F_{IS}	0.801	0.556	0.916
P_{HW}	0.0000**	0.0000**	0.0000**
Pscore	0.0000**	0.0000**	0.0000**
LDH-2*			
H obs	0.0000	0.1000	0.0000
H exp	0.2861	0.4780	0.1085
F_{IS}	1	0.792	1
P_{HW}	0.0000	0.0000	0.0000
Pscore	0.0000**	0.0000**	0.0000**
MDH*			
H obs	0.1714	0.1429	0.0429
H exp	0.4625	0.4835	0.3629
F_{IS}	0.631	0.706	0.883
P_{HW}	0.0000**	0.0000**	0.0000**
Pscore	0.0000**	0.0000**	0.0000**
ODH-2*			
H obs	0.2571	0.2286	0.0857
H exp	0.3848	0.4703	0.3392
F_{IS}	0.333	0.516	0.749
P_{HW}	0.0098*	0.0000**	0.0000**
Pscore	0.0076*	0.0000**	0.0000**
PGM*			
H obs.	0.2857	0.2000	0.3286
H exp.	0.4341	0.3704	0.4048
F_{IS}	0.343	0.462	0.189
P_{HW}	0.0056*	0.0003*	0.1384
Pscore	0.0049*	0.0003*	0.0986
SOD*			
H obs	0.2429	0.1571	0.2857
H exp	0.3136	0.4912	0.3984
F_{IS}	0.227	0.682	0.284
P_{HW}	0.1120	0.0000**	0.0305*
Pscore	0.0679	0.0000**	0.0042*

Table 12 continued			
*XDH- 1**			
H obs	0.1571	0.2571	0.2000
H exp	0.4862	0.4970	0.5887
F_{IS}	0.678	0.484	0.662
P_{HW}	0.0000**	0.0001**	0.0000**
Pscore	0.0000**	0.0000**	0.0000**
Mean overall loci			
H obs	0.1724	0.1908	0.1704
H exp	0.3465	0.3969	0.3475
$P_{(0.95)}$	0.48	0.52	0.52
$P_{(0.99)}$	0.48	0.52	0.52
A_n	1.857	2.071	2.071

H obs. = Observed heterozygosity
H exp. = Expected heterozygosity
Fis = Inbreeding coefficient
P_{HW} = Probability value of significant deviation from H W E
Pscore = Probability value of significant heterozygosity deficiency
$P_{(0.95)}$ = Polymorphism at 0.95 criteria
$P_{(0.99)}$ = Polymorphism at 0.99 criteria
A_n = Mean number of alleles per locus

Table 13. F-statistics and gene flow (Nm) for overall populations (Allozyme markers) in *H. brachysoma*

Locus	Sample Size	F_{IS}	F_{ST}	Nm
AAT-2*	210	0.4011	0.0715	3.2446
EST-1*	210	0.6764	0.7815	0.0699
EST-2*	210	0.5435	0.0453	5.2738
EST-3*	210	-0.2281	0.1320	1.6442
G_3PDH*	210	0.3615	0.0809	2.8385
G_6PDH*	210	0.5329	0.1227	1.7875
GLDH*	210	0.3402	0.2808	0.6405
GPI-2*	210	0.7436	0.0073	34.1250
LDH-2*	210	0.8846	0.0718	3.2326
MDH*	210	0.7252	0.0219	11.1841
ODH-2*	210	0.5181	0.2069	0.9581
PGM*	210	0.3218	0.0042	58.8350
SOD*	210	0.4259	0.2037	0.9775
XDH-1*	210	0.6064	0.0246	9.9061
Mean	**210**	**0.5072**	**0.1537**	**1.3760**

Table 14a. Test for genetic homogeneity- Probability tests for pairwise populations for each locus (*P<0.05, **P<0.0001; significant after Bonferroni adjustments for multiple tests). (Mn: Meenachil; Ch: Chalakkudy; Ne: Nethravathi)

Locus	Populations	P-value	S.E.
*AAT-2**	Mn & Ch	0.0000**	0.0000
*AAT-2**	Ch & Ne	0.0000**	0.0000
*AAT-2**	Mn & Ne	0.0057*	0.0003
*EST-1**	Mn & Ch	Only one genotype	
*EST-1**	Ch & Ne	0.0000**	0.0000
*EST-1**	Mn & Ne	0.0000**	0.0000
*EST-2**	Mn & Ch	0.0497*	0.0012
*EST-2**	Ch & Ne	0.2279	0.0017
*EST-2**	Mn & Ne	0.0009*	0.0001
*EST-3**	Mn & Ch	0.0000**	0.0000
*EST-3**	Ch & Ne	0.0000**	0.0000
*EST-3**	Mn & Ne	Only one genotype	
*G_3PDH**	Mn & Ch	0.2139	0.0029
*G_3PDH**	Ch & Ne	0.0000**	0.0000
*G_3PDH**	Mn & Ne	0.0005*	0.0001
*G_6PDH**	Mn & Ch	0.0000**	0.0000
*G_6PDH**	Ch & Ne	0.0102*	0.0007
*G_6PDH**	Mn & Ne	0.0012*	0.0003
*GLDH**	Mn & Ch	0.0000**	0.0000
*GLDH**	Ch & Ne	0.0000**	0.0000
*GLDH**	Mn & Ne	0.0000**	0.0000
*GPI-2**	Mn & Ch	0.2166	0.0027
*GPI-2**	Ch & Ne	0.5923	0.0027
*GPI-2**	Mn & Ne	0.5826	0.0026
*LDH-2**	Mn & Ch	0.0101*	0.0006
*LDH-2**	Ch & Ne	0.0000**	0.0000
*LDH-2**	Mn & Ne	0.0596	0.0009
*MDH**	Mn & Ch	0.6340	0.0028
*MDH**	Ch & Ne	0.0361*	0.0014
*MDH**	Mn & Ne	0.1137	0.0019
*ODH-2**	Mn & Ch	0.1116	0.0020
*ODH-2**	Ch & Ne	0.0000**	0.0000
*ODH-2**	Mn & Ne	0.0000**	0.0000
*PGM**	Mn & Ch	0.3145	0.0024
*PGM**	Ch & Ne	0.6365	0.0021
*PGM**	Mn & Ne	0.6404	0.0021
*SOD**	Mn & Ch	0.0000**	0.0000
*SOD**	Ch & Ne	0.0398*	0.0011
*SOD**	Mn & Ne	0.0000**	0.0000
*XDH-1**	Mn & Ch	0.0566	0.0016
*XDH-1**	Ch & Ne	0.0007*	0.0001
*XDH-1**	Mn & Ne	0.0065*	0.0009

Markov chain parameters: - dememorization: 1000, batches: 100 and iterations: 1000

Table 14b. Test for genetic homogeneity- Probability tests for overall populations (*P<0.05, **P<0.0001; significant after Bonferroni adjustments for multiple tests). (Mn: Meenachil; Ch: Chalakkudy; Ne: Nethravathi)

Locus	P-value	S.E.
*AAT-2**	0.0000	0.0000
*EST-1**	0.0000	0.0000
*EST-2**	0.0024	0.0006
*EST-3**	0.0000	0.0000
*G_3PDH**	0.0000	0.0000
*G_6PDH**	0.0000	0.0000
*GLDH**	0.0000	0.0000
*GPI-2**	0.4419	0.0095
*LDH-2**	0.0000	0.0000
*MDH**	0.0679	0.0052
*ODH-2**	0.0000	0.0000
*PGM**	0.5332	0.0085
*SOD**	0.0000	0.0000
*XDH-1**	0.0000	0.0000
Overall	0.0000	-----

Markov chain parameters:- dememorization: 1000, batches: 100 and iterations: 1000

Table 15. Fisher's pair wise F_{ST} (above diagonal) and their significant levels (below diagonal) for *Horabagrus brachysoma* -allozyme markers.

Populations	Meenachil	Chalakkudy	Nethravathi
Meenachil	----	0.0952	0.2640
Chalakkudy	P< 0.0001	----	0.2418
Nethravathi	P< 0.0001	P< 0.0001	----

4.1.7. Genetic relationship between populations

Nei's (1978) unbiased genetic identity and distance estimated between pairs of three populations of *H. brachysoma* are presented in Table-16. Genetic distance values between the populations of Meenachil and Chalakkudy was 0.0299; between Chalakkudy and Nethravathi was 0.0899; and Meenachil and Nethravathi was 0.0927. According to these results, the Meenachil and Chalakkudy populations are closely related when compared to Nethravathi population. The genetic distance values agree with the geographic distances between the populations (Table-16).

Table 16. Genetic identity (above diagonal) and Genetic distance (below diagonal) (Nei, 1978) - allozyme markers, Geographical distances (in KM) are given in bracket

Populations	Meenachil	Chalakkudy	Nethravathi
Meenachil	****	0.9705	0.9115
Chalakkudy	0.0299 (140)	****	0.9140
Nethravathi	0.0927 (520)	0.0899 (380)	****

4.1.8. Dendrogram

On the basis of Nei's (1978) genetic distance values the phylogenetic relationships between three populations of *Horabagrus brachysoma* were made through a dendrogram (Fig.17) following unweighted pair group method using arithmetic averages (UPGMA, Sneath and Sokal, 1973). The bootstrap values suggested the populations have robust clusters.

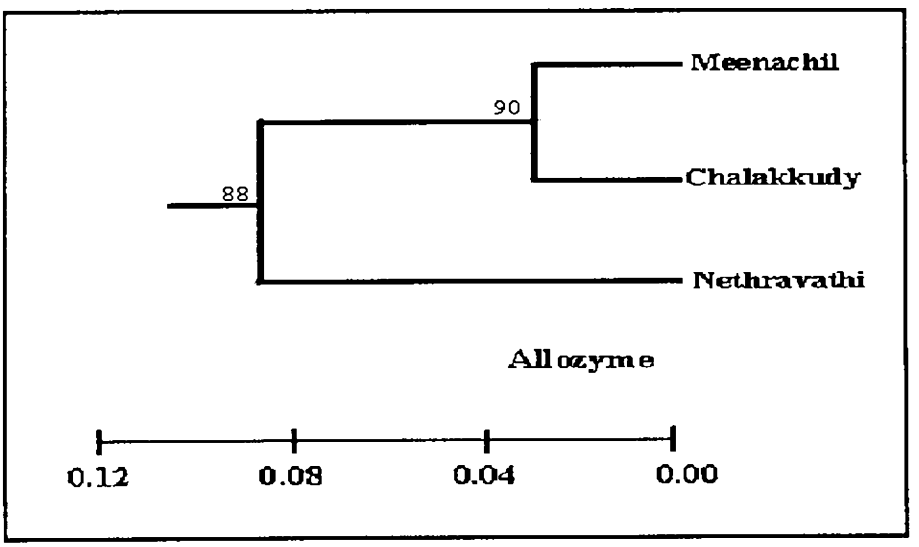

Fig. 17. UPGMA dendrogram for allozyme analysis in *Horabagrus brachysoma* from three locations. Values at nodes represent bootstrap confidence levels (1000 replications).

4.2. RAPD analysis

4.2.1. Isolation of DNA

The DNA was isolated from each sample collected from three locations by the method mentioned in section 3.4.1. The extracted DNA was electrophoresed through 0.7% agarose gel containing ethidium bromide (Fig. 18).

4.2.2. Quantification and Purity of DNA

The quantity and quality of the extracted DNA was checked by taking the optical density (OD) using spectrophotometer at wavelength 260nm and 280nm. Most of the extracted DNA had very high concentration; therefore, the samples were diluted with sterile double distilled water to get appropriate concentration for PCR reactions. The OD ratio (260nm to 280nm) of the each sample was in between 1.7 and 1.9. Therefore, the samples were in pure condition without contamination of protein and RNA.

4.2.3. Selection of primers

Thirty two oligonucleotide primers were selected from 80 primers (4 kits- 20 primers each from kit OPA, OPAA, OPAC & OPAH) on primary screening, however, only 10 primers were selected *viz,* OPA-07, OPA-09, OPA-11, OPA-20, OPAC-14, OPAH-01, OPAH-02, OPAH-04, OPAH-08 and OPAH-09 for population genetic analysis (Table-3).

4.2.4. Reproducibility of RAPD pattern

Reproducibility of the RAPD pattern was also tested in the present investigation at various stages of process, leading to consistent banding pattern with all amplified primers. The amplification results were routinely repeatable even after the DNA was stored at -20^{0}C for more than 6 months, demonstrating the robustness of the technique.

4.2.5. Genetic variability

4.2.5.1. Number of amplified fragments

A total of 124 different randomly amplified DNA fragments from specimens of *H. brachysoma* were detected consistently with all 10 decamer primers in three

populations. The size of the fragments ranged from 150 to 3000 bp. The number of fragments generated per primer varied from 6 to 19. The number of fragments amplified and the size of each fragment for each primer is given in Table-17 and Table-18.

With OPA-07, 9 RAPD fragments were expressed. Out of the 9, six fragments were polymorphic (66.67%) among all the populations (Fig. 20). Nine RAPD fragments were obtained in OPA-09, and out of these, six fragments were polymorphic (66.67%) (Fig. 21). OPA-11 amplified 15 RAPD fragments and five of these were polymorphic (33.33%) (Fig. 22). OPA-20 showed the highest rate of polymorphism (91.67%) with a total 12 RAPD fragments, eleven of which were polymorphic (Fig. 23). This primer showed among all others. With OPAC-14, the maximum numbers of fragments were expressed, *i.e.,* 19 (Fig. 24) and nine fragments were polymorphic (47.37%) among the populations. OPAH-01 primer showed 85.71% polymorphism (Fig. 25). Fourteen RAPD fragments were amplified in OPAH-01. Out of these, 12 fragments were polymorphic. OPAH-02 amplified 13 RAPD fragments and ten were polymorphic (76.92%) (Fig. 26). A total 14 RAPD fragments were expressed with OPAH-04 (Fig. 27) and eight fragments were polymorphic (57.14%). With OPAH-08, 9 RAPD fragments were obtained and out of these, only three fragments were polymorphic (33.33%) (Fig. 28). With OPAH-09, a total 10 RAPD fragments were amplified and out of these, five fragments were polymorphic (50.0%) (Fig. 29). Of the 124 total RAPD fragments amplified, 49 (39.51%) were found to be shared by individuals of all three populations. The remaining 75 fragments were found to be polymorphic (60.48%) (Table-19). OPAH-08 and OPA-11 produced the least polymorphic fragments among the primers used.

Meenachil: A total of 112 amplified DNA fragments were detected consistently with all the 10 decamer primers in Meenachil population. The size of the fragments ranged from 150 to 3000 bp. In this population, 42 (37.5%) fragments were polymorphic. The remaining 70 bands were monomorphic (62.5%). The percentage of polymorphic bands ranged from 8.33% (OPA-11) to 80.0% (OPA-20). The numbers of fragments and polymorphic bands obtained with each primer are given in Table-19.

In this population, a total of 12 bands were absent in different primers compared to other populations. In OPA-07, one fragment (approximately 3000 bp); in OPA-09, three fragments (680, 650, 550 bp); in OPA-11, three fragments (1060, 890, 560 bp); in OPA-20, two fragments (2000, 950 bp); in OPAH-01, one fragment (500 bp); in OPAH-02, one fragment (1010 bp); and in OPAH-04, one fragment (1150 bp) was not amplified (Table-19).

In primer OPAH-09, the third and highly prominent fragment of 980 bp was amplified only in this population, so this fragment could be considered as stock-specific marker for this population (Table-22).

Chalakkudy: A total of 109 amplified DNA fragments were detected consistently with all the 10 decamer primers in Chalakkudy population. The size of the fragments ranged from 150 to 3000 bp. Out of the 109, forty-two (38.53%) fragments were polymorphic in this population. The remaining 67 bands were monomorphic (61.47%). The percentage of polymorphic bands ranged for each primer from 0% (OPAH-09) to 85.71% (OPAH-01). The numbers of fragments and polymorphic bands recorded with each primer are given in Table-19.

In this population, a total of 15 bands were missing in different primers compared to the other 2 populations (Meenachil and Nethravathi). In OPA-09, three fragments (680, 650 and 450 bp); in OPA-11, three fragments (1300,1060 and 890 bp); in OPA-20, three fragments (2000, 950 and 830 bp); in OPAH-01, one fragment (1010 bp); in OPAH-02, two fragments (1210 and 530 bp); in OPAH-04, one fragment (600 bp) and in OPAH-09 one fragment (980 bp) were not detected in this population (Table-19).

In primer OPAH-02, the eighth fragment of 1010 bp was specific to this population, so this fragment could be considered as stock specific marker for this population (Table-22).

Nethravathi: A total of 110 amplified DNA fragments were detected consistently with all the 10 decamer primers in Nethravathi population. The size of the fragments ranged from 150 to 3000 bp. In this population, 42 (41.81%) fragments

were polymorphic. The remaining 68 bands were monomorphic (61.81%). The percentage of polymorphic bands ranged from 0% (OPAH-09) to 85.71% (OPAH-01). The number of fragments amplified and polymorphic bands in each primer are given in Table-19.

A total of 14 bands were not recorded with different primers in this population compared to the yellow catfish stocks of Meenachil and Chalakkudy. In OPA-07, two fragments (approximately 420 and 250 bp); in OPA-09, one fragment (500 bp); in OPA-11, one fragment (1060 bp); in OPAC-14, two fragments (1150 and 820 bp); in OPAH-02, one fragment (1010bp); in OPAH-04, one fragment (1150 bp); in OPAH-08, two fragments (3000 and 1260 bp); and in OPAH-09, four fragments (1160, 980, 700 and 550 bp) were not detected (Table-19), compared to other populations.

In primer OPA-09, a prominent fragment (680 bp) and a less prominent (fifth) fragment (650 bp); in OPA-11, sixth, a prominent fragment (1060 bp) and ninth fragment (890 bp); in OPA-20, the first (a prominent) fragment (2000 bp) and sixth fragment (950 bp) were amplified in this population only, so these six fragments could be considered as stock-specific markers of Nethravathi population. (Table-22).

Table 17. Number of RAPD fragments and their size for each Operon primer

Sl. No.	Primer	Fragment No.	Approximate size of Fragment (bp)	Sl. No.	Primer	Fragment No.	Approximate size of Fragment (bp)
1	OPA-07	1	3000	19	OPA-11	1	2400
2		2	990	20		2	1590
3		3	830	21		3	1380
4		4	760	22		4	1300
5		5	690	23		5	1160
6		6	550	24		6	1060
7		7	420	25		7	1000
8		8	250	26		8	740
9		9	150	27		9	690
10	OPA-09	1	1000	28		10	640
11		2	830	29		11	560
12		3	790	30		12	500
13		4	680	31		13	380
14		5	650	32		14	340
15		6	550	33		15	170
16		7	500				
17		8	450				
18		9	300				

Table 17 continued

34	OPA-20	1	2000
35		2	1660
36		3	1580
37		4	1210
38		5	1100
39		6	950
40		7	890
41		8	830
42		9	760
43		10	690
44		11	480
45		12	360
46	OPAC-14	1	2100
47		2	1900
48		3	1580
49		4	1360
50		5	1150
51		6	1000
52		7	940
53		8	900
54		9	860
55		10	820
56		11	750
57		12	700
58		13	660
59		14	560
60		15	530
61		16	500
62		17	470
63		18	430
64		19	400
65	OPAH-01	1	1700
66		2	1580
67		3	1450
68		4	1300
69		5	1160
70		6	1050
71		7	870
72		8	770
73		9	750
74		10	540
75		11	500
76		12	450
77		13	330
78		14	300
79	OPAH-02	1	2500
80		2	2000
81		3	1700
82		4	1580
83		5	1400
84		6	1350
85		7	1210
86		8	1010
87		9	890
88		10	760
89		11	600
90		12	530
91		13	450
91		13	450
92		1	3000
93	OPAH-04	2	2500
94		3	1900
95		4	1500
96		5	1400
97		6	1150
98		7	1010
99		8	900
100		9	830
101		10	780
102		11	730
103		12	630
104		13	600
105		14	560
106	OPAH-08	1	3000
107		2	1900
108		3	1500
109		4	1260
110		5	1080
111		6	950
112		7	830
113		8	680
114		9	590
115	OPAH-09	1	1500
116		2	1160
117		3	980
118		4	830
119		5	700
120		6	670
121		7	600
122		8	550
123		9	510
124		10	400

Table 18. Number of RAPD fragments and their range of size for each Operon primer

Sl. No.	Primer Code	Sequence	No. of fragments	Range of size (bp)
1	OPA-07	GAAACGGGTG	7-9	150-3000
2	OPA-09	GGGTAACGCC	6-9	300-1000
3	OPA-11	CAATCGCCGT	12-15	170-2400
4	OPA-20	GTTGCGATCC	9-12	360-2000
5	OPAC-14	GTCGGTTGTC	17-19	400-2100
6	OPAH-01	TCCGCAACCA	13-14	300-1700
7	OPAH-02	CACTTCCGCT	11-13	450-2500
8	OPAH-04	CTCCCCAGAC	13-14	560-3000
9	OPAH-08	TTCCCGTGCC	8-9	590-3000
10	OPAH-09	AGAACCGAGG	6-10	400-1500

Table 19. The total number of RAPD fragments and % of polymorphic bands for each and overall populations

Primer Code	Meenachil		Chalakkudy		Nethravathi		Overall populations	
	Total no. of bands	No. of polymorp hic bands (p%)	Total no. of bands	No. of polymorp hic bands (p%)	Total no. of bands	No. of polymorp hic bands (p%)	Total no. of bands	No. of polymorp hic bands (p%)
OPA-07	8	2 (25.0)	9	5 (55.56)	7	4 (57.14)	9	6 (66.67)
OPA-09	6	1 (16.67)	6	1 (16.67)	8	4 (50.0)	9	6 (66.67)
OPA-11	12	1 (8.33)	12	1 (8.33)	14	2 (14.29)	15	5 (33.33)
OPA-20	10	8 (80.0)	9	3 (33.33)	12	5 (41.67)	12	11 (91.67)
OPAC-14	19	3 (15.79)	17	3 (17.65)	17	1 (5.88)	19	9 (47.37)
OPAH-01	13	9 (69.23)	14	12 (85.71)	14	12 (85.71)	14	12 (85.71)
OPAH-02	12	8 (66.67)	11	8 (72.73)	12	8 (66.67)	13	10 (76.92)
OPAH-04	13	7 (53.85)	13	6 (46.15)	13	5 (38.46)	14	8 (57.14)
OPAH-08	9	2 (22.22)	9	3 (33.33)	8	1 (12.5)	9	3 (33.33)
OPAH-09	10	1 (10.0)	9	0 (0)	6	0 (0)	10	5 (50.0)
Total	112	42 (37.5)	109	42 (38.53)	111	42 (37.84)	124	75 (60.48)

'p%' denotes the percentage of polymorphic bands (in brackets)

4.2.5.2. Observed and effective number of alleles

Meenachil: In this population, the average value of observed (na) and effective (ne) number of alleles for overall primers was 1.3468 and 1.1865 respectively. The value of na ranged from 1.0667 in OPA-11 to 1.75 in OPA-20 with mean value 1.3488. The ne ranged from 1.0355 in OPAH-09 to 1.4037 in OPAH-02 with mean value 1.19. For overall primers Ne was 1.1865 (Table-20).

Chalakkudy: In this population, the average value of observed (na) and effective (ne) number of alleles for overall primers was 1.3387 and 1.1773 respectively. The na ranged from 1.0 in OPAH-09 to 1.8571 in OPAH-01 with mean value 1.3376. The ne ranged from 1.0 in OPAH-09 to 1.5623 in OPAH-01 with mean value 1.1760. For overall primers ne was 1.1773 (Table-20).

Nethravathi: In this population, the observed (na) and effective (ne) number of alleles were 1.3468 and 1.1954 respectively. The na ranged from 1.0 in OPAH-09 to 1.8571 in OPAH-01 with mean value 1.3509. The ne ranged from 1.0 in OPAH-09 to 1.6094 in OPAH-01 with mean value 1.1944. For overall primers, ne was 1.1954 (Table-20).

Inter populations: For overall populations, the observed (na) and effective (ne) number of alleles were 1.5887 and 1.3897 respectively. The na ranged from 1.3333 in OPA-11 and OPAH-08 to 1.9167 in OPA-20 with mean value of 1.6088. The ne ranged from 1.2206 in OPA-11 to 1.6828 in OPAH-01 with mean value of 1.3932. The ne for overall primers was 1.3897 (Table-20).

4.2.5.3. Average gene diversity or heterozygosity (H)

Meenachil: The average gene diversity or heterozygosity (H) (Nei, 1987) for each primer for Meenachil population is given in the Table-20. The value of H ranged from 0.0292 (OPAH-09) to 0.2398 (OPAH-01) with mean value 0.1115. For overall primers, the average value of H was 0.1100.

Table 20. Estimates of RAPD Variations in *Horabagrus brachysoma*

RAPD Primer	Mn	Ch	Ne	Mean	Among populations
OPA-07					
na	1.2222	1.5556	1.4444	1.4074	1.6667
ne	1.1956	1.3685	1.1286	1.2309	1.4966
H	0.1040	0.2193	0.0895	0.1376	0.2751
OPA-09					
na	1.1111	1.1111	1.4444	1.2222	1.6667
ne	1.0900	1.0518	1.3231	1.1550	1.4485
H	0.0497	0.0353	0.1845	0.0900	0.2569
OPA-11					
na	1.0667	1.0667	1.1333	1.0887	1.3333
ne	1.0660	1.0072	1.0587	1.0440	1.2206
H	0.0332	0.0065	0.0326	0.0241	0.1299
OPA-20					
na	1.7500	1.2500	1.4167	1.4722	1.9167
ne	1.2328	1.1370	1.2764	1.2154	1.3407
H	0.1579	0.0751	0.1534	0.1288	0.2211
OPAC-14					
na	1.1579	1.1579	1.0526	1.1228	1.4737
ne	1.0654	1.1001	1.0339	1.0665	1.3534
H	0.0406	0.0561	0.0206	0.0223	0.1980
OPAH-01					
na	1.6429	1.8571	1.8571	1.7857	1.8571
ne	1.3950	1.5623	1.6094	1.5222	1.6828
H	0.2398	0.3216	0.3407	0.3007	0.3727
OPAH-02					
na	1.6154	1.6154	1.6923	1.6410	1.7692
ne	1.4037	1.2780	1.3514	1.3443	1.4082
H	0.2229	1.1659	0.2110	0.1999	0.2499
OPAH-04					
na	1.5000	1.4286	1.3571	1.4286	1.5714
ne	1.1992	1.1547	1.1519	1.1686	1.4000
H	0.1283	0.0998	0.0961	0.1081	0.2230
OPAH-08					
na	1.2222	1.3333	1.1111	1.2222	1.3333
ne	1.2170	1.1003	1.0102	1.2092	1.2502
H	0.1098	0.0718	0.0093	0.0636	0.1373
OPAH-09					
na	1.2000	1.0000	1.0000	1.0666	1.5000
ne	1.0355	1.0000	1.0000	1.0118	1.3310
H	0.0292	0.0000	0.0000	0.0097	0.1863
Mean Primers					
na	1.3488	1.3376	1.3509	1.3458	1.6088
ne	1.1900	1.1760	1.1944	1.1868	1.3932
H	0.1115	0.1051	0.1138	0.1101	0.1975
Overall Primers					
na	1.3468	1.3387	1.3468	1.3441	1.5887
ne	1.1865	1.1773	1.1954	1.1864	1.3897
H	0.1100	0.1053	0.1139	0.1097	0.2222

Observed number of alleles (**na**), effective number of alleles (**ne**) and average gene diversity or heterozygosity (**H**) (Nei, 1987) are listed for each primer. Mean = average of each primer for each population. Among populations = actual value for each primer across populations. Mean primers = average for each population for 10 primers. Overall primers = actual value for each population across 10 primers. Mn- Meenachil, Ch- Chalakkudy and Ne- Nethravathi.

Chalakkudy: In Chalakkudy population, the value of H ranged from 0.000 (OPAH-09) to 1.1659 (OPAH-02) with mean value 0.1051. For overall primers, the average value of H was 0.1053 (Table-20).

Nethravathi: In this population, H ranged from 0.0097 (OPAH-09) to 0.3007 (OPAH-01) with mean value 0.1101. For overall primers, the average value of H was 0.1097 (Table-20).

Inter population: The average gene diversity or heterozygosity (H) (Nei, 1987) for each primer for each population is given in Table-20. The H ranged from 0.1299 in OPA-11 to 0.3727 in OPAH-01 with mean value of 0.1975 among the populations for each primer. For overall primers, the H was 0.2222.

4.2.6. Linkage disequilibrium

RAPD loci did not show any significant linkage disequilibrium ($P>0.05$) in any of the 3 populations of yellow catfish. It was therefore assumed that allelic variation at RAPD loci could be considered independent.

4.2.7. Genetic differentiation and gene flow

The value of coefficient of differentiation (G_{ST}) was estimated for each primer across all populations (Table-21). The maximum value of G_{ST}, 0.9478, was shown by the primer OPAH-09 and the minimum value by the primer OPAH-01 *ie*, 0.1925; with mean value 0.5577. The average of the G_{ST} in overall primer among populations was 0.5060.

The values of gene flow or effective migration value (Nm) for each primer is given in Table-21. Nm ranged from 0.0275 (in OPAH-09) to 2.0968 (in OPAH-01) with mean value of 0.6731. The average value of Nm for overall primers among populations was 0.4880.

Table 21. Co-efficient of genetic differentiation (G_{ST}) and rate of gene flow (Nm) (migration) for overall populations

Primers code	G_{ST}	Nm
OPA-07	0.4999	0.5003
OPA-09	0.6503	0.2689
OPA-11	0.8147	0.1137
OPA-20	0.4176	0.6974
OPAC-14	0.8026	0.1230
OPAH-01	0.1925	2.0968
OPAH-02	0.1999	2.0010
OPAH-04	0.5155	0.4700
OPAH-08	0.5365	0.4319
OPAH-09	0.9478	0.0275
Mean	**0.5577**	**0.6731**
Overall populations	**0.5060**	**0.4880**

4.2.8. Stock-specific markers (Private alleles)

Several RAPD fragments showed fixed frequencies in a particular population. These could be used as stock specific markers to distinguish the populations. Eight RAPD fragments were detected as stock-specific markers in 5 primers. These eight fragments were exclusively observed in particular populations. The 3rd band of OPAH-09 (980 bp in size) was specific to Meenachil population; while the eighth fragment of the primer OPAH-02 (1010 bp) was seen only in Chalakkudy population. With the primer OPA-09, 4th and 5th fragments (680 and 650 bp); with OPA-11, 6th and 9th (1060 and 890 bp) and with OPA-20, 1st and 6th fragments (2000 and 950 bp) were observed only in Nethravathi population (Table-22).

Table 22. Stock- specific RAPD markers with size in bp for each population

Primer code	Fragment No.	Size (bp)	Mn	Ch	Ne
OPA-09	4th	680	-	-	+
	5th	650	-	-	+
OPA-11	6th	1060	-	-	+
	9th	890	-	-	+
OPA-20	1st	2000	-	-	+
OPAC-14	6th	950	-	-	+
OPAH-02	8th	1010	-	+	-
OPAH-09	3rd	980	+	-	-

(Mn: Meenachil, Ch: Chalakkudy and Ne: Nethravathi)

4.2.9. Similarity index and genetic distance

Nei's (1978) unbiased genetic identity and distance estimated between pairs of three populations of *H. brachysoma* are presented in Table-23. Genetic identity values obtained were 0.8740 between Meenachil and Chalakkudy, 0.7501 between Meenachil and Nethravathi and 0.8095 between Chalakkudy and Nethravathi. The genetic distance between Meenachil and Chalakkudy was 0.1347, between Meenachil and Nethravathi, 0.2876; and between Chalakkudy and Nethravathi 0.2113. This indicates Meenachil and Chalakkudy populations are genetically closer to Nethravathi population. These results are in agreement with the geographic distances between pairs of the populations.

Table 23. Nei's genetic identity (above diagonal) and genetic distance (below diagonal), the geographic distances between populations are given in bracket

Populations	Meenachil	Chalakkudy	Nethravathi
Meenachil	****	0.8740	0.7501
Chalakkudy	0.1347 (140)	****	0.8095
Nethravathi	0.2876 (520)	0.2113 (380)	****

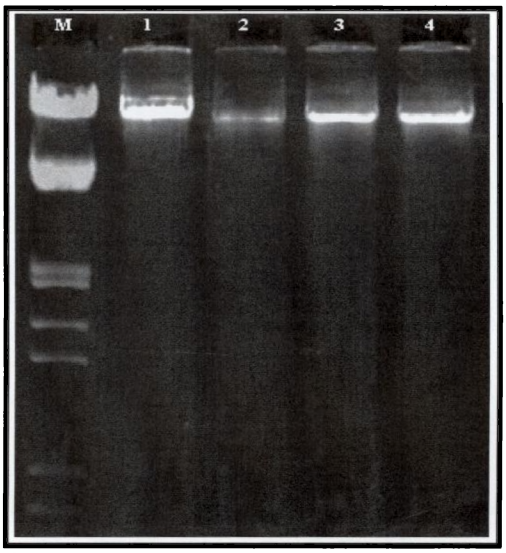

Fig. 18. DNA extracted from blood tissues.

Lanes 1, 2, 3 and 4 DNA extracted from 0.25 ml of blood tissues and dissolved in 1 ml of TE buffer. M- molecular weight marker (λDNA with EcoR1 & Hind111 double digest)

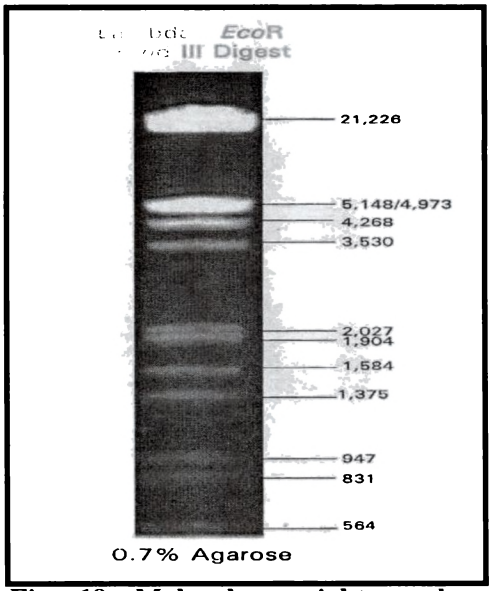

Fig. 19. Molecular weight marker (λDNA with EcoR1 & Hind111 double digest) used to analyze the size of RAPD fragments

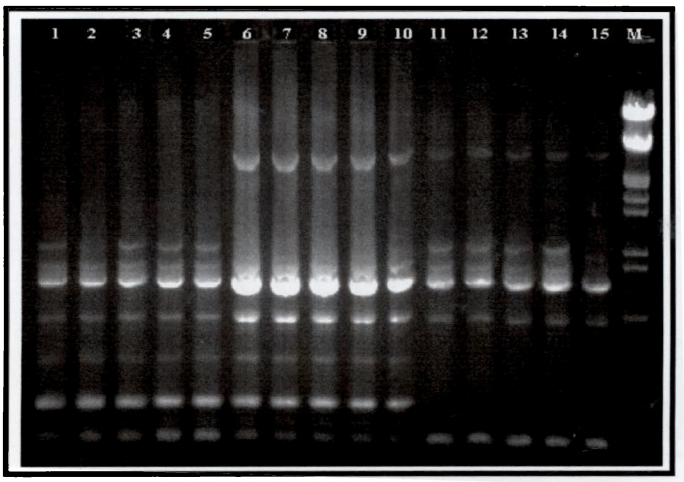

Fig. 20. RAPD pattern of *H. brachysoma* with primer OPA-07.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River. M-molecular weight marker (λDNA with *Eco*R1 & *Hind*111 double digest)

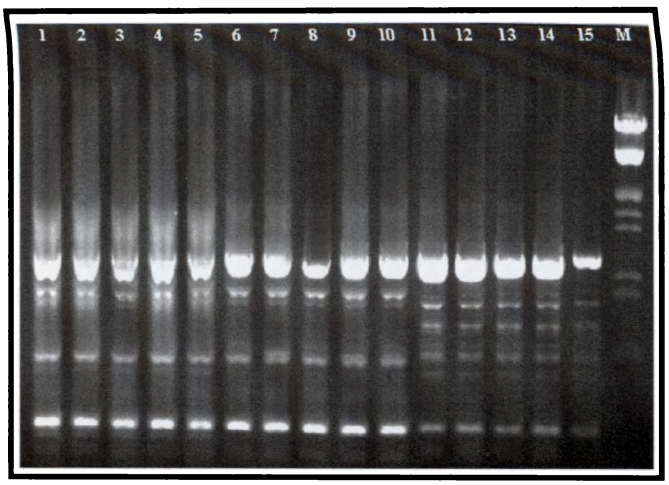

Fig. 21. RAPD pattern of *H. brachysoma* with primer OPA-09.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River. M-molecular weight marker (λDNA with EcoR1 & Hind111 double digest)

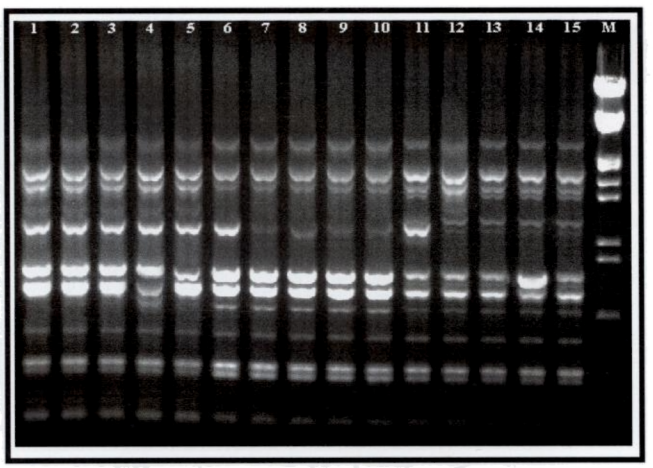

Fig. 22. RAPD pattern of *H. brachysoma* with primer OPA-11.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River. M-molecular weight marker (λDNA with *Eco*R1 & *Hind*111 double digest)

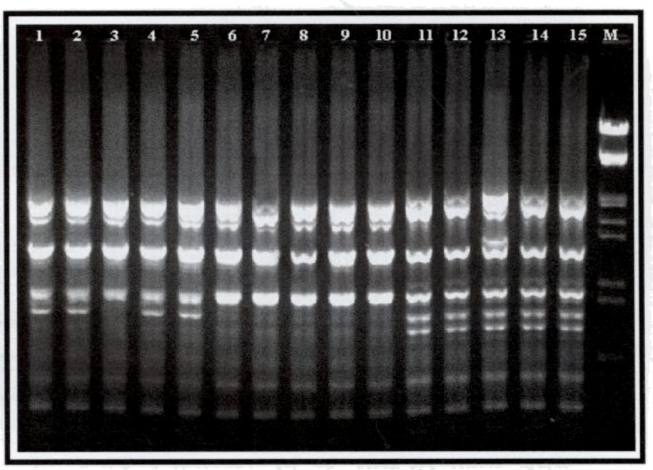

Fig. 23. RAPD pattern of *H. brachysoma* with primer OPA-20.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River. M-molecular weight marker (λDNA with *Eco*R1 & *Hind*111 double digest)

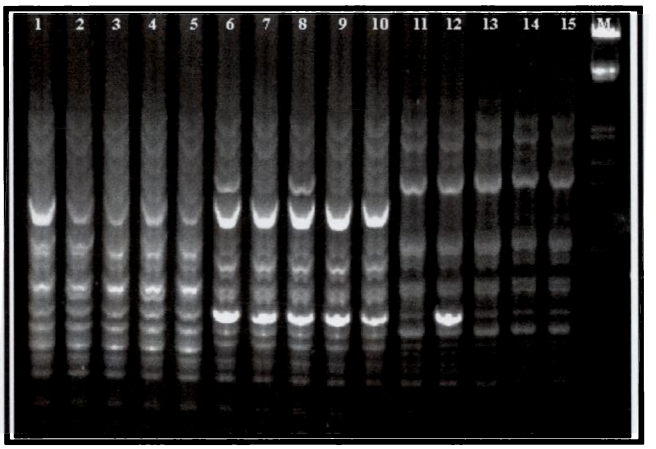

Fig. 24. RAPD pattern of *H. brachysoma* with primer OPAC-14.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River. M-molecular weight marker (λDNA with *Eco*R1 & *Hind*111 double digest)

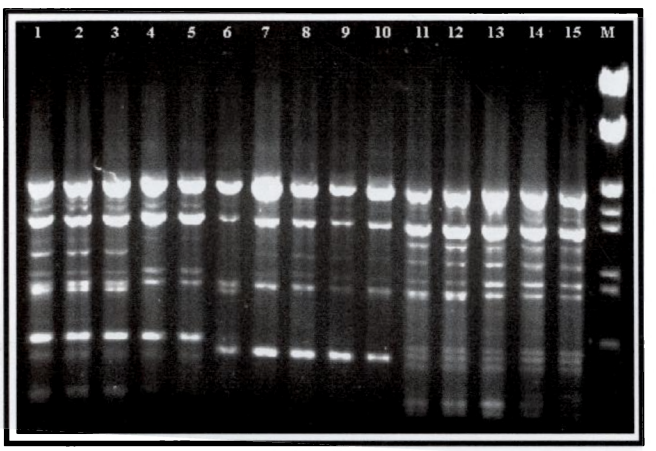

Fig. 25. RAPD pattern of *H. brachysoma* with primer OPAH-01.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River. M-molecular weight marker (λDNA with *Eco*R1 & *Hind*111 double digest)

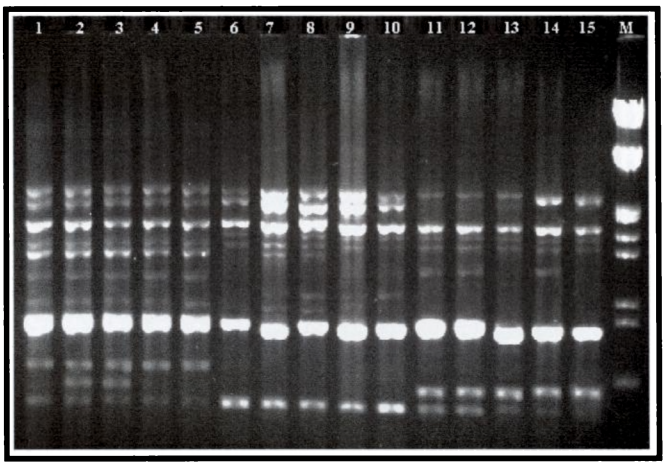

Fig. 26. RAPD pattern of *H. brachysoma* with primer OPAH-02.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River. M-molecular weight marker (λDNA with *Eco*R1 & *Hind*111 double digest)

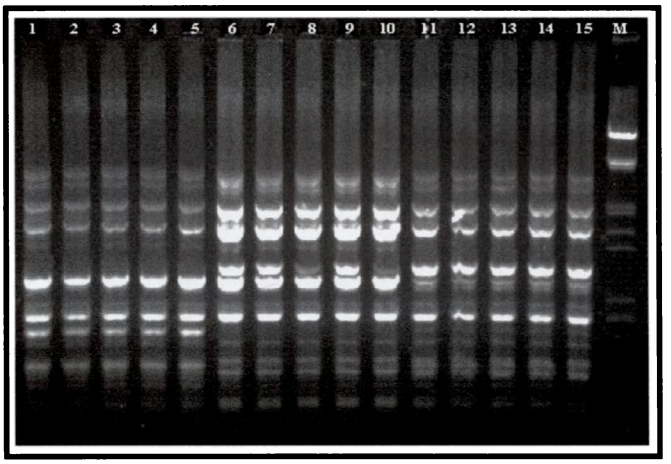

Fig. 27. RAPD pattern of *H. brachysoma* with primer OPAH-04.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River. M-molecular weight marker (λDNA with *Eco*R1 & *Hind*111 double digest)

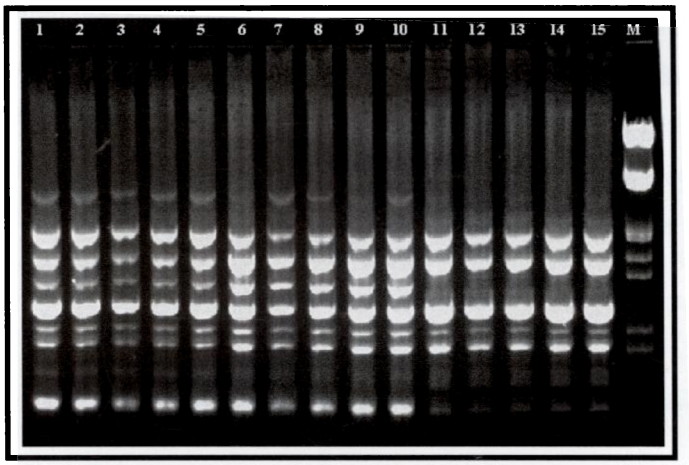

Fig. 28. RAPD pattern of *H. brachysoma* with primer OPAH-08.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River. M-molecular weight marker (λDNA with *Eco*R1 & *Hind*111 double digest)

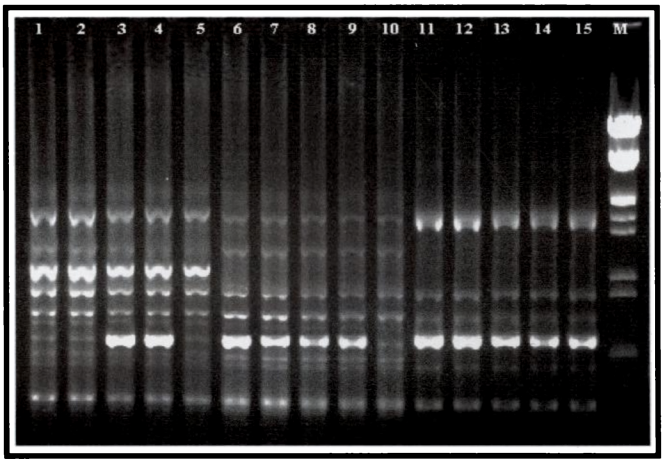

Fig. 29. RAPD pattern of *H. brachysoma* with primer OPAH-09.

Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River. M-molecular weight marker (λDNA with *Eco*R1 & *Hind*111 double digest)

4.2.10. Dendrogram

Phylogenetic relationships among three populations of *H. brachysoma* were made based on RAPD data following unweighted pair group method using arithmetic averages (UPGMA, Sneath and Sokal, 1973) implemented in PHYLIP (Felsenstein, 1993), using POPGENE ver.1.31 (Yeh *et al.*, 1991). The binary data matrix was bootstrapped 1000 times and the bootstrap values suggested the populations had a robust cluster (Fig.30).

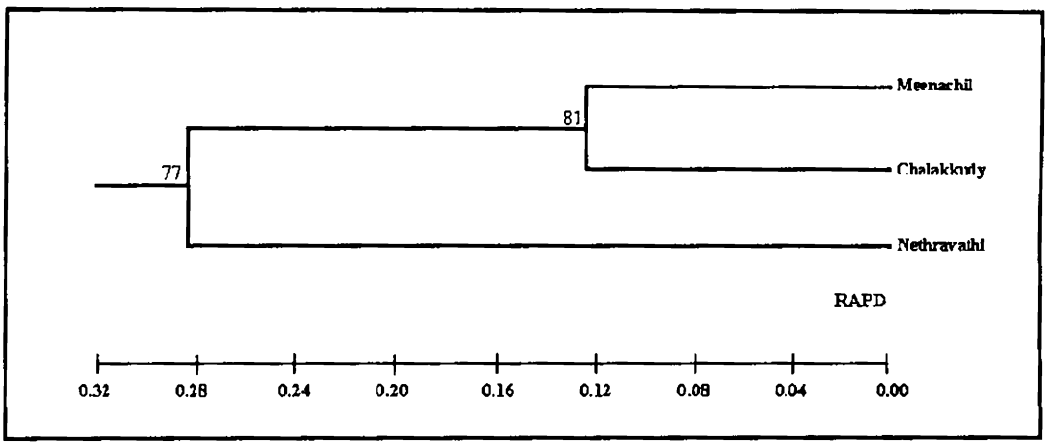

Fig. 30. Dendrogram pattern of RAPD analysis in *Horabagrus brachysoma*. Values at nodes represent bootstrap confidence levels (1000 replications).

4.3. Microsatellite analysis

4.3.1. Selection of primers

Twenty-five primers (microsatellite flanking regions) from different siluriform and osteoglossiform species (downloaded from NCBI site), were used to study the cross-species amplification of microsatellites in *H. brachysoma* (Table-24). The annealing temperature of these primers in the resource species; and the same in *H. brachysoma* at which successful amplification without stutter band occurred are given in Table-25. Only eight primers out of 25 gave scorable banding patterns after PCR amplification. These 8 primers produced 13 presumptive polymorphic microsatellite loci *viz., Phy 01, Phy 05, Phy 07-1, Phy 07-2, Cma 3, Cma 4-1, Cma 4-2, Cga 06-1, Cga 06-2, D-33-1, D-33-2 D-38-1*and *D-38-2*. To confirm the occurrence of repeats, PCR products were sequenced.

4.3.2. Confirmation of microsatellites

The occurrence of microsatellites in PCR products were confirmed by sequencing after cloning them in a TA vector (Invitrogen, Carlsbad, USA (Fig. 41). The transformed competent cells (100 µl) produced blue and white colonies on LB plate (90 mm) containing 50 µg/ml ampicillin coated with 40 µl X-gal (20 mg/ml) and 4µl IPTG (200 mg/ml). The blue colonies did not contain the insert in the plasmid while white colonies contained the inserts.

4.3.2.1. Confirmation of cloning

i) Through PCR

The DNA from both blue and white colonies was amplified with specific primers for the particular microsatellites locus. The DNA from white colonies containing the insert only was PCR amplified and produced bands in 1.5% agarose gels (Fig. 43).

ii) By comparing the size of plasmid

The plasmids isolated from recombinant colonies and non-recombinant colonies were compared in 0.8% agarose gels. The plasmids from recombinant colonies (white colonies) had higher molecular weight than the normal non-recombinant colonies (Fig.44).

Table 24. The sequence, concentration and the annealing temperature of microsatellite primers

No:	Microsatellite primers		Sequence 5'-3'	Conc. (nmol)	Ta for each primer
1	*Phy 01*	F	CGAACACGCCACAGAGAGTA	49.5	57°C
		R	CCACACCCACAACACCATAA	51.4	55°C
2	*Phy 05*	F	CCAGCAACCCACATAATTGA	43.2	53°C
		R	CAGCTCAGGGCCAAAAGTAG	45.1	57°C
3	*Phy 07*	F	AGTCACTTCAGCACCTGCCT	38.4	57°C
		R	ATCTCTGTGATGGTGAGCCA	53.9	55°C
4	*Cma 3*	F	TTCGGATTGTTTCTGTGG	53.1	47°C
		R	ACACTCTTTACACTGATT	50.2	43°C
5	*Cma 4*	F	TTTCGCCACGCAGGTTT	46.9	47°C
		R	TGGATTTTGACTGTGTATT	50.7	45°C
6	*Cga 06*	F	CAGCTCGTGTTTAATTTGGC	79.6	53°C
		R	TTGTACGAGAACCGTGCCAGG	52.0	61°C
7	*D-33*	F	CACATGCATGGAATTATGGC	47.7	53°C
		R	GAGCCAGAAGCAGGACTGAC	42.0	59°C
8	*D-38*	F	AATGCTGATGGACCTGCTCT	56.3	55°C
		R	CAAACAGGGAACCCACAGAT	53.3	55°C

4.3.2.2. Microsatellite loci confirmed after sequencing

After sequencing, the following loci were confirmed as microsatellites containing the repeat sequences *viz, Phy01, Phy05, Phy07-1, Cma3, Cma4-2, Cga06-1, D33-2 and D38-1.* These loci alone were further considered for population genetic analysis of *H. brachysoma*. The repeat sequences of each locus are given in Fig. 45a and 46b and Table-25.

4.3.2.3. Type and relative frequency of microsatellites

Of the 8 amplified loci, three (37.5%) were perfect (GT) *viz, Phy01, Phy05* and *Cga06–1* and their sequence information is presented in figures 45a and b. Rest of the loci exhibited imperfect repeats (Table-29). The length of the repeats (is equal to number of repeats) varied from 13 (Phy07-1) to 33 (Phy01) and the average length of repeats was 20.375. The tandem repeats of 37.5% of the microsatellite loci (*viz, Phy01, Phy05 and D33-2*) were same as that of the resource species, while repeat motifs of all other loci differed from that of the resource species (Table-25).

Table 25. Microsatellite loci and repeat sequence in *Horabagrus brachysoma*

Sl. No.	Locus in resource species	Repeat units in resource species	Repeat motif in *H. brachy-soma*	Annealing temp.		Monomorphic / Polymorphic
				In resource species (in ^{0}C)	In target species (in ^{0}C)	
1	*Phy01*	(GT)n	(GT)n	65	56	Polymorphic
2	*Phy05*	(GT)n	(GT)n	60	55	Polymorphic
3	*Phy07-1*	(CA)n..... (CT)n	(TC)n	68	55	Polymorphic
4	*Cma3*	(CA)n	(TC)n	50	45	Polymorphic
5	*Cma4-2*	$(CA)_n$	(TG)n	48	46	Polymorphic
6	*Cga06-1*		(GT)n	--	57	Polymorphic
7	*D33-2*	(CA)n	(CA)n	55	53	Polymorphic
8	*D38-1*	(GT)n	(TC)n	50	55	Polymorphic

4.3.3. Variations in microsatellite band pattern

In *Phy01*, 7 alleles were observed. The size of the alleles ranged from *162* to *196* bp (*i.e., 162, 170, 176, 180, 184, 190* and *196* bp). The fragment *176* bp was most common in all populations. In the Nethravathi population, the *196* bp fragment was absent.

In *Phy05*, a total of 6 alleles were observed. The sizes of the alleles were *146, 150, 155, 162, 166* and *170* bp. The allele having the size *162* bp was the most common in all populations. The *170* bp fragment was totally absent in Nethravathi samples.

In *Phy07-1*, only 4 alleles were observed. The sizes of the alleles were *270, 275, 280* and *285* bp. All the 4 alleles were present in all the populations. The fragment *275* bp allele was most common in all the populations.

In *Cma3* there were seven alleles and the size of the alleles were *147, 151, 155, 159, 163, 166* and *170* bp. The fragment *159* bp was most common in all the three populations. In Nethravathi population, *170* bp fragment was absent.

In *Cma4-2*, there were four alleles and their sizes were *172, 175, 178* and *182* bp. The fragment *178* bp was most common in all the populations. All four alleles were recorded in all the three populations.

In *Cga06-1*, five alleles were recorded. The size of the alleles ranged from *218* to *244* bp (*i.e., 218, 226, 234, 240* and *244* bp). Overall the fragment *234* bp was most common. In Meenachil, the allele *218* bp was not recorded, the allele *226* was not observed in Chalakkudy whereas all the alleles were present in Nethravathi population.

In *D33-2* locus, only three alleles were observed - *192, 200* and *212* bp and the fragment *212* bp was most common. The fragment *192* bp was observed only in Meenachil population, hence this allele was considered as a private allele.

In *D38-1* locus, there were only four alleles. The size of the alleles ranged from *252* to *310* bp (*i.e., 252, 272, 295* and *310* bp). The fragment *295* bp was most common in all populations. In Meenachil and Nethravathi populations, the allele *310* bp was not amplified and this allele was only present in Chalakkudy population, hence considered as a private allele of this population

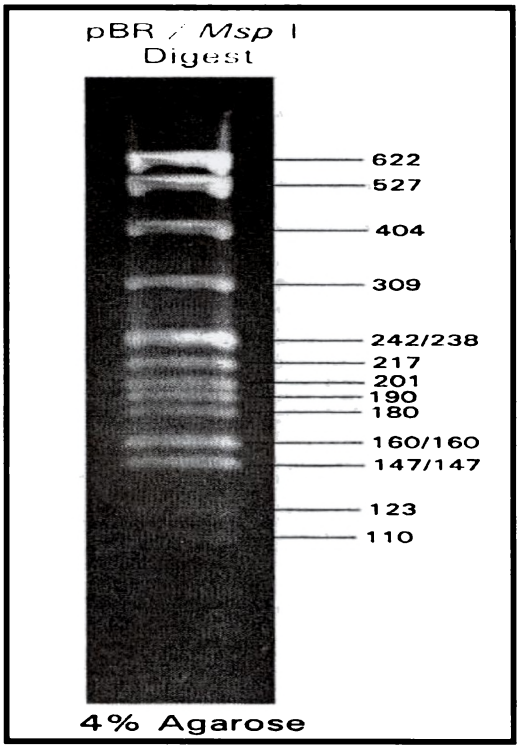

Fig. 31. Molecular weight marker (pBR322 with *Msp*I cut) used to analyze the size of microsatellite alleles

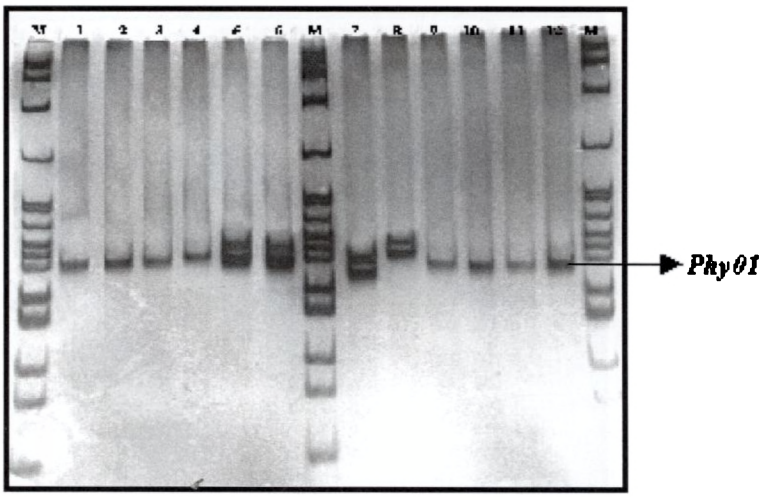

Fig. 32. Microsatellite pattern of locus *Phy01 in H. brachysoma*.

Lanes 1-4 samples from Meenachil River, 5-8 Chalakkudy River and 9-12 Nethravathi River. M-molecular weight marker (*pBR*322 with *Msp*I cut)

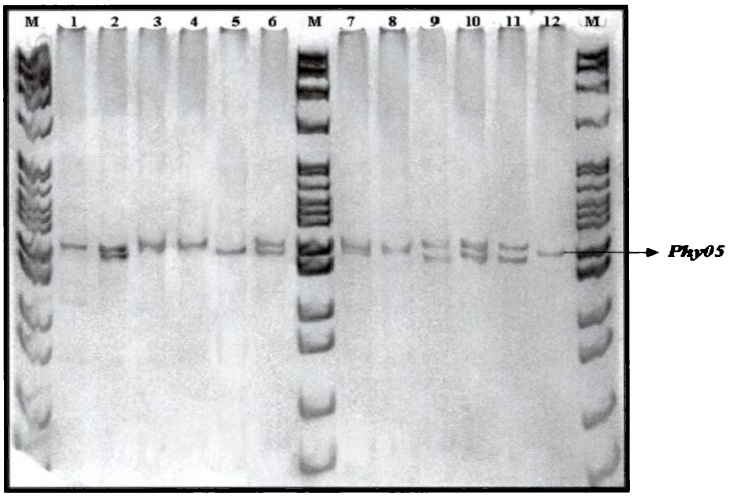

Fig. 33. Microsatellite pattern of locus *Phy05 in H. brachysoma*.

Lanes 1-4 samples from Meenachil River, 5-8 Chalakkudy River and 9-12 Nethravathi River. M-molecular weight marker (*pBR*322 with *Msp*I cut)

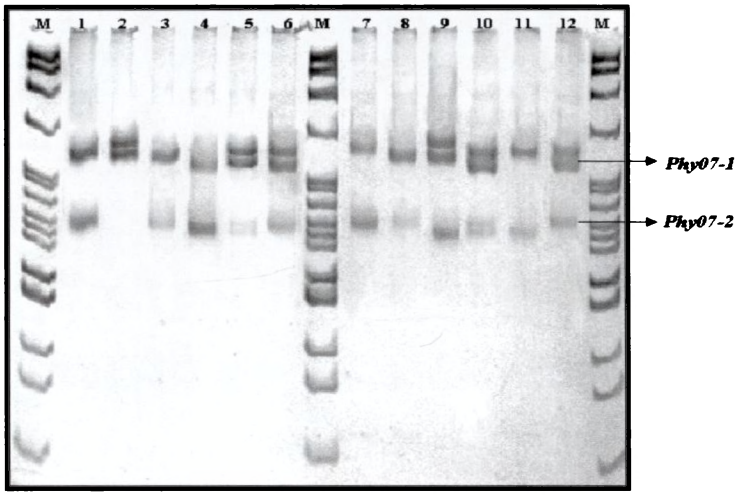

Fig. 34. Microsatellite pattern of loci *Phy07-1* and *Phy07-2 in H. brachysoma*.

Lanes 1-4 samples from Meenachil River, 5-8 Chalakkudy River and 9-12 Nethravathi River. M-molecular weight marker (*pBR*322 with *Msp*I cut)

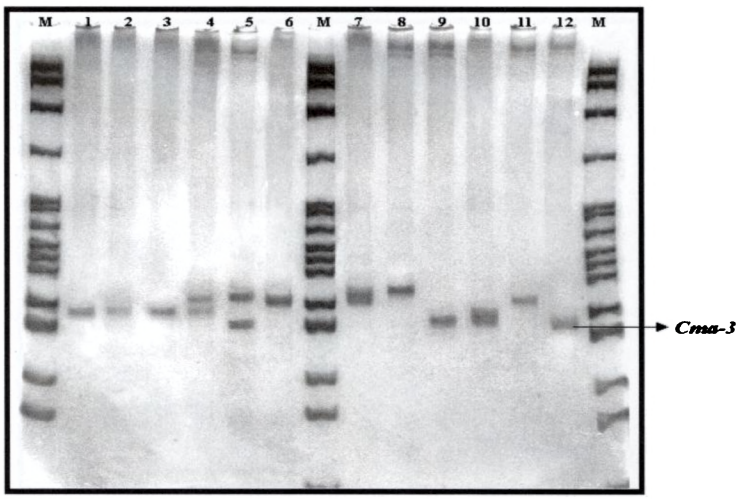

Fig. 35. Microsatellite pattern of locus *Cma-3* in *H. brachysoma*.

Lanes 1-4 samples from Meenachil River, 5-8 Chalakkudy River and 9-12 Nethravathi River. M-molecular weight marker (pBR322 with *Msp*I cut)

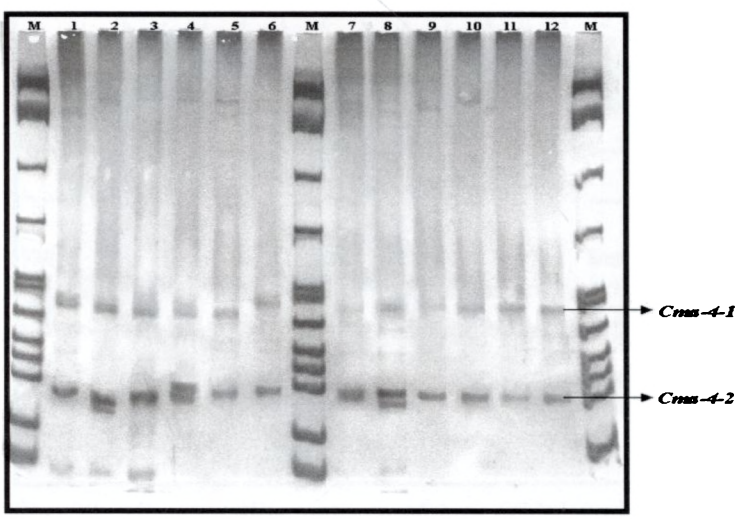

Fig. 36. Microsatellite pattern of loci *Cma-4-1* and *Cma-4-2* in *H. brachysoma*.

Lanes 1-4 samples from Meenachil River, 5-8 Chalakkudy River and 9-12 Nethravathi River. M-molecular weight marker (pBR322 with *Msp*I cut)

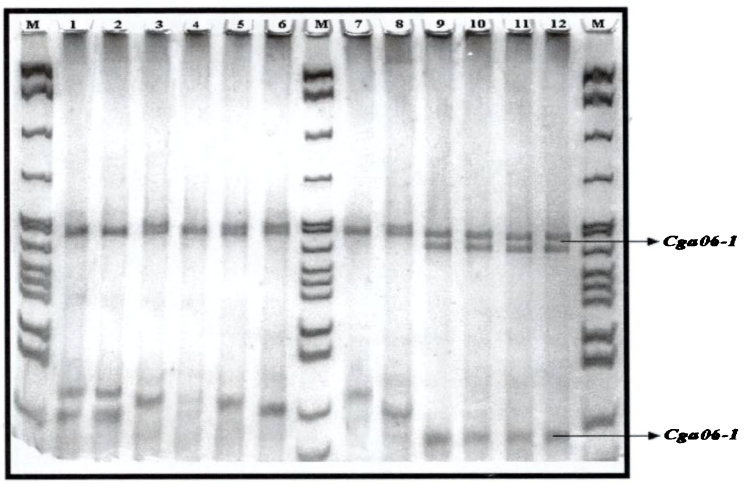

Fig. 37. Microsatellite pattern of loci *Cga06-1* and *Cga06-2* in *H. brachysoma*.

Lanes 1-4 samples from Meenachil River, 5-8 Chalakkudy River and 9-12 Nethravathi River. M- molecular weight marker (pBR322 with *Msp*I cut)

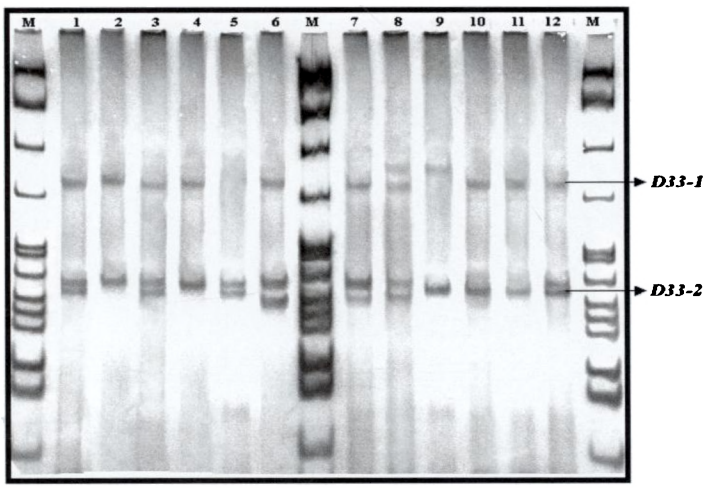

Fig. 38. Microsatellite pattern of loci *D33-1* and *D33-2* in *H. brachysoma*.

Lanes 1-4 samples from Meenachil River, 5-8 Chalakkudy River and 9-12 Nethravathi River. M-molecular weight marker (pBR322 with *Msp*I cut)

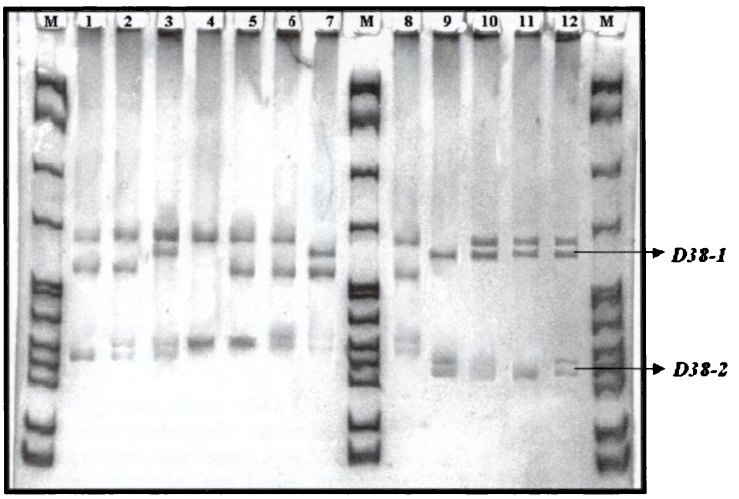

Fig. 39. Microsatellite pattern of loci *D38-1* and *D38-2* in *H. brachysoma*.

Lanes 1-4 samples from Meenachil River, 5-8 Chalakkudy River and 9-12 Nethravathi River. M-molecular weight marker (*pBR*322 with *Msp*I cut)

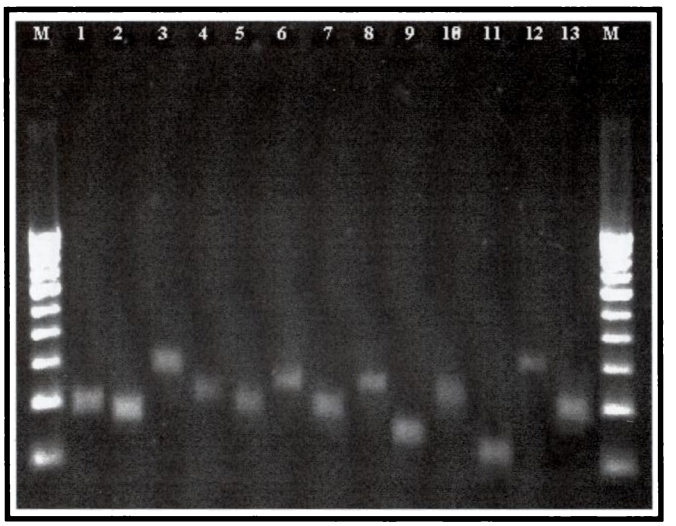

Fig. 40. Samples purified from gel for cloning.

Lane 1: Phy01, 2: Phy05, 3: Phy07-1, 4: Phy07-2, 5: Cma-3, 6: Cma-4-1, 7: Cma-4-2, 8: Cga06-1, 9: Cga06-2, 10: D33-1, 11: D33-2, 12: D38-1, 13: D38-2 and M-molecular weight marker (100 bp ladder)

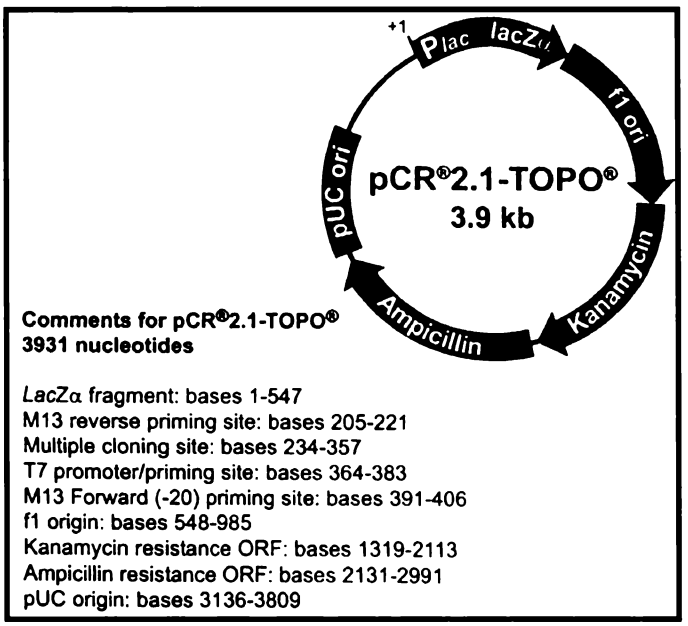

Fig. 41. Topo vector (T-vector) used for cloning of the microsatellite PCR products.

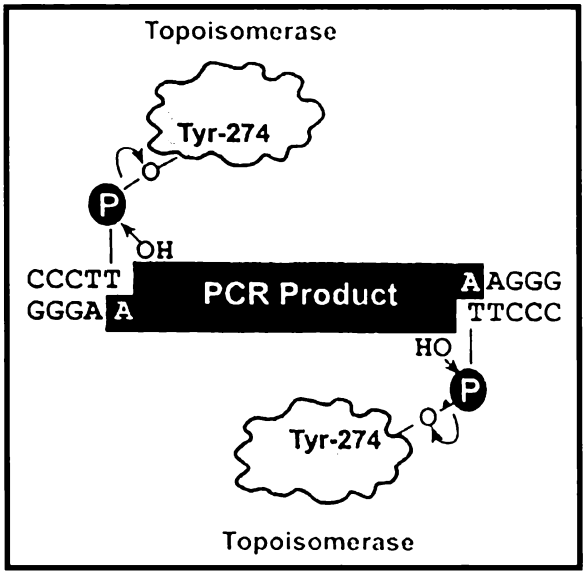

Fig. 42. Schematic diagram of cloning of PCR products in T-vector.

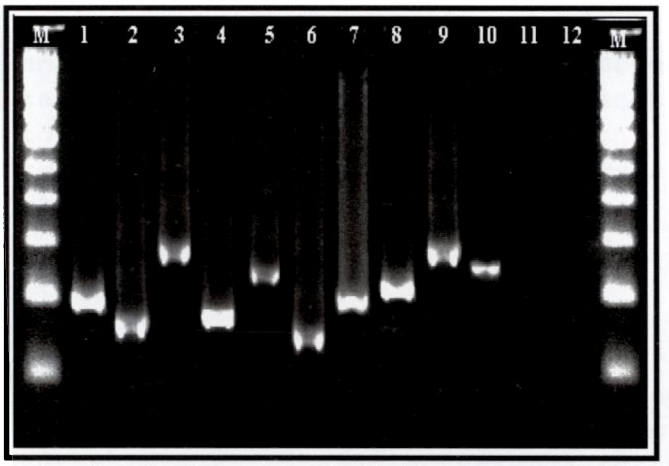

Fig. 43. Confirmation of cloning through PCR amplification.

Lane 1: Phy01, 2: Phy05, 3: Phy07-1, 4: Cma-3, 5: Cma-4-1, 6: Cma-4-2, 7: Cga06-1, 8: D33-2, 9: D38-1, 10: D38-2, 11 and 12: Plasmids extracted from blue colonies and M- molecular weight marker (100 bp ladder)

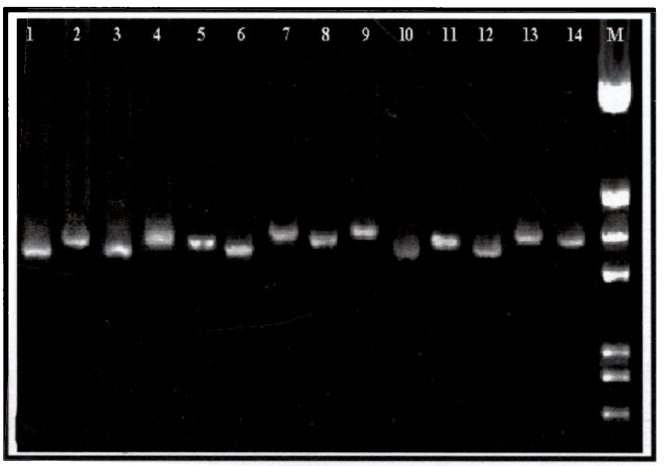

Fig. 44. Confirmation of cloning by comparing the plasmid size.

Lane 1: Plasmid (TOPO vector) without inserts, Lane 2: Phy01, 3: Phy05, 4: Phy07-1, 5: Phy07-2, 6: Cma-3, 7: Cma-4-1, 8: Cma-4-2, 9: Cga06-1, 10: Cga06-2, 11: D33-1, 12: D33-2, 13: D38-1, 14: D38-2 and M-molecular weight marker (λDNA with *Eco*R1 & *Hind*111 double digest)

Phy01

cgaacacgcc acagagagta aacagagcca cctgcggcat
taaggccggg ctttaactgt gtaataataa taataacgtg
tgtgtgtgtg tgtgtgtgtg tgtgtgtgtg tgtgtgtgtg
tgtgtgtgtg tgtgtgtgtg tgttaagcct gacattgaga
gttatggtgt tgtgggtgtg g

Phy05

cagcaaccca cataattgaa taacacaaat aggactagag
gtgcagggtg tgtgtgtgtg tgtgtgtgtg tgtgtgtgtg
tgtgtgtgtg tgtgtgtgtg tgttcaaaga tgcttgcggg
tgtgtgagca atataggttg gctacttttg gccctgagct
ga

Phy7-1

atctctgtga tggtgagcca gtcgtcagtg tagaccccaa
gtgcatctct ctctctctca tctctctctc tccagatgca
aaataactcg gggtcaacca cagccactat gaacactaat
aataatctac tgaatgcagt tataagcaag caggaaacct
catccacaat gagcagctga tctgatcaca attacacttc
tgtatataaa tgtgtatctg gccgtgggta tatattaaag
ggctcactct tcctcaggca ggtgctgaag tgacta

Cma3

tacactcttt acactgattc actgattctg ttcgctctgg
tttcccattt accaaagaca ctccccccag atatctctct
ctctctcctc actcacactc tctctctctc tctctctctc
tctctctctc tctccagcca gctcaggaac cacagaaaca
atccga

Fig 45a. The nucleotide sequence of each microsatellite loci amplified in *H. brachysoma*. Repeat sequences are given in red colour and primer sequences are in blue colour.

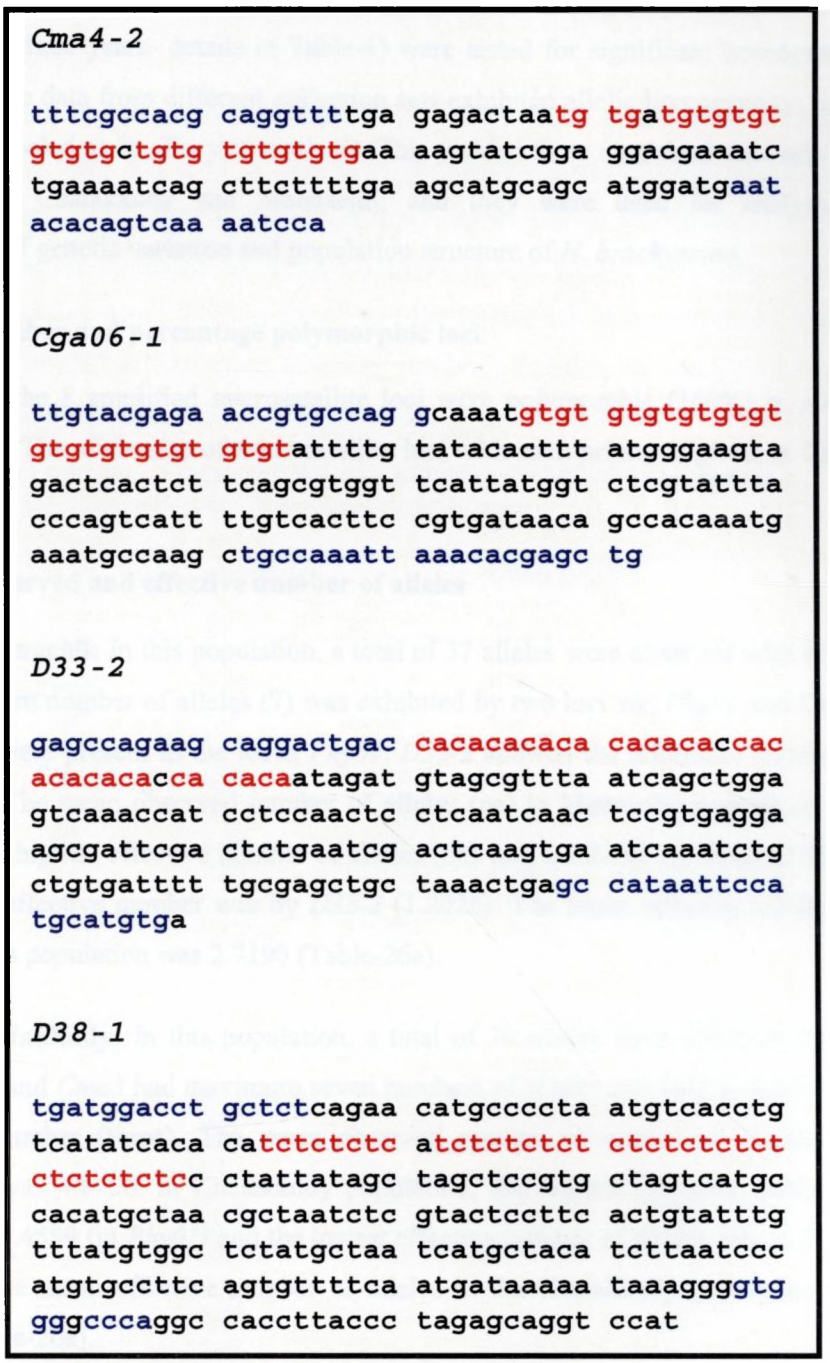

Fig 45b. The nucleotide sequence of each microsatellite loci amplified in *H. brachysoma*. Repeat sequences are given in red colour and primer sequences are in blue colour.

4.3.4. Genetic Variability

The allele frequencies of microsatellite loci from multiple collections of the same river (three years- details in Table-1) were tested for significant homogeneity. The genotype data from different collection sets exhibited allelic homogeneity; hence they were pooled as in allozyme analysis. This yielded three combined data sets *viz.*, Nethravathi, Chalakkudy and Meenachil; and they were used for analysis of parameters of genetic variation and population structure of *H. brachysoma.*

4.3.4.1. Number and percentage polymorphic loci

All the 8 amplified microsatellite loci were polymorphic (100%) in all the populations. The allele size of microsatellite loci with each primer is given in Table-27.

4.3.4.2. Observed and effective number of alleles

Meenachil: In this population, a total of 37 alleles were observed with 8 loci. The maximum number of alleles (7) was exhibited by two loci *viz, Phy01* and *Cma3.* Six alleles were present in the locus *Phy05. D33-2* showed the minimum number of alleles (3). The mean observed number of alleles (na) in Meenachil population was 4.6250. The highest effective number of alleles (ne) was exhibited by *Cma3* (5.5840) and lowest effective number was by *D33-2* (1.2026). The mean effective number of alleles in this population was 2.7190 (Table-26a).

Chalakkudy: In this population, a total of 39 alleles were observed with 8 loci. *Phy01* and *Cma3* had maximum seven numbers of alleles and *D33-2* showed the minimum number (three). The mean observed number of alleles in Chalakkudy population was 4.8750. In Chalakkudy population, the highest effective number of alleles was 6.4559 (in *Phy01)* and the lowest effective number of alleles was in *D33-2* (1.3712). The mean effective number of alleles in the Chalakkudy population was 3.2502 (Table-26a).

Nethravathi: In this population, 35 alleles were observed with 8 loci. *Phy01* and *Cma3* had maximum number of alleles, *i.e.,* six and *D33-2* showed the minimum number of alleles (two). The mean observed number of alleles in this population was

4.3750. The highest effective number of alleles in this population was 4.6214 with *Phy01* and lowest effective number with *D33-2* (1.9333). The mean effective number of alleles was 2.6548 in this population (Table-26b).

Overall populations: Among the three populations, a total of 40 alleles were observed in 8 loci. The maximum number of alleles (7) was recorded in *Phy01* and *Cma3*, while, *D33-2* showed the minimum number (three) of alleles. The mean observed number of alleles for overall population was 5.000. The highest effective number of alleles was 5.7474 in *Cma3* and the lowest effective number (1.5271) of alleles was in *D33-2*. The mean effective number of alleles for overall populations was 3.3137 (Table-26b).

Table 26a. Observed (Na) and Effective (Ne) number of alleles for Meenachil and Chalakkudy populations

Locus	Meenachil		Chalakkudy	
	na	ne	na	ne
Phy01	7.00	2.9456	7.00	6.4559
Phy05	6.00	2.6827	6.00	4.1385
Phy07-1	4.00	2.3300	4.00	2.4014
Cma3	7.00	5.5840	7.00	4.1473
Cma4-2	4.00	2.8267	4.00	2.1610
Cga06-1	4.00	1.9931	4.00	2.9509
D33-2	2.00	1.2026	3.00	1.3712
D38-1	3.00	2.1870	4.00	2.3752
Total	37	---	39	---
Mean	**4.6250**	**2.7190**	**4.8750**	**3.2502**
S.D.	1.8468	1.2836	1.5526	1.6108

Table 26b. Observed (Na) and Effective (Ne) number of alleles for Nethravathi and overall populations

Locus	Nethravathi		Overall	
	na	ne	na	ne
Phy01	6.00	2.1836	7.00	4.6214
Phy05	5.00	3.0482	6.00	4.1843
Phy07-1	4.00	3.2058	4.00	2.6724
Cma3	6.00	3.8522	7.00	5.7474
Cma4-2	4.00	2.0847	4.00	2.5802
Cga06-1	5.00	2.6681	5.00	2.7879
D33-2	2.00	1.9333	3.00	1.5271
D38-1	3.00	2.2628	4.00	2.3889
Total	35	---	40	---
Mean	**4.3750**	**2.6548**	**5.000**	**3.3137**
S.D.	1.4079	0.6669	1.5119	1.3971

4.3.4.3. Frequency of alleles

The allelic frequencies of 8 polymorphic microsatellite loci are given in Table-31. In Meenachil population, the allelic frequencies ranged from 0.014 (*Cma3* and *Cma4-2*) to 0.9071 (*D33-2*). The 3 alleles (one each in *Cga06-1*, *D33-2* and *D38-1*) were totally absent. In Chalakkudy population, the allelic frequencies ranged from 0.0071 (*Phy05, Cga06-1* and *D38-1*) to 0.8429 (*D33-2*). Only one allele (*Cga06-1*) was totally absent in this population. In Nethravathi population, the allelic frequencies ranged from 0.0143 (*Phy01* and *Phy05*) to 0.6500 (*Cma4-2*). Five alleles (one each in *Phy01, Phy05, Cma3, D33-2* and *D38-1*) were totally absent in the samples from Nethravathi. The overall allele frequency value ranged from 0.0024 (*D38-1*) to 0.7810 (*D33-2*) (Table-27).

Table 27. The sizes of alleles and the allele frequencies in individual population and overall populations

Locus	Allele size (bp)	Meenachil	Chalakkudy	Nethravathi	Overall populations
Phy01	162	0.0643	0.1071	0.0143	0.0619
	170	0.0286	0.1214	0.0357	0.0619
	176	0.1500	0.1500	0.6500	0.3167
	180	0.5357	0.2143	0.0643	0.2714
	184	0.1500	0.1714	0.1500	0.1571
	190	0.0357	0.1571	0.0857	0.0929
	196	0.0357	0.0786	0.0000	0.0381
Phy05	146	0.0286	0.1643	0.4214	0.2048
	150	0.0429	0.1071	0.1286	0.0929
	155	0.1857	0.3071	0.3571	0.2833
	162	0.5643	0.3143	0.0786	0.3190
	166	0.1143	0.1000	0.0143	0.0762
	170	0.0643	0.0071	0.0000	0.0238
Phy07-1	270	0.2071	0.1786	0.3143	0.2333
	275	0.6071	0.6000	0.4214	0.5429
	280	0.0786	0.1143	0.1500	0.1143
	285	0.1071	0.1071	0.1143	0.1095
Cma3	147	0.0857	0.0500	0.0786	0.0714
	151	0.1071	0.0286	0.3857	0.1738
	155	0.1929	0.0357	0.2643	0.1643
	159	0.2143	0.3071	0.1643	0.2286
	163	0.1571	0.2643	0.0214	0.1476
	166	0.2286	0.2643	0.0857	0.1929
	170	0.0143	0.0500	0.0000	0.0214

Table 27 continued					
Cma4-2	172	0.2286	0.0286	0.0357	0.0976
	175	0.4643	0.4357	0.2143	0.3714
	178	0.2929	0.5214	0.6500	0.4881
	182	0.0143	0.0143	0.1000	0.0429
Cga06-1	218	0.0000	0.0071	0.2571	0.0881
	226	0.0143	0.0000	0.0643	0.0262
	234	0.6429	0.4000	0.5429	0.5286
	240	0.2929	0.3357	0.0857	0.2381
	244	0.0500	0.2571	0.0500	0.1190
D33-2	192	0.0000	0.0214	0.0000	0.0071
	200	0.0929	0.1357	0.4071	0.2119
	212	0.9071	0.8429	0.5929	0.7810
D38-1	252	0.2929	0.1929	0.0786	0.1881
	272	0.1071	0.2214	0.3857	0.2381
	295	0.6000	0.5786	0.5357	0.5714
	310	0.0000	0.0071	0.0000	0.0024

4.3.4.4. Frequency of null alleles

Seven of the 8 primer pairs in *H. brachysoma* indicated positive F_{IS} values in different populations (Table-30). The expected frequency of null alleles was calculated using MICRO-CHECKER and all the genotypes of the loci showing deviation from Hardy-Weinberg equilibrium were tested for null alleles. The estimated null allele frequency was not significant ($P< 0.05$) at all 7 tested loci using different algorithms, indicating the absence of null alleles and false homozygotes. There was also the absence general excess of homozygotes over most of the allele size classes in all the 7 loci in three populations. In addition, there was no instance of non-amplified samples in repeated trials with any of the primer pairs. Therefore, for population genetic analysis, information from all the 8 loci was considered.

Table 28. Summary statistics of null allele frequencies in *H. brachysoma*

Locus	Populations showing high F_{IS} values	Null allele frequency* (from MICRO-CHECKER)		
		Van Oosterhout	Chakraborty	Brooksfield
Phy01	Meenachil	0.0032	0.0034	0.0032
Phy05	Meenachil	0.0053	0.0061	0.0058
	Chalakkudy	0.0028	0.0021	0.0036
Phy07-1	Meenachil	0.0096	0.0087	0.0104
	Chalakkudy	0.0076	0.0094	0.0082
	Nethravathi	0.0116	0.0101	0.0147

Table 28 continued				
Cma03	Meenachil	0.0063	0.0069	0.0075
	Chalakkudy	0.0045	0.0051	0.0051
	Nethravathi	0.0076	0.0092	0.0083
Cma04-2	Meenachil	0.0098	0.0113	0.0099
	Chalakkudy	0.0088	0.0087	0.0082
	Nethravathi	0.0169	0.0183	0.0199
Cga06-1	Meenachil	0.0056	0.0067	0.0041
	Chalakkudy	0.0049	0.0052	0.0081
	Nethravathi	0.0006	0.0012	0.0021
D33-2	Meenachil	0.0016	0.0023	0.0018

4.3.4.5. Observed (H_{obs}) and expected (H_{exp}) heterozygosities

Meenachil: In Meenachil population, the range of observed heterozygosity (Hobs) was from 0.1286 (*D33-2*) to 0.7143 (*D38-1*) and the mean was 0.4179 (SD: 0.1832). The expected heterozygosity (Hexp) for this population ranged from 0.8268 (*Cma3*) to 0.1697 (*D33-2*), with a mean of 0.5710 (SD: 0.1893) (Table-30).

Chalakkudy: In this population the range of observed heterozygosity (Hobs) was from 0.2143 (*Cma4-2*) to 0.9286 (*Phy01*) with a mean value of 0.5018 (SD: 0.2569). The expected heterozygosity (Hexp) for this population ranged from 0.2727 in *D33-2* to 0.8512 for *Phy01*, with a mean value of 0.6287 (SD: 0.1796) (Table-30).

Nethravathi: In this population, the observed heterozygosity ranged from 0.0714 (*Phy07-1*) to 0.7429 (*D38-1*) with a mean value of 0.4964 (SD: 0.2380). The expected heterozygosity for this population ranged from 0.4862 (*D33-2*) to 0.7457 (*Cma3*) with a mean value of 0.6079 (SD: 0.0920) (Table-30).

Inter-population: In overall population, the observed heterozygosity values ranged from 0.2048 (*Phy07-1*) to 0.6857 (*D38-1*), with the mean value of 0.4720 (SD: 0.1923). The expected heterozygosity for this population ranged from 0.3460 (*D33-2*) to 0.8280 (*Cma3*) with a mean value of 0.6486 (SD: 0.1519) (Table-30).

4.3.5. Private alleles (Stock-specific markers)

There were only two private alleles and both the alleles were found in Chalakkudy population. These include the *192* bp fragment in the locus *D33-2*, and

the *310* bp fragment in *D38-1* locus. The frequencies of these alleles were 0.0071 and 0.0214 respectively (Table-29).

Table 29. Private alleles and their frequencies

Locus	Private alleles	Meenachil	Chalakkudy	Nethravathi
D33-2	*192* bp	----	0.0214	----
D38-1	*310* bp	----	0.0071	----

4.3.6. Linkage disequilibrium

There was no significant association indicative of linkage disequilibrium ($P>0.05$) between any pair of microsatellite loci for any population ($P > 0.05$); 84 pair-wise comparisons, comprising 28 pair comparisons for 3 populations). It was therefore assumed that allelic variation at microsatellite loci could be considered independent.

4.3.7. Agreement with Hardy-Weinberg expectations

The probability test revealed that the observed allele frequencies in most of the loci showed significant deviation ($P<0.05$) from Hardy-Weinberg equilibrium except for *D33-2* in Meenachil population, *D33-2* and *D38-1* in Chalakkudy population and *Phy05* in Nethravathi population after sequenctial Bonferroni correction was made to the probability levels (Table-30). Wright's (1978) fixation index (F_{IS}) is a measure of heterozygote deficiency or excess (inbreeding co-efficient) and the significant values for each locus in each population and overall population are given in Table-30 and 31. The values ranged from –0.2246 for *D38-1* to 0.6666 for *Phy07-1* and with an average of 0.5072. In most of the loci, the value of F_{IS} was found to deviate significantly from zero, indicating a deficiency of heterozygotes, except in *D33-2* and *D38-1*.

4.3.8. Genetic differentiation and gene flow

The coefficient of genetic differentiation, F_{ST} ranged from 0.0824 for *Cga06-1* to 0.1390 for *Phy01*, with a mean of 0.1055, indicating that 10.55% of the total

genetic variation exists among 3 populations (Table-31). Pair-wise F_{ST} estimates between populations differed significantly ($P < 0.0001$) from zero for all the pairs of riverine locations (Table-32). The loci exhibiting significant heterogeneity in genotype proportions between different population pairs and for overall population are depicted in Table-33a and b. Out of the possible 24 tests, 20 had significant genotypic heterogeneity at least at one locus. For overall populations, all the eight loci showed significant genotypic heterogeneity Table-33b. The gene flow or migration rate (Nm) for each locus for overall population is given in Table-31. Nm ranged from 1.5085 (*Phy01*) to 2.7839 (*Cga06-1*) with the mean value of 1.5386.

Table 30. Summary of genetic variation and heterozygosity statistics of 8 microsatellite loci in *H. brachysoma*

Locus	Populations (n=70 each)		
	Meenachil	Chalakkudy	Nethravathi
Phy01			
H obs	0.5571	0.9286	0.5571
H exp	0.6653	0.8512	0.5459
Fis	+0.164	-0.092	-0.021
P_{HW}	0. 0038	0.0000	0.0244
Pscore	0.1926	0.9704	0.0393
Phy05			
H obs	0.4571	0.7571	0.6857
H exp	0.6318	0.7638	0.6768
Fis	+0.278	+0.009	-0.013
P_{HW}	0.0002	0.0015	0.1803
Pscore	0.0013	0.7164	0.4038
Phy07-1			
H obs	0.3000	0.2429	0.0714
H exp	0.5749	0.5878	0.6930
Fis	+0.480	+0.589	+0.898
P_{HW}	0.0000	0.0000	0.0000
Pscore	0.0002	0.0000	0.0000
Cma3			
H obs	0.5286	0.5571	0.4857
H exp	0.8268	0.7643	0.7457
Fis	+0.362	+0.273	+0.350
P_{HW}	0.0000	0.0004	0.0000
Pscore	0.0000	0.0000	0.0000
Cma4-2			
H obs	0.3143	0.2143	0.2000
H exp	0.6509	0.5411	0.5240
Fis	+0.519	+0.606	+0.620
P_{HW}	0.0000	0.0000	0.0000
Pscore	0.0026	0.0102	0.0000

Table 29 continued			
Cga06-1			
H obs	0.3429	0.4286	0.6143
H exp	0.5018	0.6659	0.6297
Fis	+0.318	+0.358	+0.025
P_{HW}	0.0000	0.0000	0.0006
Pscore	0.0064	0.0000	0.0002
D33-2			
H obs	0.1286	0.2857	0.6143
H exp	0.1697	0.2727	0.4862
Fis	+0.244	-0.048	-0.266
P_{HW}	0.0938	1.0000	0.0457
Pscore	0.0938	0.7617	0.9940
D38-1			
H obs	0.7143	0.6000	0.7429
H exp	0.5467	0.5831	0.5621
Fis	-0.310	-0.029	-0.325
P_{HW}	0.0002	0.9211	0.0006
Pscore	0.9651	0.5632	0.9859
Mean Overall Loci			
H obs	0.4179	0.5018	0.4964
H exp	0.5710	0.6287	0.6079
Fis	-	-	-
$P_{(0.95)}$	1.000	1.000	1.000
$P_{(0.99)}$	1.000	1.000	1.000
A_n	4.6250	4.8750	4.3750

H obs = Observed heterozygosity
H exp = Expected heterozygosity
Fis = Inbreeding coefficient
P_{HW} = Probability value of significant deviation from HWE
Pscore = Probability value of significant heterozygosity deficiency
$P_{(0.95)}$ = Polymorphism at 0.95 criteria
$P_{(0.99)}$ = Polymorphism at 0.99 criteria
A_n = Mean number of alleles per locus

Table 31. F-statistics and gene flow (Nm) for overall populations

Microsatellite Locus	Sample Size	F_{IS}	F_{ST}	Nm
Phy01	210	0.0023	0.1390	1.5085
Phy05	210	0.0766	0.1088	2.0477
Phy07-1	210	0.6666	0.0886	2.7934
Cma3	210	0.3227	0.1137	1.9487
Cma4-2	210	0.5724	0.0827	2.7729
Cga06-1	210	0.2235	0.0824	2.7839
D33-2	210	-0.1157	0.1196	1.8403
D38-1	210	-0.2246	0.0869	2.6268
Mean	**210**	**0.2110**	**0.1055**	**1.5386**

Table 32. Fisher's pair wise F_{ST} (above diagonal) and their significant levels (below diagonal) for *Horabagrus brachysoma* -microsatellite markers

Populations	Meenachil	Chalakkudy	Nethravathi
Meenachil	----	0.04544	0.21892
Chalakkudy	P<0.0001	-----	0.18650
Nethravathi	P<0.0001	P<0.0001	----

Table 33a. Probability tests for pairwise populations for each locus (*P<0.05,P<0.0001). (Mn: Meenachil; Ch: Chalakkudy; Ne: Nethravathi)**

Locus	Populations	P-value	S.E.
Phy01	Mn & Ch	0.0000**	0.0000
Phy01	Ch & Ne	0.0000**	0.0000
Phy01	Mn & Ne	0.0000**	0.0000
Phy05	Mn & Ch	0.0000**	0.0000
Phy05	Ch & Ne	0.0000**	0.0000
Phy05	Mn & Ne	0.0000**	0.0000
Phy07-1	Mn & Ch	0.8447	0.0054
Phy07-1	Ch & Ne	0.1098	0.0072
Phy07-1	Mn & Ne	0.0830	0.0067
Cma3	Mn & Ch	0.0016*	0.0008
Cma3	Ch & Ne	0.0000**	0.0000
Cma3	Mn & Ne	0.0000**	0.0000
Cma4-2	Mn & Ch	0.0000**	0.0000
Cma4-2	Ch & Ne	0.0040*	0.0010
Cma4-2	Mn & Ne	0.0000**	0.0000
Cga06-1	Mn & Ch	0.0000**	0.0000
Cga06-1	Ch & Ne	0.0000**	0.0000
Cga06-1	Mn & Ne	0.0000**	0.0000
D33-2	Mn & Ch	0.0900	0.0042
D33-2	Ch & Ne	0.0000**	0.0000
D33-2	Mn & Ne	0.0000**	0.0000
D38-1	Mn & Ch	0.0086*	0.0013
D38-1	Ch & Ne	0.0006*	0.0002
D38-1	Mn & Ne	0.0000**	0.0000

Markov chain parameters:- demomorization: 1000, batches: 100 and iterations: 1000

Table 33b. Test for genetic homogeneity- Probability tests for overall populations (*P<0.05, **P<0.0001; significant after Bonferroni adjustments for multiple tests). (Mn: Meenachil; Ch: Chalakkudy; Ne: Nethravathi)

Locus	P-value	S.E.
Phy01	0.0000	0.0000
Phy05	0.0000	0.0000
Phy07-1	0.0006	0.0003
Cma3	0.0000	0.0000
Cma4-2	0.0000	0.0000
Cga06-1	0.0000	0.0000
D33-2	0.0000	0.0000
D38-1	0.0000	0.0000
Overall	0.0000	-----

Markov chain parameters:- demomorization: 1000, batches: 100 and iterations: 1000

4.3.9. Genetic distance and similarity

Nei's (1978) unbiased genetic identity and distance estimated between pairs of three populations of *H. brachysoma* are presented in Table-34. The genetic distance between Meenachil and Chalakkudy was 0.0756, between Chalakkudy and Nethravathi was 0.2144 and Meenachil and Nethravathi were 0.3055. These results agreed with the geographic distances between the populations (Table-34).

Table 34. Genetic identity (above diagonal) and Genetic distance (below diagonal) using microsatellite markers in *H. brachysoma* (Nei, 1978), Geographical distances (in KM) are given in bracket

Populations	Meenachil	Chalakkudy	Nethravathi
Meenachil	****	0.9272	0.7368
Chalakkudy	0.0756 (140)	****	0.8070
Nethravathi	0.3055 (520)	0.2144 (380)	****

4.3.10. Dendrogram

On the basis of Nei's genetic distance values and UPGMA dendrogram was constructed. The cluster values indicated distinct relationship between the 3 populations of *H. brachysoma* (Fig.46). The bootstrap values suggested the populations have a robust cluster.

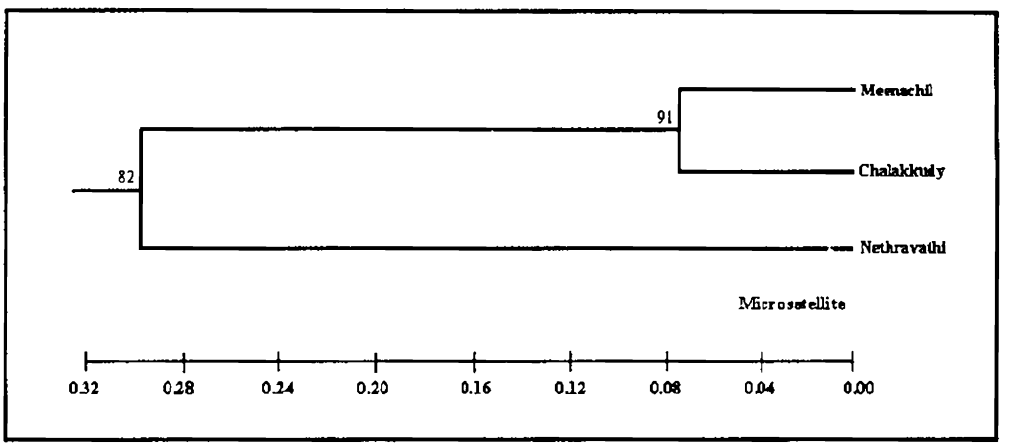

Fig. 46. Dendrogram resulting from UPGMA analysis of microsatellite of 3 populations of *H. brachysoma* based on pair-wise values for Nei's unbiased genetic distance and following 100 bootstrapping replications. Bootstrap estimate (as percentage) is indicated left side of each branch.

4.4. Comparative assessment of results of three markers

The results derived by using three markers *viz,* allozymes, RAPD and microsatellites in *H. brachysoma* are compared as follows:

4.4.1. Number of loci and alleles

The number of loci obtained was 25 in allozymes; 124 in RAPD; and 8 in microsatellites. Altogether 44 alleles were obtained in allozymes and 40 alleles in microsatellites (Table-35).

4.4.2. Percentage of polymorphic loci

In allozymes, 14 loci out of the 25 were polymorphic (56%); in RAPD analysis, 75 (60.48%) were polymorphic and in microsatellites, all the 8 loci were polymorphic (100%) (Table-35).

4.4.3. Observed and expected heterozygosities

In allozymes, the observed heterozygosity (Hob) was 0.1779 and expected heterozygosity (Hex) was 0.4276. For microsatellites, Hob and Hex were 0.4720 and 0.6486 respectively. In RAPD, the average heterozygosity or gene diversity (H) was 0.2222 (Table-36).

4.4.4. Private alleles

In allozyme analysis, 7 private alleles were identified, while in RAPD, 8 alleles were specific to different populations. In microsatellites, only two private alleles (both in Chalakkudy population) were obtained.

4.4.5. Genetic differentiation and gene flow (Nm)

The co-efficient of genetic differentiation (F_{ST} or G_{ST}) values for allozymes, RAPD and microsatellites were 0.1537, 0.5060 and 0.1055 respectively (Table-36).

The gene flow or migration rate (Nm) for allozymes, RAPD and microsatellites were 1.3760, 0.4880 and 1.5386 respectively (Table-36).

Table 35. Comparative assessment of results with three markers in *H. brachysoma*

Markers	No. of enzymes/ primers checked	No. of enzyme/ primers used	No. of loci	No. of alleles	No of polym-orphic loci	Percentage of polymorp-hic loci
Allozymes	25	14	25	44	14	56
RAPD	80	10	124	---	75	60.48
Microsatellites	25	8	8	40	8	100

Table 36. Comparison of heterozygosity, genetic differentiation (F_{ST}/ G_{ST}) and gene flow (Nm) among populations using three markers in *H. brachysoma*

Markers	Heterozygosity		F_{ST} / G_{ST}	Nm
	Hobs	Hexp		
Allozymes	0.1779	0.4276	1.3760	0.1537
RAPD	0.2222		0.5060	0.4880
Microsatellites	0.4720	0.6486	1.5386	0.1055

4.4.6. Genetic distance and similarity

The comparison of pair-wise Nei's genetic distances and F_{ST} values for allozymes and microsatellites is given in Table-37. The pair-wise genetic distance values between Meenachil and Chalakkudy for allozymes, RAPD and microsatellites were 0.0299, 0.1347 and 0.0756 respectively. The genetic distance values between Chalakkudy and Nethravathi for allozymes, RAPD and microsatellites were 0.0899, 0.2113 and 0.2144 respectively, while the same between Meenachil and Nethravathi for allozymes, RAPD and microsatellites were 0.0927, 0.2876 and 0.3055 respectively. The pair-wise F_{ST} values between Meenachil and Chalakkudy populations of *H. brachysoma* using allozymes and microsatellites were 0.0952 and 0.0454 respectively; between Chalakkudy and Nethravathi 0.2418and 0.1865 respectively and between Meenachil and Nethravathi 0.2640 and 0.2189 respectively.

The UPGMA based dendrogram constructed using the three different genetic markers are compared in Fig. 47.

Table 37. Comparisons of pair-wise genetic distances and F_{ST} among populations using three class of markers

Population pair	Allozyme		RAPD	Microsatellite	
	Nei's Genetic distance	Pairwise F_{ST}	Nei's Genetic distance	Nei's Genetic distance	Pairwise F_{ST}
Meenachil & Chalakkudy	0.0299	0.0952	0.1347	0.0756	0.0454
Chalakkudy & Nethravathi	0.0899	0.2418	0.2113	0.2144	0.1865
Meenachil & Nethravathi	0.0927	0.2640	0.2876	0.3055	0.2189

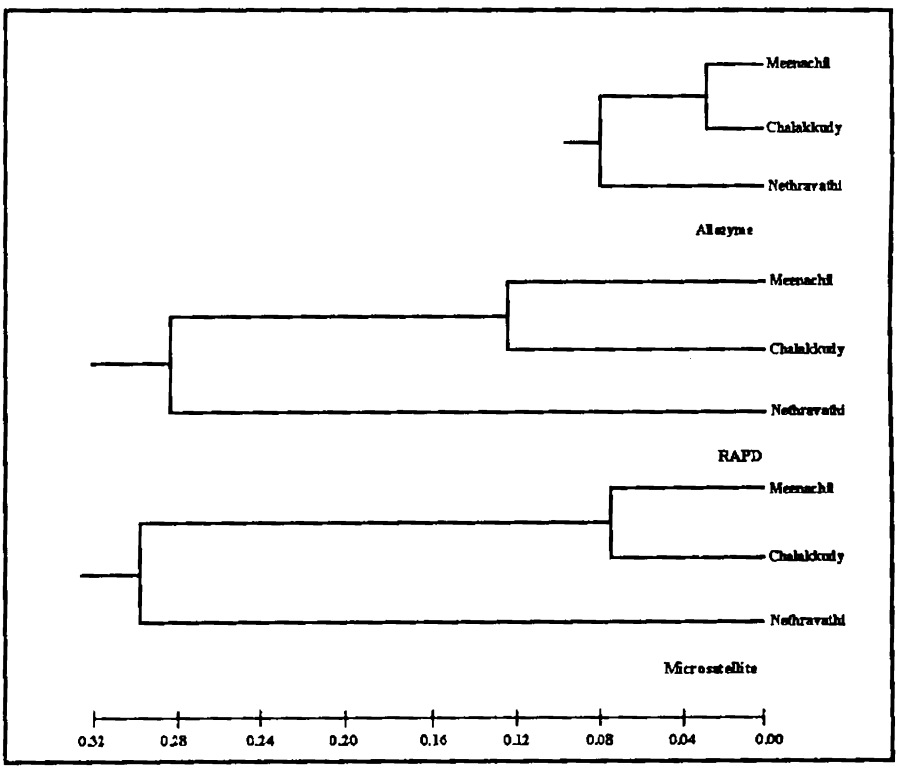

Fig. 47. Comparison of UPGMA dendrograms of allozyme, RAPD and microsatellite markers in *H. brachysoma*. The values at nodes represent bootstrap confidence levels (1000 replications).

5

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, G_6PDH, 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) G_6PDH 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 ($P_{0.95}$) across populations was 0.56 (56%) in *H. brachysoma*. The value is greater than those in other catfishes like *Clarias gariepinus* ($P_{0.95}$ = 48%) and *C. anguillaris* ($P_{0.95}$ = 28%, Rognon *et al.* (1998), but lower than that of *Pangasius* species ($P_{0.95}$ = 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 $P_{0.95}$ 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. maroceana* (Berrebi *et al.*, 1995); 27% in Pacific herring (Grand and Utter, 1984); 50% in *Cyprinus carpio* (Kohlmann and Kersten, 1999); 28% in *Alphanius facsciatus* (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 (H_{obs}) 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 H_{obs} 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 H_{obs} 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 (H_{obs}) values obtained in the present study in *H. brachysoma* are lower than that of the expected values (H_{exp}), indicating the deficiency of heterozygotes except in one or two loci in each population. All the loci except *G_3PDH**, *G_6PDH** 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 F_{IS} (inbreeding coefficient) figures were found to deviate significantly from zero in several loci in all

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 logifilis*. 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 *G_6PDH-1* specific to Parana River population and the other in *MDH-A_2* 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-1* 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,* = 0.578 (Mattagliati, 1998); and *Tor malabaricus* = 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 con-specific populations of marine and freshwater fish (D = 0.05 and Ĩ=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.*, 19980. 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 Morocco 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; Ihssen *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 a 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. brahcysoma* 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; Lehmann *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

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).

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

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 ± 9.1 for an average allele length of 23.0 ± 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 F_{IS} 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 homozygosity 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 Nm (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 Nm value indicated 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 (Donnelly *et al.,* 1999; Kang and Chung, 1997). Many authors reported a higher value of Nm in various fish and shellfish species (Taylor *et al.*, 2001; in cichlids, Nm = 3.36) and in *Anopheles arabiensis* (Nm = 3.37, Donnelly *et al.*, 1999). However, low values of Nm 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 (*eg.* 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 (*eg.* 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^{0}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.

6

SUMMARY

- *Horabagrus brachysoma* (Gunther) an endemic, cultivable catfish belonging to Family Bagridae is found in rivers originating from southern part of the biodiversity hotspot – the Western Ghats. This species is categorized as "endangered" based on latest IUCN criteria, due to restricted distribution, loss of habitat, over exploitation, destructive fishing practices and trade (Anon., 1998).

- The species is shortlisted for taking up stock-specific, propagation-assisted rehabilitation programme in rivers where it is naturally distributed. Information on the basic genetic profile and stock structure, which is essential for the fishery management, conservation and rehabilitation of this species, is lacking. Hence, the present work was undertaken to identify molecular genetic markers like allozymes, RAPD and microsatellites in *H. brachysoma* and to use them in analyzing the population genetic structure of this species, collected from three geographically isolated river systems *viz.*, Meenachil, Chalakkudy and Nethravathi in South India.

- In allozymes, the screening was carried out to detect 25 enzymes, but only 14 showed their presence with scorable activity. Out of fourteen enzymes studied, 12 enzymes were polymorphic and two enzymes were monomorphic. The fourteen enzymes yielded 25 scorable loci in all populations. A total of 33 alleles were obtained in overall populations.

- The mean observed heterozygosity (Hobs) for overall populations using allozymes was 0.1779. In Meenachil population, the mean observed and expected heterozygosities were 0.1724 and 0.3465 respectively. Whereas, in Chalakkudy population, the mean of observed heterozygosity was 0.1908 and the expected

heterozygosity was 0.3969. In Nethravathi population, the observed and the expected heterozygosities were 0.1704 and 0.3475 respectively.

- Seven private alleles were recorded in allozyme analysis. Out of these seven private alleles, Chalakkudy population represented 3 and the rest represented by Nethravathi population. No private alleles were obtained in Meenachil population.

- In all the three populations, the probability test provided the evidence that the observed heterozygosities in most of the loci significantly deviated ($P<0.05$) from that expected under Hardy-Weinberg equilibrium, except in *G_3PDH**, *G_6PDH** and *SOD** in Meenachil population; *EST-3** in Chalakkudy population and *EST-2** and *PGM** in Nethravathi population. The F_{IS} (inbreeding coefficient) deviated from zero in most of the loci in all the 3 populations, indicating deficiency of heterozygotes. No allozyme locus showed linkage disequilibrium.

- In F-statistics F_{ST} represents the standardized genetic differentiation among the populations. F_{ST} for overall populations was 0.1537, indicating that 15.37 genetic variation exists among populations. The 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.

- Genetic identity value between the populations Meenachil and Chalakkudy was 0.9705; between Chalakkudy and Nethravathi 0.9140; and between Meenachil and Nethravathi 0.9115. This indicates the Meenachil and Chalakkudy populations are closely related compared to Nethravathi population. The genetic distance values agreed with the geographic distances. On the basis of Nei's (1978) genetic distance values, phylogenetic relationships among three populations of *Horabagrus brachysoma* were established by constructing a dendrogram.

- In RAPD analysis, 32 oligonucleotide primers were selected from 80 primers (4 kits-20 primers each from kit OPA, OPAA, OPAC and OPAH) in primary screening, however, only 10 primers were selected based on repeatability,

sharpness and intensity of bands *viz.*, OPA-07, OPA-09, OPA-11, OPA-20, OPAC-14, OPAH-01, OPAH-02, OPAH-04, OPAH-08 and OPAH-09 for population genetic analysis. A total of 124 were detected consistently with all 10 decamer primers in three populations. The size of the fragments ranged from 150 to 3000bp. The number of fragments generated per primer varied from 6 to 19. Of the 124 RAPD fragments, 49 (39.51%) were found to be shared by individuals of all three populations. The remaining 75 fragments were found to be polymorphic (60.48%). In Meenachil population, a total of 112 different fragments were detected consistently; in Chalakkudy population 109 fragments and in Nethravathi population a total of 110 fragments were detected.

- Eight RAPD fragments were identified as stock specific markers in 5 primers. These eight fragments were exclusive to a particular population.

- The average gene diversity or heterozygosity (H) was 0.1975 among the populations for each primer and for overall primers, it was 0.2222. For Meenachil population, the mean H was 0.1115 for each primer and for overall primers the average value of H was 0.1100. The mean value of H in Chalakkudy population was 0.1051 for each primer and for overall primers, the average value was 0.1053. In Nethravathi population, the values of mean H were 0.1101 and 0.1097 for each and overall primers respectively. No RAPD loci showed linkage disequilibrium.

- The average value of coefficient of differentiation (G_{ST}) was 0.5060 for overall primers among populations. The average value of gene flow (Nm) for overall primers was 0.4880.

- Nei's (1978) unbiased genetic identity and distance were estimated between pairs of three populations of *H. brachysoma*. Based on the genetic distance value, a dendrogram depicting the phylogenetic relationships among three populations of *H. brachysoma* were constructed following unweighted pair group method using arithmetic averages (UPGMA, Sneath and Sokal, 1973). The results and pattern of

dendrogram were concordant with that obtained from allozyme analysis in this species.

- For Microsatellite analysis, twenty-five primers, from various resource species belonging to orders Siluriformes and Osteoglossiformes, were used for cross-species amplification in *H. brachysoma.* Eight primers out of 25 gave scorable banding pattern after PCR amplifications. These 8 primers produced 13 presumptive microsatellite loci. These 8 loci *viz., Phy01, Phy05, Phy07-1, Cma3, Cma4-2, Cga06-1, D33-2* and *D38-1* were confirmed to contain repeats after sequencing. All the 8 microsatellite loci were polymorphic (100%).

- A total of 40 alleles were produced in microsatellite analysis across all the populations. In Meenachil population, the number of observed alleles was 37 whereas in Chalakkudy it was 39. In Nethravathi stock, 35 alleles were exhibited.

- There were two private alleles in microsatellites. These two alleles were found in Chalakkudy population.

- The mean observed and expected heterozygosities in overall population were 0.4720 and 0.6486 respectively. In Meenachil population, the mean value of observed heterozygosity (H_{obs}) was 0.4179 and the mean expected heterozygosity (H_{exp}) for this population was 0.5710. In Chalakkudy population, the mean value of observed and expected heterozygosities were 0.5018 and 0.6287 respectively. In Nethravathi population, the mean observed heterozygosity was 0.4964 and the mean expected heterozygosity was 0.6079. None of the microsatellite loci showed linkage disequilibrium.

- The probability test provided the evidence that the observed heterozygosity values in most of the loci showed significant deviation ($P<0.05$) from Hardy-Weinberg Equilibrium except for *Phy01* and *D33-2* in Meenachil population; *D33-2* and *D38-1* in Chalakkudy population; and *Phy05* in Nethravathi population as a result of heterozygote deficiency. The mean value of F_{IS} for overall populations was

0.5072. The positive value of F_{IS} at almost all the loci indicated inbreeding in populations of *H. brachysoma*. Analysis of data using MICRO-CHECKER indicated absence of general excess of homozygotes over most of the allele size classes and ruled out occurrence of null alleles in all the 3 populations for the 7 primer pairs.

- The pair-wise F_{ST} estimates between populations and the overall value (0.1055) were highly significant ($P<0.0001$) indicating a significant level of genetic differentiation among the populations. The mean value of gene flow or migration rate (Nm) for overall population was 1.5386.

- Nei's (1978) unbiased genetic identity and distance were estimated between pairs of three populations of *H. brachysoma* and on the basis of these values, a UPGMA dendrogram was constructed. The genetic distance values and the pattern of dendrogram were consistent with that obtained from allozyme and RAPD analyses using the same samples.

- In conclusion, the genetic markers (allozyme, RAPD and microsatellite) were found to be powerful tools to analyze the population genetic structure of the yellow catfish. These three classes of markers demonstrated striking genetic differentiation between pairs of fish populations examined. Geographic isolation by land distance is likely to be the factor that contributed to the restricted gene flow between the river systems. The inbreeding as a result of over-exploitation might be one of the reasons for the deficiency of heterozygosity revealed by the two co-dominant markers, allozyme and microsatellites. The study emphasizes the need for stock-wise, propagation assisted-rehabilitation of the natural populations of yellow catfish.

7

CONCLUSION

7.1. Suggestions for conservation and management of natural populations of *Horabagrus brachysoma* based on the present findings

The present study has generated important information on the genetic variation and stock structure of the endangered yellow catfish, *Horabagrus brachysoma,* endemic to the Western Ghats. Three genetically discrete stocks of the species have been identified for the first time using allozymes, RAPD and microsatellite markers and it is a significant step towards realizing the goal of management of fishery and conservation of the yellow catfish populations in the rivers of the Western Ghats region. The differentiation of a species into genetically distinct populations is a fundamental part of the process of evolution and it depends upon, physical and biological forces such as migration, selection, genetic drift, geographic barriers etc. Endangered species will have small and / or declining populations, so inbreeding and loss of genetic diversity are unavoidable in them. Since inbreeding reduces reproduction and survival rates, and loss of genetic diversity reduces the ability of populations to evolve to cope with environmental changes, Frankham (2003) suggested that these genetic factors would contribute to extinction risk especially in small populations of threatened species. With the loss of a population/ genetic stock, a species also loses its members adapted and evolved to survive in particular habitat. Hence, conservation and fishery management strategy need to be stock-specific. The results of the present study point out the need to identify the most suitable conservation and management prescriptions for the genetically distinct populations of endangered *H. brachysoma.*

Low genetic variability (heterozygote deficiency and deviation from Hardy-Weinberg equilibrium) coupled with inbreeding (positive value of F_{IS}) was observed in this study in all the three populations of yellow catfish with different types of

genetic markers, which can be the consequence of genetic bottleneck, resulting from over-exploitation and habitat (Anon., 1998). As these factors would lead to a reduction in reproductive fitness (Padhi and Mandal, 2000), efforts to increase the genetic diversity of yellow catfish should be given high priority for conservation of the species, based on genetic principles as mentioned below:

- The effective population size (Ne) should be maintained as large as possible to maximize the contribution of a large number of adults for reproduction so as to maintain natural genetic variability.
- The causative factors that reduce the effective population size such as over-exploitation should be controlled at the earliest.
- No artificial gene flow between distinct stocks should be created by means of haphazard stocking and rehabilitation programmes.
- The rehabilitation strategy should also include means (screening the population, using genetic markers) to monitor impact of such programme.

To attain these objectives, it is essential (i) to protect the populations and habitat against anthropogenic stress and (ii) enhance the population through propagation assisted stock-specific rehabilitation programmes.

7.1.1. Approaches for *in-situ* conservation

- Regulation of human activities either self-imposed (public understanding and awareness through education) or state imposed (formulation and implementation of suitable laws).

- State imposed-law should have following measures
  i. Imposing ban on fishing practices targeted for yellow catfish, particularly during breeding seasons.

  ii. Stock assessment of *H. brachysoma* in different rivers and imposing quota systems for maintaining the population size.

  iii. Banning the sale of under-sized yellow catfish specimens.

iv. Restrict the fishing gear for not catching small and immature yellow catfish and prevent the use of explosives and chemicals for fishing.

v. Maintaining minimum water level in the rivers (in case there are dams and weirs) and declaring certain stretches of rivers as sanctuaries.

7.1.2. Action plan suggested for 'propagation-assisted, stock-specific restocking' of yellow catfish with the help of 'supportive breeding programme'

The natural populations of this endangered species can be enhanced by 'supportive breeding'. In this programme, a fraction of the wild parents is bred in captivity and the progeny are released in natural waters.

1. Brood stock of yellow catfish collected from different rivers must be tagged and maintained in separate ponds in the holding facility.

2. The existing farm and hatchery facilities at the Regional Agricultural Research Station (RARS) of Kerala Agricultural University, Kumarakom, Kerala can be used, for the supportive breeding of Chalakkudy and Meenachil stocks of yellow catfish. Similar facility at the College of Fisheries, Kankanadi (Mangalore), Karnataka can be utilized for breeding the Nethravathi stock.

3. Effective breeding population size and sex ratio should not be restricted. To achieve this, collection of different size / year classes at different time intervals to be preferred over the same size / year class.

4. Use of cryopreserved milt, collected from different males and pooled, would be useful for increasing the effective population size and recovery of endangered populations of yellow catfish. In comparison to the captive breeding programme, the gene banking through sperm cryopreservation is relatively cheaper, easy to maintain, less prone to risk due to system failure or mortality due to diseases. Therefore, it should serve as a useful adjunct to the captive breeding programme.

5. Different genetic stocks should be bred separately and ranched in the same rivers from where they are collected.

6. Stretches of rivers harbouring resident population or that can serve as a potential sanctuary, may be selected for ranching of yellow catfish populations.

7. Assessing the impact of ranching through monitoring the parameters like catch per unit effort / area through experimental fishing.

8. Changes in genetic variation *i.e.* allele frequencies especially the occurrence of rare alleles over a course of time. It will be useful to keep base genetic profile of representative samples of fish stocked in the holding facility and those used for ranching. Microsatellite markers and the baseline data generated in this study can be helpful in further assessing the impact of genetic variation.

7.2. Current status of *ex-situ* conservation of *H. brachysoma*

- *H. brachysoma* collected from wild was successfully bred in captivity for the first time by NBFGR in collaboration with the RARS, Kumarakom. The experiments gave high percentage of hatching and larval survival. The fingerlings and the spawners were reared in captivity and were successfully used in the consecutive years again for breeding.

- Protocol for successful milt cryopreservation of yellow catfish was developed by the NBFGR - RARS team that gave high hatching rates (92% of control) and larval survival (81% of control). Milt collected from more than 300 healthy males were pooled (population wise), cryopreserved and maintained in the gene bank. (Milt cryopreservation as a means of *ex-situ* conservation is not an ideal option for majority of the catfishes and especially for the endangered species. Males have to be sacrificed, as milt can not be collected by stripping even after hormonal administration. Interestingly, *H. brachysoma* may be the only catfish in the world that will release copious amount of milt on stripping the males, a couple of hours after hormonal intervention!).

- The life history traits of the species were also studied in detail.

In conclusion, the markers and stock structure data generated in the study can provide an essential component for formulating meaningful conservation strategies of yellow catfish as mentioned above. This, along with the existing technology on captive breeding and sperm cryopreservation can be integrated into a package for conserving genetic diversity and rehabilitation of the natural populations of *H.brachysoma.*

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8

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