MOLECULAR GENETIC CHARACTERIZATION OF ENDEMIC RED - TAILED BARB,

Gonoproktopterus curmuca (Hamilton - Buchanan, 1807)

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

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by

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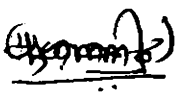
April, 2008

DECLARATION

I hereby declare that this Ph.D thesis entitled "**MOLECULAR GENETIC CHARACTERIZATION OF ENDEMIC RED - TAILED BARB, *Gonoproktopterus curmuca* (Hamilton - Buchanan, 1807)**" is the authentic and bonafide record of the research work done by me at National Bureau of Fish Genetic Resources (NBFGR) Unit, Central Marine Fisheries Research Institute (CMFRI), Cochin and it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.



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CERTIFICATE

This is to certify that the thesis entitled, "**MOLECULAR GENETIC CHARACTERIZATION OF ENDEMIC RED - TAILED BARB, *Gonoproktopterus curmuca* (Hamilton - Buchanan, 1807)**" is an authentic record of the original and bonafide research work carried out by **Sri. K.K. Musammilu (Reg. No. 2582)** at National Bureau of Fish Genetic Resources (NBFGR) Unit, Central Marine Fisheries Research Institute (CMFRI), Cochin 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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Kochikkaran Kunjumohammed **MUSAMMILU**
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CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page No.

LIST OF ABBREVIATIONS

µg	Micrograms
µl	Microlitre
AAT	Aspartate Amino Transferase
AFLP	Amplified Fragment Length Polymorphism
AK	Adenylate Kinase
AMOVA	Analysis of Molecular Variance
APS	Ammonium persulphate
bp	Base pairs
BPB	Bromo Phenol Blue
CAGE	Cellulose Agarose Gel Electrophoresis
CAMP	Conservation Assessment Management Plan
CK	Creatine Kinase
CMFRI	Central Marine Fisheries Research Institute
CUSAT	Cochin University of Science and Technology
Da	Dalton
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
GPI	Glucose phosphate isomerase
H	Average gene diversity or heterozygosity
H_{obs}	Observed heterozygosity
H_{exp}	Expected heterozygosity
HWE	Hardy-Weinberg Equilibrium
IAM	Infinite allele mutation model
IUCN	International Union for Conservation of Nature and Natural Resources
KFRI	Kerala Forest Research Institute
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase
ME	Malic enzyme

MM	Mission Mode
MFRs	Microsatellite Flanking Regions
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	Nanograms
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
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
SMM	Stepwise mutation model
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
TPM	Two Phased Mutation model
UPGMA	Unweighted Pair-Group Method with Arithmetic Mean
VNTRs	Variable Number of Tandem Repeats
XDH	Xanthine dehydrogenase

Chapter 1

INTRODUCTION

Contents

The Western Ghats is 1600Km long, unbroken chain of mountains along the west coast of Peninsular India. Geographically the Western Ghats (steps of a staircase) extends from the mouth of the River Tapti (in Gujarat; about 8^0N) to the tip of south India (Kanyakumari, Tamil Nadu; about 21^0N). It has been rightly recognized as one of the 34 globally identified 'hot spot' areas of mega bio-diversity for conservation and one of the three such areas in the country. With respect to freshwater fish species, the streams and rivers originating from the Western Ghats have been identified as one of the few sites in the world exhibiting high degree of endemism and exceptional bio-diversity (Myers *et al.*, 2000). There are around 326 species of primary and secondary freshwater fishes in the Western Ghats of which nearly 69% (228 species) are endemic to the region (Gopalakrishnan & Ponniah, 2000).

The family Cyprinidae is the largest of freshwater fishes and, with the possible exception of Gobiidae, the largest family of vertebrates (Nelson, 1994). The common name for the family most frequently used in North America is minnow, while in Eurasia it is carp. Various members of this family are important as food fish, as aquarium fish, and in biological research (Nelson, 1994). In this study, a fish species from this family exclusively found in the west flowing rivers originating from the Western Ghat region – *Gonoproktopterus curmuca* – was taken for population genetic analysis.

In spite of rich piscine diversity in the Western Ghats region, practically no attention has been paid for the stock assessment, sustainable utilization and conservation of these species. Several endemic food and ornamental fishes of the region have been enlisted as endangered, either due to over exploitation, gratuitous 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 of the situation. These waters are also considered as the gold mine for nearly 110 endemic ornamental fishes like loaches, bagrid catfishes and cyprinids. But, recent

surveys reported their alarming rate of depletion due to over-exploitation and clandestine export (Ponniah and Gopalakrishnan, 2000). It is noteworthy that steps have been initiated to conserve the endemic food and ornamental teleosts of the region through propagation assisted rehabilitation programme by the National Bureau of Fish Genetic Resources (NBFGR), Lucknow (Annamercy *et al.*, 2007). *G. curmuca* is one of the prioritized species for the rehabilitation programme.

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 was an urgent need for restoration ecology by 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). These natural units of a species can otherwise be called as 'stocks'. A **stock** (Shaklee *et al.*, 1990b) can be defined as "a panmictic population of related individuals within a single species that is genetically distinct from other such populations".

The study of genetic variation in fishes has proven valuable in aquaculture and fisheries management, for identification of stocks, in selective breeding programmes, restoration ecology 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 process and differentiate into genetically distinct sub-populations or stocks in 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 the 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. The dearth of knowledge about the genetic structure of the 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 investigation 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 developed different methodologies to distinguish and characterize the fish stocks and evaluate the genetic variation. One of the traditional methods of distinguishing fish stocks has been the comparative examination of morphological characters (Hubbs and Lagler, 1947). But the conventional morphometric measurements have been graded as inefficient and biased, as they often produced 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 as truss network analysis, where the shape of the body forms of fish or shellfish also was taken into account along with the size (Humphries *et al.*, 1981; Winans, 1984). However, the application of truss network analysis for the identification of stock 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 method (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 co-dominant type (Ayala, 1975). The results of a limited number of studies using allozyme electrophoresis demonstrated that 15-30% of structural gene loci were detectably variable within populations, and that even closely related species showed extensive genetic divergence (Hubby and Lewontin, 1966; Harris, 1966). 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 free to evolve much more rapidly than the coding sequences.

With the advent of thermocyclers the amplification of small fragment of DNA through Polymerase Chain Reaction (PCR) gained popularity. This enabled the users to screen the polymorphism in the DNA of the individuals without sacrificing them. One such technique (Williams *et al.*, 1990 and Welsh and McClelland, 1990) was Random Amplified Polymorphic DNA (RAPD) based on PCR using short single primers of arbitrary nucleotide sequence typically of length of ten (deca(pri)mers) nucleotides that amplified random segments of the genome. The amplified fragments are also inherited in Mendelian fashion, like allozyme markers (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.*, 1995).

Variable Number of Tandem Repeats (VNTRs) include minisatellites and microsatellites. Minisatellites are DNA sequences usually 10-200 bp long that are repeated in tandem at variable number of times. 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 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 between populations of species (O'Connell *et al.*, 1997) and also at individual level especially in forensics and paternity disputes.

The mitochondrial DNA (mtDNA) is another type molecular marker, which revealed 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). Mitochondrial DNA is smaller, 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 followed by sequencing the PCR products have made the examination of mtDNA variations considerably easier and faster. The slow-evolving regions of mtDNA such as 16SrRNA are used to discriminate species and higher levels of taxa while fast evolving zones such as control region (D-loop) and ATPase genes are used in population genetic analysis. Universal vertebrate primers can be used to amplify various mtDNA regions and with the advent of recent mtDNA sequences for several fish species being available, more fish specific primers can be designed.

In brief, the techniques available to screen the variability at different levels of the species organization are many ranging from simple morphometric to molecular genetic methods that can reveal polymorphism at the DNA level. The species that is selected in the present investigation for applying three molecular genetic markers (allozymes, RAPD and microsatellites) is the red-tailed barb, *Gonoproktopterus curmuca* from three rivers (viz., Periyar River, Chalakkudy River, and Chaliyar River) originating from the Western Ghats. The major reasons for selecting this particular species are given below.

Gonoproktopterus curmuca (Figure 01 & 02) belongs to Family Cyprinidae and is endemic to the rivers originating from southern part of the biodiversity hotspot –

the Western Ghats. The species enjoys a good market value as a food fish and fetches Rs.70-100/Kg in Kerala. Owing to its fast growth rate (maximum size 70cm total length), it is one of the potential candidate species for aquaculture practices in the region. Its attractive colour makes it an ideal species for aquarium keeping in India and abroad (fetches US $ 10 per live fish in international market). Till date, stock assessment of the species has not been made in different rivers; hence there is no information about the current exploitable potential of red-tailed barb. However, there has been a massive hunt for the species from wild for aquarium trade since last few years and its drastic decline was recorded in 1997 itself in field surveys. 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 latest IUCN criteria due to restricted distribution, loss of habitat, over exploitation, destructive fishing practices and trade (Anon., 1998). The species was finally short-listed as one of the candidates for stock-specific, propagation assisted rehabilitation and management programme in rivers where it is naturally distributed. In connection with this, captive breeding and milt cryopreservation techniques of the species have been developed by the National Bureau of Fish Genetic Resources (NBFGR), Lucknow. However, for a scientific stock-specific rehabilitation programme, information on the stock structure and basic genetic profile of the species are essential and that is not available in case of *G. curmuca*. In view of the above facts and reasons, the present work was taken up (1) to identify molecular genetic markers like allozymes, microsatellites and RAPDs in *G. curmuca* and, (2) to use these markers to discriminate the distinct populations of the species, if any, in areas of its natural distribution.

1.2 Objective of the study

Population genetic analysis of natural populations of *Gonoproktopterus curmuca* from its distributional range using allozymes, microsatellites and RAPDs.

1.3 Technical programme of the study

a. Identification of allozyme and RAPD markers to be used for stock discrimination of *Gonoproktopterus curmuca.*

b. Identification of microsatellite marker by cross-species amplification of primer sequences of other closely related fish species (derived from available accessions in GenBank or from available literature) for using them as potential genetic markers in *G. curmuca.*

c. The population structure analysis of *G. curmuca* using allozymes, microsatellites and RAPDs.

1.4 Description of the species

1.4.1 Taxonomic status

G. curmuca (Figure 01 & 02) is a freshwater barb described by Hamilton-Buchanan in 1807. The species has following synonyms: *Barbus curmuca, Hypselobarbus curmuca, Puntius curmuca.* The current taxonomic position of *G. curmuca* 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	Cypriniformes
Family	Cyprinidae
Genus	*Gonoproktopterus*
Species	*curmuca*

1.4.2 Confusion over the scientific name of the species

Hamilton-Buchanan (1807) described *Barbus curmuca* from Vedawati River of the Tungabhadra drainage in Mysore, with two barbels, 39 scale rows along the lateral line and a weak and articulated last undivided dorsal ray. Sykes (1840) described *B. kolus* also with the same characteristics from Deccan. Specimens from South Canara with four barbels and the caudal tipped with black, Day (1878) considered as a local variety of *B. curmuca*. But subsequent workers like Hora and Law (1941), Silas (1951) Talwar & Jhingran (1991), Jayaram (1997, 1999) treated the species with four barbels and weak last undivided dorsal ray and 41 - 43 lateral line scales as *Puntius curmuca* (later renamed as *Gonoproktopterus curmuca*) and the other species with 2 barbels and 39 scale rows along the lateral line and slate-colouration as *G. kolus*. This classification was widely followed in the standard taxonomic books (Jayaram, 1999; Talwar & Jhingran, 1991; Shaji *et al.*, 2000). Menon & Rema Devi (1995) later renamed the red-tailed barb from Kerala and South Canara as *Hypselobarbus kurali* which was earlier referred to as *G. curmuca*.

To avoid confusion, in the present study the species name of red-tailed barb (4 barbels, 41-43 scales in the lateral line and caudal tipped black) is retained as *Gonoproktopterus curmuca* (Hamilton – Buchanan, 1807) following the standard fish taxonomy books (Jayaram, 1999; Talwar & Jhingran, 1991; Shaji *et al.*, 2000) and the species is having following diagnostic characters.

1.4.3 Distinguishing Characters

D iv 9; A iii 5; P i 15; V i 8.

Body fairly deep, the dorsal profile convex and the ventral profile nearly horizontal, its depth about four times in standard length. Snout conical; a band of pores on cheeks. Eyes moderate, its diameter about 4.3 times in head. Mouth sub-terminal; barbels two maxillary pairs, lower ones as long as orbit, upper ones half as long. Dorsal fin inserted anterior to origin of pelvic fins, its last un-branched

ray osseous but weak. Scales medium; lateral line with 41 to 43 scales; lateral transverse 3 ½ to 4 ½; pre-dorsal scales 9.

1.4.4 Colour

In life, silvery, lightest on flanks and belly. Caudal fin with blackish tip; in young middle-third of caudal fin orange, tipped with black.

1.4.5 Common names

The species is commonly known as "red-tailed barb" in English and locally called as "*Kooral*" or "*Chundan*" in Malayalam.

1.4.6 Habitat and distribution

Gonoproktopterus curmuca is confined to selected west flowing rivers originating from the Western Ghats in the states of Kerala and Karnataka (South Canara). The species once found in abundance has recorded a sharp decline in the catches due to over-exploitation for ornamental fish trade and for human consumption and is now restricted to a few rivers *viz.,* Nethravathi River, Chaliyar River, Bharathapuzha River, Chalakkudy River, Periyar River, Kallada River, Achankovil River. It is usually recorded from the upper middle stretches of these rivers.

Chapter 2

REVIEW OF LITERATURE

Contents

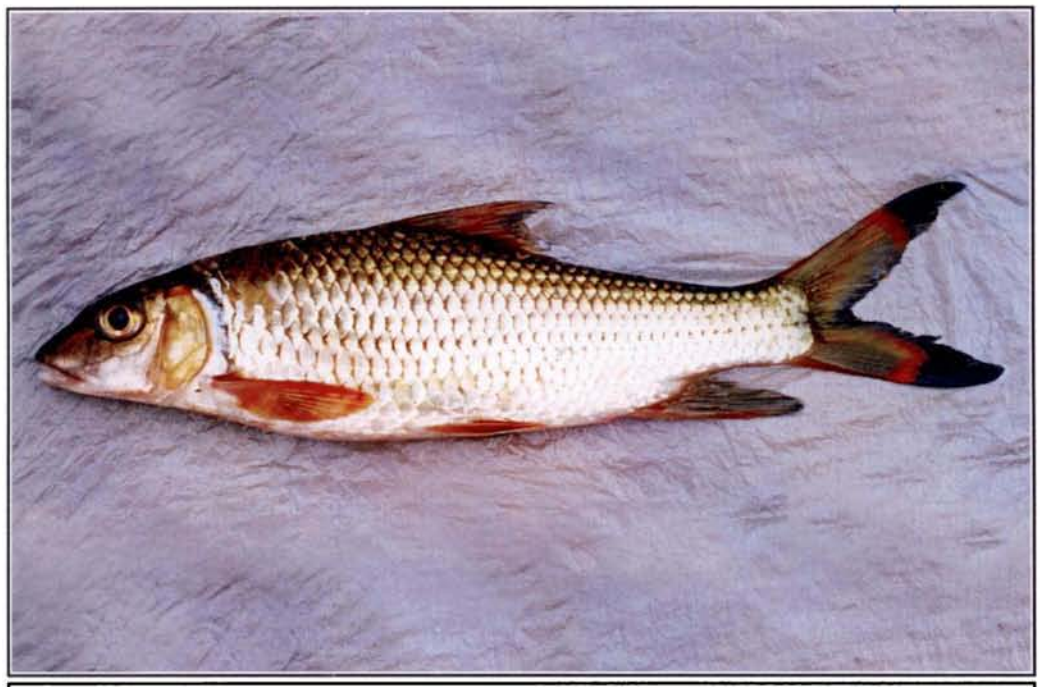

Fig. 1 *Gonoproktopterus curmuca* (Adult)

Fig. 2 *Gonoproktopterus curmuca* (Juvenile)

Population genetics is the study of genetic variation within species and it attempts to understand the processes that result in adaptive evolutionary changes in species through time using the application of Mendel's laws and other genetic principles to entire populations of organisms (Hartl and Clark, 1997). 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 the 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 stocks, 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, 1997).

The micro and macro evolutionary processes both at molecular and organismal levels are incessantly undergoing in all organisms. 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 intra-specific groups were called as 'stocks' and fishery biologists started using these stocks as a basis to manage commercially important marine organisms. Shaklee *et al.* (1990b) 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 stock becomes a critical element (Ihssen *et al.*, 1981a; Fetterolf, 1981).

The genetic variation in population 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, 1997). For the identification of stock structure and genetic variation in populations, Ihssen *et al.* (1981b) suggested that the population parameters and physiological, behavioral, morphometric, meristic, calcareous, cytogenetic and biochemical characters are useful.

Of these, the morphometric investigations are based on a set of measurements of the body form (Hubbs and Lagler, 1947). The 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 the individuals of different stocks (Ihssen *et al.*, 1981b), though the branching patterns for the morphometrics versus the biochemical variation were different. For the selection of the brood stock in genetic improvement programmes of certain penaeids, one or two morphometric variables could be identified, giving accurate estimate of the tail weight (Lester, 1983; Goswami *et al.*, 1986). A study on the Pacific white shrimp, *Penaeus vannamei* from different commercial hatcheries could find significant differences in all the morphometric traits between sites, indicating that the environmental differences affected growth as well as shape of the shrimps (Chow and Sandifer, 1992). But, in a study on the use of canonical discriminant function analysis (DFA) of morphometric and meristic characters to identify cultured tilapias, the results did not support the use of morphometric characters for differentiating the tilapia strains and introgressed hybrids (Pante *et al.*, 1988). These conventional data sets are 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 by 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 the better tool than the former 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 been directly related to particular differences in the genome always (Clayton, 1981). Therefore, new techniques using **genetic markers** came to practice to detect the stock structure and genetic variation among organisms.

2.1 Type 1 Molecular markers

The need to detect genetic variation has fueled the development of novel genetic marker systems in fishery 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 **genetic markers**. 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. They are also used to identify different species in the event of taxonomic disputes and also 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 (**MUs**) or stocks (Moritz, 1994). The relevance of genetic information to species conservation planning has long been recognized (e.g. Lande & Barrowclough, 1987; Simberloff, 1988), and population genetic information has assumed an important role in conservation biology. Estimates of genetic variation within and between populations can provide important information on the level of interaction between local populations and permit assessment of the contribution of a metapopulation structure to regional persistence (reviewed in Hanski, 1999). Genetic markers are also an important tool for identifying population units that merit separate management and high priority for conservation. The definition of independent units for conservation of most widespread use in the last few years is the one of Moritz (1994), although recently it has become a point of debate (Paetkau, 1999; Crandall *et al.*, 2000; Goldstein *et al.*, 2000). Moritz distinguished two types of conservation units, namely management units (MUs), representing populations that are demographically independent, and evolutionary significant units (ESUs), which represent historically isolated sets of populations that are on independent evolutionary trajectories. ESUs are recognized by reciprocal monophyly for mitochondrial DNA (mtDNA) alleles, whereas MUs are recognized by significant divergence in allele frequencies. 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 most 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 (Ne)-contemporary and historical), amount of gene flow (Nm: 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).

In general, genetic markers basically are of 2 types – **protein** (type I) and **DNA** (type II). In 1960's initial studies used 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). New techniques, based on molecular characters to identify the stocks, were also developed in early nineties using arbitrary/conserved primers (Williams *et al.*, 1990, Welsh and McClelland, 1990; Penner *et al.*, 1993; Jeffreys *et al.*, 1985; Tautz, 1989).

Molecular markers can again be classified into two categories: **type I** are markers associated with genes of known function, while **type II** markers are associated with anonymous genomic segments. Under this classification, more RFLP

markers are type I markers because they were identified during analysis of known genes. Like wise, allozyme markers are type I markers, being the protein they encode has known function. RAPD markers and majority of the microsatellite markers are type II markers as these markers are amplified from anonymous genomic regions via the polymerase chain reaction (PCR).

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). The 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 all the 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 the Mendelian fashion (Ayala, 1975). A comprehensive review by Ligny (1969, 1972) shows that the use of isozyme or allozyme study has become useful for the analysis of the 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 are called "allozymes" (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. Sometime non-genetic causes like post-transnational modification and conformational changes also lead to the change in pattern of isozymes (Padhi and Mandal, 2000). Several investigations in the last 25 years have made the use of allozyme analysis to measure parameters such as genetic variability in natural populations, gene flow among populations, process of natural hybridization, species dispersion and phylogenic analysis in animals, plants and microorganisms (Ferguson *et al.*, 1995). Allozyme electrophoresis can give independent estimate level of variation between different populations without an extensive survey of morphological and other quantitative traits (Menezes *et al.,* 1993). Reports on the efficiency of biochemical genetic techniques in revealing the intraspecies allozyme polymorphism and existence of heterogeneous or homogeneous stocks in various species including teleosts are also many (Richardson *et al.,* 1986).

Studies have been successfully carried out to assess levels of genetic differentiation and gene flow at 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 indicated 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 [*Canadian Journal of Fisheries and Aquatic Sciences,* Volume 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 necessary information to adjust regulations according to the stock structure.

Many authors studied the extent of genetic differentiation and population structure using allozyme markers in many 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 in Asian stocks; Phelps *et al.*(1994) in Pacific North West populations; Kondzela *et al.* (1994) compared the stocks of South East Alaska and Northern British Columbia. Allozymes markers were employed in other salmons also 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 the genetic variability in *Hoplias malabaricus* in fluvial and lacustrine environments in the upper Paranas flood plain. Musyl and Keenan (1992) 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 of 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 the populations of *Craterocephalus stercusmuscarum fulvus* and *Craterocephalus stercusmuscarum 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). Haniffa *et al.* (2007) used allozymes to investigate the genetic variability of three *Channa punctatus* populations collected from three south Indian rivers. Gopalakrishnan *et al.* (2006) identified fourteen polymorphic allozyme loci in yellow catfish, *Horabagrus brachysoma.* The genetic variation detected at each allozyme locus was assessed for samples collected from three west flowing rivers of the Western Ghats. The identified loci, which are potential to analyze stock structure of natural

populations of *H. brachysoma*. Engelbrecht and Mulder (1999) examined populations of *Mesobola brevianalis* (the river sardine occurs in Southern Africa) for genetic variation using horizontal starch gel electrophoresis. Gene products of 27 protein coding loci were consistently resolved and revealed polymorphism at five loci.

Survey of relevant literature reveals that work on biochemical genetics of Indian fishes is scanty in comparison to the work done in the rest of the world. Chandrasekhar (1959) has studied the profile of blood proteins of five Indian carps. Krishnaja and Rege (1977, 1979) made 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*. The examples of other important biochemical genetic studies at intraspecific level in India are that of mullet, *Mugil cephalus* (Vijayakumar, 1992; Menezes *et al.*, 1990); oil sardine, *Sardinella longiceps* (Venkitakrishnan, 1992; Menezes, 1994a, b); mackerel (Menezes *et al.*, 1990); Pomfret (Menezes, 1993) hilsa from Ganges (above and below Farakka barrage) and Brahmaputhra Rivers (Lal *et al.*, 2004a) and *Lactarius lactarius* (Gopalakrishnan *et al.*, 2004b). 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 the biochemical genetic techniques are efficient in differentiating the 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 the modern molecular genetic analysis. First of all, the numbers of polymorphic enzyme loci examined are always much less than 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 even variations in nucleotide sequences in very sub samples of DNA fragments (Ayala and Keiger, 1984). Hence, the analysis of base sequences of the DNA is the better alternative in the study of population genetics. Thus, DNA results may have greater implications in fisheries management and conservation of the genetic resources than that provided by biochemical genetic method.

2.2 Type 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). By using the restriction enzymes to cut in the sequence of mtDNA at specific sites, restriction fragment length polymorphisms (RFLP) or sequence analysis of different genes of mtDNA can be used to detect the

phylogenetic relationships, pedigree analysis and population differentiation in many species.

Detection of polymorphisms at 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. 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 the 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 the 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 had added attraction of the need of only extremely small amounts of DNA that led to the usage of this technique in aquaculture and fisheries.

2.2.1 Random amplified polymorphic DNA (RAPDs)

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 inherited in a Mendelian fashion and can be used as genetic markers (Bardakci and Skibinski, 1994; Liu *et al.*, 1999a; Appleyard and Mather, 2002). It 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 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). This 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 the 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 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 decamers and polymers complementary to random repeats. The RAPD technique apart from single copy fraction, also amplifies highly repetitive regions that may accumulate more nucleotide mutation compared with those encoding allozyme, offering a wider potential in 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 been proven to have 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 into the behavior 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). The technique of RAPD has 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; Meneses *et al.*, 1999; Balakrishana, 1995). 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. Some authors have also employed this technique in studies of systematics of numerous plant and animal species (Sultmann *et al.*, 1995; Stothard and Rollinson, 1996).

The technique of RAPD has been used extensively in aquatic organisms such as the penaeid prawn, *Penaeus monodon* as markers for breeding programs (Garcia and Benzie, 1995); in freshwater 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 in the tropical oyster, *Crassostrea belcheri* and in mud crabs (*Scylla spp.*). McCormack *et al.* (2000) reported a comparative analysis of two populations of the Brittle star (*Amphiura filiformis*) by RAPD.

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); intra-specific genetic variation in red mullet (*Mullus barbatus*) (Mamuris *et al.*, 1998) and monitoring of genetic polymorphism in sea bass after acclimatation 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); in the variations between African and American Cichlids (Goldberg *et al.*, 1999); in the Pacific cod *Gadus macrocephalus* to identify its 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). There is now increasing evidence that the RAPD technique, which has been used in different fields, can detect nuclear variation in fish (Borowski *et al*, 1995; Naish *et al*, 1995; Sultmann *et al*, 1995; Bielawski and Pumo, 1997; Caccone *et al*, 1997; Callejas and Ochando, 1998; Mamuris *et al*, 1999; Allendorf and Seeb, 2000). These studies have shown that RAPD is an extremely sensitive method for detecting DNA variation and for establishing genetic relationships in closely related organisms. Nagarajan *et al* (2006) studied the genetic variations between *Channa punctatus* populations collected from three rivers of south India were examined using randomly amplified polymorphic DNA (RAPD). The results of the study demonstrated that Thirunelveli and Quilon populations are more related to each other than to the Coimbatore population.

RAPD analysis has several advantages over the other protocols. These include relatively shorter time (1-2 days) required to complete analysis after standardization, ability to detect extensive polymorphisms, inexpensive,

simplicity, rapidity, need for minute amounts of genomic DNA (≈25ng), random primers required for analysis, simpler protocol and involvement of non - invasive sampling for tissue analysis. There is no need for molecular hybridization and especially it allows the 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. RAPD markers are inherited in Mendelian fashion and usually dominant since polymorphisms are detected as presence or absence of bands after PCR amplification. Polymorphisms result from either size changes in the amplified region or base changes that alter primer binding.

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 of 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 one copy of an allele (homozygotes). In the application of RAPD, it is assumed that populations are under the Hardy-Weinberg Equilibrium, which may not 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). Due to all these factors, many laboratories discontinued work involving RAPDs, even though this marker is still effectively used in microbes, plants, crustaceans and fishes.

2.2.2 Microsatellites

Recently, attention has been 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 is repeated from 2 to several 100 times at a locus. Minisatellites discovered in human insulin gene loci with repeat unit lengths between 10 and 64bp were also referred to as 'Variable Number of Tandem Repeats' (VNTRs) DNA (Nakamura *et al.*, 1987). Microsatellites have a unique length of 1 to 6bp 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 is usually due to increase or decrease of repeat unit copy numbers. These 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 more neutral and variable than allozymes (Queller *et al.*, 1993). Like allozymes, microsatellites alleles are inherited in 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 GenBanks.

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 fishery biology. They are a powerful DNA marker 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 confirm to the assumption of neutrality and have proved 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 polymorphism and sequences in some marine and freshwater fish species for the 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 in population structure in Pacific herring, *Clupea pallasi* collected from 6 sites in Kodiak Island. Similarly, many others reported the polymorphic microsatellite loci to evaluate the 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 of Lake Tanganyika that led to the formation of new species. This is evidenced by the analysis of flanking regions of the single copy nuclear DNA locus (Tmo M27) and 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* and 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 the

microsatellite variation in marine, fresh water and anadromous fishes compared with other animals. Microsatellite DNA variation was used for stock identification of north Atlantic populations of Whiting (Rico *et al.*, 1997); *Oncorhynchus kisutch* (Small *et al.*, 1998a & b); Atlantic salmon (Beacham and Dempson, 1998) and Ayu, *Plecoglossus altivelis* (Takagi *et al.*, 1999a). Microsatellite markers have been studied in cyprinids also. Naish and Skibinski (1998) studied tetranucleotide (TCTA) repeat sequences in Indian major carp, *Catla catla* as potential DNA markers in stock identification. Das and Barat (2002a, b, c) carried out characterization of dinucleotide microsatellite repeats in *Labeo rohita.* Kirankumar *et al.* (2002; 2003) reported that the complete sequence of repeat like region in Indian rosy barb (*Puntius conchonius*).

Although microsatellite DNA analysis through 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 of microsatellite primers. However, there are reports which point to the fact that flanking sequences of some microsatellite loci are conserved within 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 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 cannid species (Gotelli *et al.*, 1994). Similarly, primers developed for in passerine birds were used in studies of a variety of 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 salmon genomic libraries and used for cross-species amplification and population genetic applications in salmon species. May *et al.* (1997) reported the microsatellite genetic variation through cross-species amplification in sturgeons *Acipenser* and *Scaphirhynchus*. Takagi *et al.* (1999b) reported that microsatellite primers isolated from one tuna might be used to amplify microsatellite loci of other tuna 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.*, 1998a & b; 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 fontinalis* 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); whitefish, *Coregonus nasus* (Salmonidae) by using primers of rainbow trout (family: salmonidae) (Patton *et al.*, 1997) and primers of goldfish, *Carassius auratus* in nine species of cyprinids (Zheng *et al.*, 1995). Yue and Orban (2002) developed that 15 polymorphic microsatellite loci in silver crucean 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 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 *L. calbasu*, and sequenced the loci in these species. 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. The cross-species amplification of microsatellite in *Puntius denisonii* by using the primers 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 of other cyprinid fishes (Lal *et al.*, 2004b; Gopalakrishnan *et al.*, 2004a). Twenty-five primers developed for four fish species belonging to the Orders Siluriform and Osteoglossiform were tested and eight primers amplified microsatellite loci in *Horabagrus brachysoma*. The results demonstrate that cross-priming between fish species belonging to different families and even to different orders can yield microsatellite loci (Gopalakrishnan *et al.*, 2006; Muneer, 2005)

The advantages of microsatellites such as short size range, uninterrupted stretches of identical repeat units, high proportion of polymorphism, the insight gained in understanding the mutational process which helps in developing statistical procedures of inter-population comparisons, their abundance in fish genomes, the availability of methodologies in cloning of microsatellites, have all resulted in their abundant use in fisheries research. The tetranucleotide microsatellites are very much useful in 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 genotyping, fluorescent-imaging devices *etc.* (O'Connell and Wright, 1997). Disadvantages of microsatellites are that of the appearance of shadow or stutter

bands, presence of null alleles (existing alleles that are not observed using standard assays); homoplasy; and too many numbers of 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 might produce identical length variants that could compromise microsatellite population level links (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). The genetic analyses of eight microsatellite loci in European bullhead (*Cottus gobio* L.) revealed strong genetic similarities between populations of both sides of the Rhine–Rhône watershed in the Lake Geneva area (Vonlanthen *et al.*, 2007).

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 genotyping using fluorescent-labeled DNA and co-amplification of multiple loci in a single PCR (O'Connell and Wright, 1997; Smith *et al.*, 1997).

2.3 Genetic markers in cyprinids

Genetic markers have been widely used to distinguish stock structure in Cyprinids. Population genetic and phylogenetic analysis using **allozymes** were initiated in cyprinids especially in European barbs in early 1980s. Based on substrate-specificity, Gopalakrishnan *et al.* (1997) characterized different esterases in Indian major carps through polyacrylamide gel electrophoresis (PAGE) and identified species-specific esterase profiles in rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*). Hanfling and Brandl (2000) used allozyme in 23 central European cyprinid taxa to segregate the two sub-families - Alburninae and Leuciscinae - in Cyprinidae. This study suggested

that Alburninae and Leuciscinae should be merged into one sub-family, since allozyme and DNA gave evidence that together this large group may be monophyletic. Carmona *et al.* (2000) reported significant genetic divergence among 10 populations of the Iberian endemic cyprinid *Chondrostoma lemmingii* using 26 loci encoding 19 enzymes and the complete nucleotide sequence of the mitochondrial cytochrome b gene. Carmona *et al.* (1997) also examined nuclear (allozymes) and cytoplasmic genes (mtDNA) to assess the evolutionary origins, relationships, and reproductive modes of diploid and polyploidy forms of *Tropidophoxinellus alburnoides* from western Spain. The multi-locus allozyme data clearly revealed the hybrid nature of all polyploid forms of this fish and some diploid forms as well. Mitochondrial DNA and allozymes were used to determine patterns of genetic variation in populations of *Lepidomeda vittata*, a cyprinid fish native to the Little Colorado River in Arizona by Tibbets *et al.* (2001). *Aphyocypris chinensis* has drastically decreased in Japan and has been designated as an endangered species. Using 12 loci encoding eight allozyme analysis, Ohara *et al.* (2003) tested the genetic diversity of *A. chinensis* strains maintained by five institutions in Japan for conservation purposes. The genetic variations of rohu (*Labeo rohita*, Hamilton) sampled from five hatchery populations (Arabpur, Brahmaputra, Comilla, Kishorganj and Natore of Bangladesh) and three major river populations (the Halda, the Jamuna and the Padma of Bangladesh) were analysed by Khan *et al.* (2006) using electrophoretic analysis of 10 allozymes. The relationship between shoaling behaviour and the genotypic structure of 13 European minnow, *Phoxinus phoxinus*, collected from Dorset and North Wales, UK., was examined by Naish *et al.* (1993) using 13 allozymes. Konishi (2003) investigated the genetic relationships among the three *Pseudorasbora* species and two endangered subspecies, *P. pumila pumila* and *P. pumila subsp.* found in Japan using allozyme analyses and indicated that the level of genetic differentiation between *Pseudorasbora parva* and *Pseudorasbora pumila* was greater than that between the two subspecies.

Microsatellite loci are now commonly used as genetic markers for population genetic studies and to resolve the phylogenetic relationships between different

populations, to classify individuals by relatedness, and for finding quantitative trait loci (QTL). In a study by Das *et al.* (2005) isolated 12 microsatellite loci in rohu by genomic enrichment. These markers great potential in terms of studying genetic variation within and between populations, selective breeding programs as well as gene mapping in fish species were carried out in *L. rohita*, and were amplified and were found 12 polymorphic loci in *L. rohita*. Crooijmans *et al.* (1997) isolated clones containing (CA) repeat from common carp (*Cyprinus carpio* L.) genomic libary and sequenced the clones and described microsatellite markers of the poly (CA) type. The number of repeats found was high compared to mammals but comparable with other teleost fishes. A total of 41 primer sets were designed and thirty two markers were found to be polymorphic and were found to be useful in determining the stock strucuture of *Cyprinus carpio*. Several polymorphic microsatellite markers have been developed and successfully employed to score intra-specific variation in cyprinids. Markers were either generated by microsatellite-enriched genomic libraries or through cross-species amplification. These classes of markers were found to be superior over other and have become extremely popular in a wide-variety of genetic investigations in carps in the recent past (Liu & Cordes, 2004). Mohindra *et al.* (2005) tested 54 primers published for six cyprinid fishes to amplify homologous microsatellite loci in *Labeo dyocheilus*. Fifteen primers yielded successful amplification and seven were polymorphic with 3–9 alleles. The genetic variation detected at these loci exhibit promise for use in fine level population structure analysis of *L. dyocheilus*. Gopalakrishnan *et al.* (2004a) demonstrated successful cross-priming of microsatellite loci in red-tailed barb, *G. curmuca* and identified five polymorphic loci that exhibit promise to determine genetic divergence in natural populations of this species. This will also provide monitoring mechanism against the possible genetic bottlenecks; the populations may be facing and help to plan strategy for rehabilitation of declining natural resources. Saillant *et al.* (2004) examined allelic variation at 22 nuclear-encoded markers (21 microsatellites and one anonymous locus) and mitochondrial (mt)DNA in two geographical samples of the endangered cyprinid fish *Notropis mekistocholas* (Cape Fear shiner).

Genetic diversity was relatively high in comparison to other endangered vertebrates, and there was no evidence of small population effects despite the low abundance reported for the species. Significant heterogeneity (following Bonferroni correction) in allele distribution at three microsatellites and in haplotype distribution in mtDNA was detected between the two localities. Tong *et al.* (2005) in their study, proved a microsatellite locus, MFW1, originating from common carp is highly conserved in flanking nucleotides but variable in repeat length in some fishes from different sub-families of the Cyprinidae. This polymorphic orthologous locus was proved to be a potentially good marker in population genetics of cyprinid species, where no microsatellite markers were available. Chauhan *et al.* (2007) studied the population structure of *Cirrhinus mrigala* from ten rivers belonging to Indus, Ganges, Brahmaputra and Mahanadi basins in India was investigated using allozyme and microsatellite loci. Both markers types demonstrated concordant results and various estimates revealed genetic variability within the subpopulations but surprisingly low level (θ=0.015 to 0.02) of genetic differentiation among *C. mrigala* from different river samples.

RAPD markers were also employed in cyprinids mainly to study genetic variation among isolated riverine populations as well as to generate species-specific molecular signatures (Das *et al.*, 2005). Barman *et al.* (2003) used these markers for studying genetic relationships and diversities in four species of Indian major carps (IMCs: Family-Cyprinidae). Thirty-four arbitrary primers were screened to identify species-specific RAPD markers among rohu (*Labeo rohita*), kalbasu (*L.calbasu*), catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*). Distinct and highly reproducible RAPD profiles with a great degree of genetic variability were detected among species. Genetically heterogeneous populations of *Rutilus rutilus caspicus*, a cyprinid fish species from two geographical areas (Gorgan Bay and Anzali Wetland) in Iran were identified by Keyvanshokooh and Kalbassi (2006) using 10 decamer primers. Callejas and Ochando (2002) used amplified polymorphic DNA (RAPD) markers to estimate the population structure and phylogenetic relationships among the eight species of the genus *Barbus* that

inhabit the Iberian Peninsula. Wolter *et al.* (2003) in their study, using RAPD, analyzed the genetic structure of populations for seven common cyprinid fish species within a 120Km long stretch of the lowland Elbe River, northern Germany. Islam & Alam (2004) used RAPD to assess the genetic variation in three rivers: the Halda, the Jamuna and the Padma (in India) as well as in one hatchery population of the commercially important Indian major carp, *Labeo rohita* and they concluded that the RAPD system may be more useful to generate molecular markers for genetic characterization in the Indian major carp, *L. rohita.* The genetic variation of the endangered freshwater fish *Ladigesocypris ghigii,* endemic to the island of Rhodes (Greece), was investigated for nine populations, originating from seven different stream systems using Random Amplified Polymorphic DNA analysis and the study revealed high levels of inter-population genetic structuring (Mamuris *et al.*, 2005). The work by Basavaraju *et al.* (2007) dealing with the genetic diversity of six stocks of common carp of diverse origin showed that despite the varied origins of the stocks assessed in this study, the level of genetic variation within each stock is low. The whole brood stock of two Hungarian common carp farms—80 and 196 individuals—was analyzed by using random amplified polymorphic DNA (RAPD) assay and microsatellite analysis. Ten polymorphic RAPD markers and four microsatellites were selected to genotype both of the stocks. As expected, microsatellite analysis revealed more detailed information on genetic diversities than RAPD assay (Bartfai *et al.*, 2003). RAPD markers were also used in Spanish barb for identification of 3 endemic species (Callejas and Ochando, 1998) and in the common carp, *Cyprinus carpio* for the study of heterosis (Dong and Zhou, 1998).

Since the existence of natural population subdivisions may imply adaptation to local conditions, genetic assessments of the degree of population structuring and gene flow are necessary not only to preserve the existing biodiversity, but also to keep valuable adaptive resources. The assessment of the degree of genetic differentiation between cultivated and wild populations, as well as monitoring of the changes in genetic composition of the receiving populations after release,

should constitute an integral part of any translocation or restocking programme. *Gonoproktopterus curmuca*, the species selected for present study was enlisted as 'endangered' according to the latest IUCN categorization in the NBFGR-CAMP workshop held in 1997 (Anon., 1998). The species was short listed for taking up 'stock-specific propagation assisted rehabilitation programme' in rivers where it is naturally distributed. Liu *et al.* (2007) confirmed that it is important to detect the genetic variability of the selected population for the conservation of natural resources. However, no attempts have been made to study the stock structure and basic genetic profile of the species that are essential for the fishery management, conservation and rehabilitation of this species.

Hence the present study was taken up with a view to obtain a detailed population structure of the species – *Gonoproktopterus curmuca* - distributed in three rivers (Periyar River, Chalakkudy River, and Chaliyar River) in the Western Ghats using polymorphic allozyme, microsatellite and RAPD markers.

Chapter 3

MATERIALS AND METHODS

Contents

3.1 Fish Specimen Collection

Live specimens of *Gonoproktopterus curmuca* (70 specimens from each population, ranging from 15cm - 41cm in total length; ~ 45g - 325g total weight) (**Figure 01** & **02**) were collected from its natural distribution range - three west flowing rivers along the Western Ghats in Kerala state, India, *viz.*, 1) Periyar River at Bhoothathankettu, Ernakulam, (10^0 08' 06" N; 76^0 39' 40" E; 520m above MSL); 2) Chalakkudy River at Athirampilly, Trichur (10^0 17' 23" N; 76^0 32' 49" E; 680m above MSL) and 3) Chaliyar River at Manimooli, Nilambur, Malappuram (11^0 20' 59" N; 76^0 18' 39" E; 950m above MSL) (**Figure 03**). The riverine locations were chosen to cover geographically distinct populations of *G. curmuca*. Fishes were collected using cast nets and other conventional methods and their total length, total weight were recorded. The details of fish samples collected in different periods are given in **Table 01**.

Table 01. Sample size of *G.curmuca* and sampling period at three riverine locations

Sl. No.	River system	Collection Site	Sampling Date	No. of specimens	Total samples (N)
1.	Periyar River	Bhoothathankettu, Ernakulam 10^0 08' 06" N 76^0 39' 40" E	16.07.2002	09	70
			17.09.2002	18	
			15.11.2002	18	
			11.07.2003	15	
			06.10.2003	10	
2.	Chalakkudy River	Athirampilly, Trichur 10^0 17' 23" N 76^0 32' 49" E	14.02.2002	07	70
			26.04.2002	25	
			27.04.2002	18	
			29.06.2002	04	
			06.02.2003	16	
3.	Chaliyar River	Manimooli, Nilambur, Malappuram 11^0 20' 59" N 76^0 18' 39" E	04.07.2002	34	70
			30.12.2002	26	
			19.05.2003	10	

3.2 Collection of tissue samples

3.2.1 Collection of blood samples for DNA marker studies

Blood samples (circa 0.25mL) for DNA extraction were collected using minimal-invasive method from the live fish immediately after capture by puncturing the caudal vein, using sterile syringes rinsed with anticoagulant Heparin (1000units/1mL; Biological E. Limited, India.). The blood samples were immediately poured into sterile 1.5mL microfuge tubes containing 1.25mL 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 for allozyme analysis

Liver and abdominal muscle tissues were dissected out from the specimens at the sampling site itself, 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

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

- Approximately 100-250mg tissue (liver or muscle) was taken in a labelled 1.5mL 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 14000 rpm at 4°C for 1 hour in 'Heraeus - Biofuge Stratos'.
- 100-200µL 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 30minutes in 'Heraeus - Biofuge Stratos'

- The clear solution was taken from middle portion for allozyme analysis.

Extracting solution (to be prepared afresh)		
For liver (250mg/mL)	50% sucrose	2mL
	EDTA (64mg/100mL)	5mL
	0.2M Tris-HCl (pH7.2)	0.5mL
	Double distilled water	2.5mL
	Total volume	**10mL**
For muscle (125mg/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 *Gonoproktopterus curmuca*. The name of enzyme loci, enzyme commission numbers and quaternary structure are given in **Table 02**. The selected fourteen enzymes were Aspartate amino transferase (AAT, 2.6.1.1), Creatine kinase (CK, 2.7.3.2), Esterase (EST, 3.1.1.1), Glucose-6-phosphate dehydrogenase (G_6PDH, 1.1.1.49), Glucose phosphate isomerase (GPI, 5.3.1.9), α-Glycerophosphate (Glycerol 3-phosphate) dehydrogenase (α-G_3PDH, 1.1.1.8), Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH, 1.2.1.12), Lactate dehydrogenase (LDH, 1.1.1.27), Malate dehydrogenase (MDH, 1.1.1.37), Malic Enzyme (ME, 1.1.1.40), Phosphogluconate dehydrogenase (6PGDH, 1.1.1.44), Phosphoglucomutase (PGM, 5.4.2.2), Superoxide dismutase (SOD, 1.15.1.1) and Xanthine dehydrogenase (XDH, 1.1.1.204). Among these **12 were polymorphic** and rest **two enzymes were monomorphic**, *viz.* Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1.2.1.12) and Malic enzyme (ME, 1.1.1.40)

The enzymes that did **not give scorable activity** and hence discarded were Acid phosphatase (ACP), Adenylate kinase (AK), Alcohol dehydrogenase (ADH), Alkaline phosphate (ALP), Fumerase (FUM), Glutamate dehydrogenase (GDH),

Glucose dehydrogenase (GLDH), Hexokinase (HK), Isocitrate dehydrogenase (ICDH), Octanol dehydrogenase (ODH), and Pyruvate kinase (PK).

Table 02. Name of enzymes with their enzyme commission (E.C.) number used in allozyme analysis in *Gonoproktopterus curmuca*

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-Glycerol-3-phosphate dehydrogenase	**α-G_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

The supernatant of the tissues was analysed PolyAcrylamide Gel Electrophoresis (PAGE) using 7.25% gel. The band patterns (zymogram) were detected by specific

enzyme substrate staining procedures of Shaw and Prasad (1970) and Shaklee *et al.* (1990a). Since the liver tissue produced sharp and reproducible band patterns without trailing, it was selected for further studies. Electrophoresis was carried out in the vertical slab gel apparatus (100mm height X 100mm wide X 1mm thick; Amersham Biosciences, USA). The gel composition for PAGE is given below.

PAGE gel composition		Volume
Acrylamide (40%)	:	3.5mL
Bis acrylamide (2.1%)		2.5mL
Double distilled water		6mL
Tank buffer (1X)		5mL
Ammonium persulphate (0.28%)		3mL
TEMED	:	20µL

Two buffer systems, TBE (90mM Tris-borate and 2mM EDTA, pH8) and TG (5 mM Tris-HCl and 0.038M Glycine, pH8.3) 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 mentioned above. To increase the resolution of the bands in the gel mixture 0.34mL of NAD (stock solution: 15mg/mL double distilled water) or 100µL NADP (stock solution: 4mg/mL double distilled water) was added in the gel based on the nature of the enzyme (Gopalakrishnan *et al.*, 1997). The gel mixture was loaded and the combs were kept to make wells in the gel. The 1X TBE or Tris-glycine (TG) buffer was poured in upper and lower chambers. For all the enzymes, except CK, ME, 6PGDH, PGM and SOD, 2.34mL of NAD stock solution was added in upper chamber during electrophoresis. For CK, ME, 6PGDH and PGM 700µL of NADP stock solution was added instead of NAD. Approximately 6µL of sample (clear supernatant) was loaded in each well at the cathodal end and the run was carried out at a constant current of 30mA (150V) for 50minutes to 130minutes at 20^0C, till the indicator dye (aqueous bromophenol blue; final concentration 0.05%) reached the anodal end of the gel. After completion of run, the gel was stained for specific allozymes using standardized protocols.

3.3.4 Staining and Imaging

The staining recipe used for allozyme detection was slightly modified from that of Shaw and Prasad (1970), Shaklee *et al.* (1990a) and Gopalakrishnan *et al.* (1997). The zones of activity of 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). The details of the staining recipe for these fourteen enzymes are given below.

Aspartate Amino Transferase (AAT) 2.6.1.1, Dimer		**Creatine Kinase (CK) 2.7.3.2, Dimer**	
α -ketoglutaric acid (20mg/mL)	1mL	Creatine phosphate	10mg
L-Aspartic Acid (50mg/mL)	1mL	ADP (2.0mg/mL)	3mL
Pyriodoxal 5-phosphate (1mg/mL)	0.5mL	Glucose	250mg
BB salt (40mg/mL)	0.5mL	NADP (4mg/mL)	1.6mL
0.2MTris-HCl buffer (pH8)	2.5mL	MTT or NBT (8mg/mL)	0.4mL
Distilled water	4.5mL	$MgCl_2$ (20mg/mL)	2mL
		0.2M Tris-HCl buffer (pH8)	2.5mL
		Hexokinase (1000units/mL)	20µL
		PMS (1.7mg/mL)	0.2mL
		G_6PDH (1000units/mL)	20µL
		Distilled water	2mL
		1% Agar overlay (Optional)	
Running buffer	TBE	Running buffer	TBE
Running time	65min	Running time	50min

Esterase (EST) 3.1.1.- , Monomer		**Glucose –6-Phosphate Dehydrogenase (G_6PDH) 1.1.1.49, Dimer**	
α-naphthyl acetate (20mg in 0.5mL acetone + 0.5mL H_2O)	0.5mL	Glucose-6-PO_4(50mg/mL)	0.6mL
β-Naphthyl acetate (20mg in 0.5mL acetone + 0.5mL H_2O)	0.5mL	NADP (4mg/mL)	1.6mL
Fast Blue RR (5mg/mL)	0.5mL	NBT (8mg/mL)	0.4mL
0.2M Tris-HCl buffer (pH8)	2.5mL	PMS (1.7mg/mL)	0.4mL
Distilled water	6mL	0.2M Tris-HCl buffer (pH8)	2mL
		$MgCl_2$ (20mg/mL)	0.4mL
		Distilled water	4.6mL
Running buffer	TBE	Running buffer	TBE
Running time	50min	Running time	90min

Glucose Phosphate Isomerase (GPI) 5.3.1.9, Dimer		**α-Glycerol-3-Phosphate Dehydrogenase (α-G_3PDH) 1.1.1.8, Dimer**	
Fructose-6-phosphate (20mg/mL)	1mL	α-DL-glycerophosphate	260mg
NADP (4mg/mL)	1.6mL	NAD (15mg/mL)	0.6mL
$MgCl_2$ (20mg/mL)	0.5mL	NBT (8 mg/mL)	0.4mL
G_6PDH (1000units/mL)	20µL	PMS (1.7mg/mL)	0.3mL
NBT (8mg/mL)	0.4mL	0.2M Tris-HCl buffer (pH8)	3.5mL
PMS (1.7mg/mL)	0.2mL	0.1M $MgCl_2$ (20mg/mL)	0.4mL
0.2M Tris-HCl buffer (pH8)	2.5mL	Distilled water	4.8mL
Distilled water	3.8mL		
Agar 2% overlay.			
Running buffer	TBE	Running buffer	TBE
Running time	90min	Running time	90min

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) 1.2.1.12, Tetramer		**Lactate Dehydrogenase (LDH) 1.1.1.27, Tetramer**	
Fructose - 1, 6- diphosphate (Sodium salt; 20mg/mL)	2.5mL	Lithium lactate (40mg/mL)	1.6mL
Aldolase (1000 units)	220µL	NAD (15mg/mL)	0.4mL
NAD (15mg/mL)	0.4mL	NBT (8mg/mL)	0.4mL
NBT (8mg/mL)	0.4mL	PMS (1.7mg/mL)	0.2mL
PMS (1.7mg/mL)	0.2mL	0.2M Tris-HCl buffer (pH8)	2.5mL
Arsenate (Sodium salt; 20mg/mL)	1.5mL	Distilled water	4.7mL
0.2M Tris-HCl buffer (pH8)	2.5mL		
Distilled water	2.5mL		
Running buffer	TBE	Running buffer	TBE
Running time	110min	Running time	90min

Malate Dehydrogenase (MDH) 1.1.1.37, Dimer		**Malic Enzyme (ME) 1.1.1.40, Tetramer**	
Sodium malate (50mg/mL; Malic acid sodium salt)	2mL	Sodium malate (50mg/mL; Malic acid sodium salt)	1mL
NAD (15mg/mL)	0.4mL	NADP (4mg/mL)	1.6mL
NBT (8mg/mL)	0.4mL	NBT (8mg/mL)	0.4mL
PMS (1.7mg/mL)	0.2mL	PMS (1.7mg/mL)	0.2mL
0.2M Tris-HCl buffer (pH8)	2.5mL	0.2M Tris-HCl buffer (pH8)	2.5mL
Distilled water	4.5mL	Distilled water	3.5mL
		$MgCl_2$ (20mg/mL)	0.5mL
		Oxaloacetic acid (to inhibit MDH)	9mg
Running buffer	TBE	Running buffer	TBE
Running time	90min	Running time	90min

Phospogluconate Dehydrogenase (6PGDH) 1.1.1.44, Dimer		**Phosphoglucomutase (PGM) 5.4.2.2, Monomer**	
Phosphogluconate tri sodium salt	5mg	Glucose-1-phosphate (50mg/mL)	1mL
NADP (4mg/mL)	1.6mL	NADP (4mg/mL)	1.6mL
$MgCl_2$ (20mg/mL)	0.5mL	$MgCl_2$ (20mg/mL)	1mL
NBT (8mg/mL)	0.4mL	G_6PDH (1000units/mL)	20µL
PMS (1.7mg/mL)	0.2mL	NBT(8mg/mL)	0.4mL
0.2M Tris-HCl buffer (pH8)	2.5mL	PMS (1.7mg/mL)	0.2mL
Distilled water	4.8mL	0.2M Tris-HCl buffer (pH8)	2.5mL
		Distilled water	6mL
		Agar 2% overlay	
Running buffer	TBE	Running buffer	TBE
Running time	65min	Running time	90min

Superoxide Dismutase (SOD) 1.15.1.1, Dimer		**Xanthine Dehydrogenase (XDH) 1.1.1.204 - Dimer**	
NBT (8mg/mL)	0.4mL	Hypoxanthine (100mg/mL)	1.6mL
PMS (1.7mg/mL)	0.4mL	NAD (15mg/mL)	0.4mL
NAD (15mg/mL)	0.4mL	0.2M Tris-HCl buffer (pH7.5)	7.5mL
0.2M Tris-HCl buffer (pH8)	3mL	NBT (8mg/mL)	0.4mL
Distilled water	6mL	PMS (1.7mg/mL)	0.2mL
Keep in dark for 20min sharply without PMS & NBT. Then expose to sunlight (or ordinary bulb) with NBT & PMS.		Distilled water	0.4mL
		Pyruvate (to inhibit LDH)	150mg
Running buffer	TG	Running buffer	TBE
Running time	80min	Running time	130min

3.3.5 Scoring of alleles

The enzyme activity obtained in the gel was differentiated into specific zones of enzyme loci. Nomenclature of loci and alleles as recommended by Shaklee *et al.* (1990a) was followed. 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). 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 heterozygous in polymorphic loci, confirmed 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 as in AAT enzyme in this study with alleles 100, 108 and 117 (**Figure 04**). 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 in 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 frequency of most common allele was equal to or less than 0.99 (Nei, 1987). The mean number of alleles per locus; observed and expected heterozygosities (H_{obs} and H_{exp}) 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 *G. curmuca.*

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 locus. 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 test validity (Raymond and Rousset, 1995a & b). 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 through estimator of Weir and Cockerham, (1984). Estimation of average F_{ST} and determining whether the values are 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 some times known as fixation index. Positive values of 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 as an inbreeding coefficient.

3.3.6.5 *Genetic similarity and distance*

Genetic similarity/identity and distance between pairs of populations of *G. curmuca* were estimated using POPGENE Version 1.31 (Yeh *et al.*, 1999). Nei and Li's (1979) pair-wise genetic similarity (SI) among these 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.

3.3.6.6 *Analysis of Molecular Variance (AMOVA)*

The partitioning of genetic variation among and within populations of *G. curmuca* was calculated by hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) at 1000 permutations. The hierarchical components of genetic variation include (1) variance due to differences between individuals within a river; and (2) variance due to differences among populations. The AMOVA calculations were performed using ARLEQUIN v2.0 (Schneider *et al.* 2000; http://lgb.unige.ch/arlequin/).

3.3.6.7 *Dendrogram*

Phylogenetic relationships based on genetic distance values generated from allozyme data among three populations of *G.curmuca* were made and dendrogram plotted, following unweighted pair group method using 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).

3.3.6.8 *Bottleneck*

Populations that have experienced a recent reduction of their effective population size exhibit a correlative reduction of the allele numbers (k) and gene diversity (He or Hardy – Weinberg heterozygosity) at polymorphic loci. But the allele numbers are reduced faster than the gene diversity. Thus in a recently bottlenecked population, the observed/measured gene diversity is higher than the expected equilibrium gene diversity (Heq), which is computed from the observed number of alleles (k) under the assumption of constant- size (mutation-drift equilibrium) population.

The programme BOTTLENECK ver 1.2.02 (Cornuet and Luikart, 1996) was used to detect recent effective population size reduction (assess the impact of population decline) using data from the 14 allozyme. BOTTLENECK detects past population reductions by testing for a transient (~ 0.2- 4.0; Ne generations) excess in measured heterozygosity compared with the heterozygosity expected at mutation-drift equilibrium (He>Heq). This excess in heterozygosity is generated because rare alleles are quickly lost due to drift during a bottleneck, but they contribute little to the expected heterozygosity (Luikart and Cornuet, 1998). To determine whether the 3 riverine populations of *G. curmuca* exhibited a significant number of loci with gene diversity excess, "Wilcoxon Sign-Rank Test" was employed in BOTTLENECK. For allozymes loci, data were analysed under the infinite allele model (IAM). In addition, a qualitative descriptor of the allele frequency distribution ("mode-shift" indicator) which discriminates bottlenecked populations from stable populations was also employed to determine the occurrence of bottleneck.

3.4 Microsatellites analysis

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.

- 500µL of blood samples (0.25mL stored in 1mL of 95% ethanol) from each specimen separately was taken in 50mL autoclaved centrifuge tube. Ethanol was decanted by centrifugation at 10000rpm for 10minutes at 4^0C.
- The blood sample was washed by mixing with 1mL of high molar TE buffer (0.1MTris-HCl and 0.04M EDTA).
- The buffer was decanted by centrifugation at 10,000rpm for 10minutes at 4^0C, and repeated the above two steps once to get clear pellets of blood cells.
- To lyse the blood cells, 3mL of incubation buffer (given in box) was added to each tube and incubated at 56^0C for 60minutes in a water bath.

Incubation buffer	
Tris-HCl (pH8.3)	: 10mM
EDTA (pH8)	: 1mM
NaCl	: 0.4M (2.337g/100mL)
Proteinase K(20 mg / mL)	: 10µL/mL
SDS	: 10%.

- The sample was taken out from water bath and 2mL of lysis buffer (10mM Tris-HCl (pH8.3), 1mM EDTA (pH8) and 0.4M (2.337g/100mL) 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 10minutes (protocol for saturation of phenol was given in box below).

Saturation of Phenol with Tris-HCl (pH8)

If phenol is transparent, added 0.1% (20mg) 8-hydroxy-quinoline (to avoid the oxidation of phenol) to 200mL of water saturated phenol

Covered the flask containing phenol with aluminium foil to avoid light reaction.

200mL of 0.5M Tris-HCl was added.

Stirred the solution using magnetic stirrer for 15minutes

Kept the solution for 30minutes to allow the phenol to settle.

The supernatant (Tris) was decanted.

200mL of 0.1M Tris-HCl was added.

Repeated the above four steps once.

200mL of 0.1M Tris-HCl was added to phenol

Stored at 4^0C

- The sample was then centrifuged at 12,000rpm for 15minutes at 4^0C and aqueous phase was transferred to a fresh autoclaved tube by using 1mL 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 12,000rpm for 15minutes 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 3M sodium acetate (pH5.2) was added to the separated aqueous phase and the DNA was precipitated with 02.5 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 12,000rpm for 10minutes at 4^0C and ethanol was decanted and the DNA pellet was marked in tube.
- To wash the DNA pellet 3mL of 70% ethanol was added, and mixed. The solution was centrifuged at 12,000rpm for 10minutes 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 100µL TE buffer (10mM: 1mM, pH8).
- RNA in the sample was degraded by incubating at 37^0C for 60minutes after the addition of 5.0µL of DNAase free RNAase (10mg/mL-Genei, Bangalore, India).
- The DNA samples were stored at -20^0C for further use.

To analyse yield and quality, the extracted DNA was checked through 0.7% agarose gel electrophoresis with ethidium bromide incorporated in 1.0X TBE buffer (90.0mM Tris-borate and 2mM EDTA, pH8).

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 260nm and 280nm. The quality was checked by measuring the ratio of absorbance at 260nm and 280nm (260/280). The value between 1.7 - 1.9 indicates the good quality of DNA without protein contamination. DNA quantification was done according to the following calculation: sample showing 1.0 OD at 260nm is equivalent to 50µg of DNA/mL. The OD of each DNA sample at 260nm was measured and quantified accordingly.

3.4.3 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 through the software Primer3 (Rozen and Skaletsky, 1998) and the primers custom synthesized for use.

3.4.4 Development of microsatellite markers through cross-species amplification

Available microsatellite information in the closely related species was collected from the GenBank (National Centre for Biotechnology Information – NCBI; www.ncbi.nlm.nih.gov). For cross-species amplification of microsatellite loci, a total of 40 microsatellite primers from *Catla catla* (1 : Naish and Skibinkski, 1998); *Cyprinus carpio* (10 : Crooijmans *et al.*, 1997; 2 : Yue *et al.*, 2004); *Barbodes gonionotus* (5 : Chenuil *et al.*, 1996); *Campostoma anamalum* (9 : Dimsoski *et al.*, 2000); *Labeo rohita* (6 : Das *et al.*, 2005) and *Pimephales promelas* (7 : Bessert *et al.*, 2003) were used (Table 03).

Table 03. Microsatellite primers of related species tested for cross-species amplification in *Gonoproktopterus curmuca*

Sl. No.	Donor species	No. of primer pairs tested	Loci / Primer	GenBank Accession No.	References
1	*Catla catla*	1	*CcatG1*	AF045380	Naish and Skibinski, (1998)
2	*Cyprinus carpio*	10	***MFW 01*** *MFW 02* *MFW 09* ***MFW 11*** *MFW 15* *MFW 17* ***MFW 19*** *MFW 20* *MFW 24* ***MFW 26***	-----	Crooijmans *et al.*, (1997)
		2	***CCa72**** *CCa80*	AY169249 AY169250	Yue *et al.*, (2004)
3	*Barbodes gonionotus*	5	*Bgon 22* *Bgon 69* *Bgon 75* *Bgon 79* *Bgon 17*	-----	Chenuil *et al.*, (1999)
4	*Campostoma anamalum*	9	*Ca 03* *Ca 05* *Ca 06* *Ca 08* *Ca 10* *Ca 11* *Ca 12* *Ca 16* *Ca 17*	AF277575 AF277577 AF277578 AF277580 AF277582 AF277583 AF277584 AF277588 AF277589	Dimsoski *et al.*, (2000)
5	*Labeo rohita*	6	*R 01* *R 02* *R 03* *R 05* *R 06* *R 12*	AJ507518 AJ507519 AJ507520 AJ507521 AJ507522 AJ507524	Das *et al.*, (2005)
6	*Pimephales promelas*	7	***Ppro 048*** *Ppro 080* *Ppro 118* ***Ppro 126*** *Ppro 132* *Ppro 168* *Ppro 171*	AY254350 AY254351 AY254352 AY254353 AY254354 AY254355 AY254356	Bessert *et al.*, (2003)
Total tested		40			

*Primer sequence of CCa72 given by Yue *et al.* (2004) was modified using PRIMER3 and renamed as MFW72 in the present study.

3.4.5 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 04**). Amplifications were performed in 25µL reaction mixture containing 1X reaction buffer (10mM Tris, 50mM KCl, 0.01% gelatin, pH9.0) with 1.5mM $MgCl_2$ (Genei, Bangalore, India), 5pmoles of each primer, 200mM dNTPs, 2U *Taq* DNA polymerase (Genei, Bangalore, India) and 25-50ng of template DNA. The volume of reaction mixture is given in box below.

PCR reaction mixture	**Volume per reaction**
Double distilled water	18.3µL
Assay buffer (10X)	02.5µL
dNTPs	02.0µL
Primers (forward and reverse)	00.5µL
Taq polymerase (Genei, Bangalore)	00.7µL
Template DNA	01.0µL
Total volume	**25.0µL**

To check DNA contamination, a reaction set up omitting the DNA from the reaction mixture (negative control). The reaction mixture was pre-heated at 94^0C for 5minutes followed by 25cycles (94^0C for 30seconds, annealing temperature depending upon the T_m value of primer (usually 50^0C - 60^0C) and 72^0C for 1minute).

The optimum annealing temperature to get scorable band pattern was determined through experimental standardization for each primer pair and it 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^{0}C below the lowest T_m value obtained for the forward/reverse primer in that combination

(**Table 04**). The reaction was then subjected to a final extension at 72^0C for 2minutes. The amplified product was checked in 10% polyacrylamide gel electrophoresis (PAGE).

3.4.6 Polyacrylamide gel electrophoresis (PAGE)

The PCR products were electrophoretically analyzed through 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 needle for approximately 1.0cm 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)	: 5mL
Double distilled water	: 2mL
5 X TBE	: 2mL
10% (Ammonium persulphate)	: 70µL
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 (8µL) were loaded with 2µL of bromophenol blue (BPB) into the wells using micropipette; and run with 1X TBE buffer (pH8) for 4hours at constant voltage of 10V/cm, at 4^0C in a cold chamber.

3.4.7 Visualization of microsatellite products

The amplified microsatellite loci were visualized through silver staining of the polyacrylamide gel. The gels were fixed in 50mL of fixing solution (diluted five times with 30.4mL double distilled water and 9.6mL ethanol) for 30minutes and silver-impregnated (with 1X staining solution) for another 30minutes. This was followed by washing the gels in double distilled water for 2minutes, after removing the staining solution. The gels were then kept in the 1X developing solution in darkness for 10minutes. 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.

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	Sodium carbonate; 12.5% w/v.
Formaldehyde; 37%	Formaldehyde; 37% w/v in water
Sodium thiosulphate; 2%	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

3.4.8 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).

3.4.9 Final selection of microsatellite primer pairs

The cross-species amplification trails were done with eight specimens of *G. curmuca* and of the 40 primer pairs tested (**Table 03**), 34 (85%) provided successful amplification of homologous loci in *G. curmuca*. These primers were again analysed with larger sample size (30 individuals from 2 rivers) to evaluate their suitability (polymorphic pattern) in quantification of genetic divergence in *G.*

curmuca. Several loci were monomorphic, few produced multiple products but 8 loci (**Table 04**) gave clear scorable products with 3-7 alleles per locus. These eight loci were finally analysed to confirm the occurrence of repeats through cloning and sequencing.

Table 04. The sequence, concentration and the annealing temperature of selected microsatellite primers

No.	Primers / Locus	F / R	Sequence 5'-3'	Conc. (nmol)	Ta for each primer (^{0}C)
1	*MFW01*	F	GTCCAGACTGTCATCAGGAG	49.0	59
		R	GAGGTGTACACTGAGTCACGC	48.7	
2	*MFW11*	F	GCATTTGCCTTGATGGTTGTG	59.1	58
		R	TCGTCTGGTTTAGAGTGCTGC	54.6	
3	*MFW19*	F	GAATCCTCCATCATGCAAAC	43.5	51
		R	CAAACTCCACATTGTGCC	40.8	
4	*MFW26*	F	CCCTGAGATAGAAACCACTG	56.3	57
		R	CACCATGCTTGGATGCAAAAG	69.4	
5	*MFW72*	F	GCAGTGGCTGGCAAGTTAAT	57.5	55
		R	GCACTACATCCACTGCACACA	44.4	
6	*CcatG1-1*	F	AGCAGGTTGATCATTTCTCC	55.8	51
		R	TGCTGTGTTTCAAATGTTCC	48.6	
7	*Ppro48*	F	TGCTCTGCTCTCCTGCGTGTCATT	74.2	51
		R	CAGCCTCGGCGGTGTTGTTGC	53.3	
8	*Ppro126*	F	CTGCGTGTCTGATAACTGTGACTG	56.0	55
		R	GTCCCGGGACTTTAAGAAGGTC	40.3	

3.4.10 Confirmation of microsatellite by cloning and sequencing

The cross-amplified polymorphic microsatellite loci (**Table 04**) were analysed by cloning them in TOPO vector (Invitrogen, Carlsbad, USA) and sequencing to confirm the occurrence of repeats. The band of the target sequence with particular primer (forward and reverse) was amplified using PCR protocol as given in section 3.4.5. The samples were electrophoresed in PAGE (section 3.4.6) to check the concentration of DNA.

3.4.11 Elution of amplified products from agarose gel

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

a. The samples (20µL) were quick spinned with 3µL bromophenol blue dye.

b. They were run in 2% agarose gels and the DNA bands were cut out from the lane after viewing the gel over long wavelength UV light quickly so as to avoid nicks.

c. The gel slices were taken in a 1.5mL micro-centrifuge tube and 1mL Tris-saturated phenol was added.

d. The sample was kept at -80^{0}C for over night. The frozen samples were centrifuged at 10,000rpm for 20minutes at 4^{0}C.

e. The supernatant was transferred to a fresh tube and DNA precipitated by adding 2.5volume of ethanol and 1/10 times 3M sodium acetate (pH5.2).

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

g. Ethanol was decanted; the DNA pellet was washed with 0.5mL of 70% ethanol and centrifuged at 10,000rpm at 4°C for 20minutes.

h. Discarded the ethanol and the DNA was vacuum dried and suspended in 15µL double distilled water.

i. For checking the concentration, DNA samples were run (4µL) in 2% agarose gel.

3.4.12 Construction of recombinant plasmid

TOPO (Invitrogen, Carlsbad, USA) vector was used for constructing the recombinant DNA. *Taq* polymerase has a non template-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).

3.4.13 Competent cell preparation

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

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

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

c. Next day, 2% of the overnight grown cultures (100μL) were inoculated to 5mL LB and grown for 3-4hours (till the OD reaches 0.3–0.5).

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

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

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

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

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

(as in section 3.4.5). The PCR products from blue and white colonies were analysed in 2% agarose gels.

3.4.17 Sequencing of microsatellite loci

The recombinant plasmids were isolated in large scale by alkaline lysis method (section 3.4.13) and were further purified through PEG precipitation for sequencing purpose. To 32µL of plasmid DNA, 8µL of 4M NaCl and 40µL of 13% PEG_{8000} were added. After thorough mixing, the sample was incubated on ice for 20minutes and the precipitated plasmid DNA was pelleted by centrifugation at 10,000rpm for 10minutes 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 water and stored at -20^0C.

The sequencing was done using forward and reverse sequencing primers with the automated DNA sequencer ABI 3730 (Applied Biosystems, USA) according to manufacturers instructions at the Department of Biotechnology, University of Delhi, South Campus, New Delhi, India

3.4.18 Population structure analysis

After sequencing, the eight polymorphic microsatellite loci (**Table 19**) were selected for further population studies in *Gonoproktopterus curmuca*. PCR reactions, PAGE and visualization were carried out as given in section 3.4.5; 6; 7.

3.4.18.1 *Scoring of alleles*

The gels having zymogram pattern of the microsatellite loci obtained following the electrophoresis and silver staining procedures (described on sections 3.4.6 and 3.4.7) 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.

3.4.18.2 *Analysis of Data*

In the analysis of microsatellites, parameters tested were as in the case of co-dominant allozymes and the softwares used were also same (pages 52 to 56). 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, plotting dendrogram, Analysis of Molecular Variance (AMOVA) and genetic bottleneck analysis using open source softwares such as GENEPOP version 3.1 (Raymond and Rousset, 1998), GENETIX version 4.0 (Belkhir *et al.*, 1997), POPGENE version 1.31 (Yeh *et al.*, 1999), ARLEQUIN v2.0 (Schneider *et al.* 2000) and BOTTLENECK ver 1.2.02 (Cornuet and Luikart, 1996) respectively. As most of the microsatellites follow step-wise mutation model (SMM), the microsatellites were mainly analyzed under the more suitable two-phased model (TPM), in addition to IAM. Compared to allozymes, microsatellites provide additional information *ie.* difference in number of repeats (variances of allele sizes) that will be helpful in measuring population sub-division. As F_{ST} takes care of only allelic frequency and does not make use of allele size differences. Hence, in addition to F_{ST}, the population differentiation was also estimated based on allele sizes of microsatellites using Slatkin's (1995) pair-wise and overall R_{ST}, assuming a step wise mutation model (SMM) using the software GENEPOP version 3.1 (Raymond and Rousset, 1998).

A null allele concerning molecular markers refers to such a marker in the case it can no longer be detected because of a mutation of annealing site, e.g., in microsatellites (in which the repeat is rather short). To do amplification, a primer or oligonucleotide aligns with either of ends of the locus, if a mutation occurs in the annealing site, then the marker can no longer be used and the allele is turned into a null allele. Occurrence of null alleles results in false homozygotes leading to genotyping errors and heterozygotes deficiency that can cause deviations from Hardy-Weinberg Equilibrium (HWE). 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 thereafter analyzed for population differentiation.

3.5 Random Amplified Polymorphic DNA (RAPD) analysis

3.5.1 Screening of RAPD primers

Eighty decamer primers (20 from each series OPA, OPAA, OPAC and OPAH) (Operon Technologies, Alameda, USA) were used for screening *Gonoproktopterus curmuca* samples. Thirty one primers out of 80 produced amplicons and they were selected for primary screening, however only 9 primers *viz.*, OPA-15, OPA-16, OPAA-07 OPAA-08, OPAC-05, OPAC-06, OPAH-03, OPAH-17 and OPAH-19 were selected for population genetic analysis taking into consideration of the repeatability, sharpness and intensity of the bands. In **Table 05**, the sequences, molecular weights and concentration of the primers are given.

Table 05. Selected primers with concentration and molecular weight, used in RAPD analysis in *Gonoproktopterus curmuca* (the primers asterisked are selected for population analysis)

Sl. No.	Primer	Sequences (5'-3')	M.W (dalton)	Conc. (pmoles/μL)
1.	OPA 05	AGGGGTCTTG	3090	5.194
2.	OPA 09	GGGTAACGCC	3044	5.160
3.	OPA 10	GTGATCGCAG	3059	5.090
4.	**OPA 15***	**TTCCGAACCC**	**2939**	**5.785**
5.	**OPA 16***	**AGCCAGCGAA**	**3037**	**4.712**
6.	OPA 17	GACCGCTTGT	3010	5.656
7.	OPA 19	CAAACGTCGG	3028	4.990
8.	**OPAA 07***	**CTACGCTCAC**	**2939**	**5.785**
9.	**OPAA 08***	**TCCGCAGTAG**	**3019**	**5.302**
10.	OPAA 11	ACCCGACCTG	2964	5.616
11.	OPAA 12	GGACCTCTTG	3010	5.656
12.	OPAA 14	AACGGGCCAA	3037	4.712
13.	OPAC 02	GTCGTCGTCT	3001	6.059
14.	**OPAC 05***	**GTTAGTGCGG**	**3090**	**5.192**
15.	**OPAC 06***	**CCAGAACGGA**	**3037**	**4.710**
16.	OPAC 07	GTGGCCGATG	3075	5.265
17.	OPAC 10	AGCAGCGAGG	3093	4.683
18.	OPAC 14	GTCGGTTGTC	3041	5.783
19.	OPAC 15	TGCCGTGAGA	3059	5.088
20.	OPAC 20	ACGGAAGTGG	3108	4.625
21.	OPAH 01	TCCGCAACCA	2948	5.413
22.	**OPAH 03***	**GGTTACTGCC**	**3010**	**5.654**
23.	OPAH 04	CTCCCCAGAC	2924	5.874
24.	OPAH 06	GTAAGCCCCT	2979	5.531
25.	OPAH 08	TTCCCGTGCC	2946	6.473
26.	OPAH 09	AGAACCGAGG	3077	4.542
27.	OPAH 11	TCCGCTGAGA	3019	5.300
28.	OPAH 14	TGTGGCCGAA	3059	5.088
29.	OPAH 16	CAAGGTGGGT	3099	4.892
30.	**OPAH 17***	**CAGTGGGGAG**	**3124**	**4.771**
31.	**OPAH 19***	**GGCAGTTCTC**	**3010**	**5.654**

3.5.2 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 05**. PCR amplifications were performed in 25µL reactions containing 1X reaction buffer (100mM Tris, 500mM KCl, 0.1% gelatin, pH9) with 1.5mM $MgCl_2$ (Genei, Bangalore, India), 6-8 pmoles of primer, 200 mM dNTPs, 2U *Taq* DNA polymerase (Genei, Bangalore, India) and 25ng of template DNA. To check DNA contamination, a negative control was made omitting template DNA from the reaction mixture. The reaction mixture was pre-heated at 95^0C for 3minutes followed by 40 cycles (94^0C for 3minutes, 40^0C for 1.30minutes and 72^0C for 2minutes). The reaction was then subjected to a final extension at 72^0C for 10minutes. The composition of PCR reaction mixture is given in box below.

PCR reaction Mixture	**Volume per reaction**
Double distilled water	17.3µL
Assay buffer (10X; Genei, Bangalore, India)	2.5µL
dNTPs (Genei, Bangalore, India)	2.0µL
Primer (Operon Technologies, USA)	1.5µL
Taq polymerase (Genei, Bangalore, India)	0.7µL
Template DNA	1.0µL
Total volume	**25.0µL**

3.5.3 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 (pH8). The gels were visualized under UV transilluminator and documented using Image Master VDS (Pharmacia Biotech, USA).

3.5.4 Analysis of Data

3.5.4.1 *Scoring of bands*

Images of gels were used to analyze the 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 6500bp) or low (below 800bp) molecular weight. The analysis was based on 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 fit the Hardy-Weinberg equilibrium, $p^2 + 2pq + q^2 = 1$, with frequencies p (dominant or band present) and q (recessive or band absent) (Clark and Lanigan, 1993; Lynch and Milligan, 1994). From the binary matrix, the total number of RAPD fragments and polymorphic ones 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.

3.4.5.2 *Allele frequencies and polymorphic loci*

Genetic variability in three populations of *Gonoproktopterus curmuca* 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).

3.5.4.3 *Average gene diversity (H)*

Average gene diversity index (Nei, 1987; Khoo *et al.*, 2002) is a measurement of genetic variation for randomly mating populations and is analogous to average

heterozygosity (H). It was calculated using the POPGENE version 1.31 (Yeh *et al.*, 1999).

3.5.4.4 *Genetic differentiation (G_{ST})*

The value of coefficient of genetic differentiation (G_{ST}) for overall population was calculated using POPGENE version 1.31 (Yeh *et al.*, 1999).

3.5.4.5 *Genetic similarity and distance*

Genetic similarity/identity and distance between pairs of populations of *Gonoproktopterus curmuca* were estimated using POPGENE Version 1.31 (Yeh *et al.*, 1999). Nei and Li's (1979) pair-wise genetic similarity (SI) among red-tailed barb 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.

3.5.4.6 *Dendrogram*

Cluster analysis was performed and dendrogram plotted based on RAPD data among three populations of *Gonoproktopterus curmuca,* 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).

Chapter 4

RESULTS

Contents

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 02**. Out of these fourteen enzymes, 12 enzymes were polymorphic and 2 enzymes were monomorphic. The polymorphic enzymes were Aspartate amino transferase (AAT), Creatine kinase (CK); Esterase (EST), Glucose-6-phosphate dehydrogenase (G_6PDH), Glucose phosphate isomerase (GPI), αGlycerophosphate dehydrogenase (αG_3PDH), Lactate dehydrogenase (LDH), Malate dehydrogenase (MDH), Phosphogluconate dehydrogenase (6PGDH), Phosphoglucomutase (PGM), Superoxide dismutase (SOD) and Xanthine dehydrogenase (XDH). The monomorphic enzymes were Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Malic enzyme (ME) (**Table 07**).

The fourteen enzymes yielded 29 scorable loci in all populations. EST exhibited maximum number of loci *i.e.*, 7 (*EST-1**, *EST-2**, *EST-3**, *EST-4**, *EST-5**, *EST-6** and *EST-7**); three loci were present in LDH (*LDH-1**, *LDH-2**and *LDH-3**) and two loci each were present in AAT (*AAT-1** and *AAT-2**), CK (*CK-1** and *CK-2**), GPI (*GPI-1* and GPI-2**) ,αG_3PDH (*αG_3PDH-1** and *αG_3PDH-2**) MDH (*MDH-1** and *MDH-2**), PGM *(PGM-1** and *PGM-2**), SOD (*SOD-1** and *SOD-2**) and all other enzymes (G_6PDH, GAPDH, ME, 6PGDH and XDH) had only single locus each. A detailed description of the polymorphic and monomorphic enzymes are given in **Table 07** and the distribution of genotypes are given in **Table 08**. Scorable activity of the following enzymes could not be detected in *Gonoproktopterus curmuca:* Acid phosphatase (ACP), Adenylate kinase (AK), Alcohol dehydrogenase (ADH), Alkaline phosphate (ALP), Fumarase (FUM), Glutamate dehydrogenase (GDH), Glucose dehydrogenase (GLDH), Hexokinase (HK), Isocitrate dehydrogenase (ICDH), Octanol dehydrogenase (ODH) and Pyruvate kinase (PK).

4.1.2 Polymorphic Enzymes

4.1.2.1 *Aspartate Amino Transferase (AAT. 2.6.1.1)*

Aspartate amino transferase is dimeric (quaternary structure) in vertebrates. The banding pattern of aspartate amino transferase patterns of *G.curmuca* are shown in **Figure 04.** 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 monomorphic 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.

4.1.2.2 *Creatine kinase (CK. 2.7.3.2)*

Creatine kinase is mostly dimeric in quaternary structure. The banding pattern of creatine kinase of *G.curmuca* is shown in **Figure 05.** It showed 2 different zones of enzyme activity, which were presumed to be under the control of 2 independent loci. They were designated as *CK-1** and *CK-2** according to their order of increasing mobility differences. *CK-1** had two allele (*A* and *B)* and heterozygous condition was also recorded in some individuals and *CK-2** had one allele (*A*) in all the populations.

4.1.2.3 *Esterase (EST. 3.1.1.-)*

Esterase enzymes are mostly monomeric in quaternary structure except the dimeric Esterase-D. The banding pattern of esterase enzyme system of *G. curmuca* is shown in **Figure 06.** It showed seven different zones of enzyme activity, which were presumed to be under the control of seven independent loci. They were designated as *EST-1**, *EST-2**, *EST-3**, *EST-4**, *EST-5**, *EST-6** and *EST-7** according to their order of increasing mobility differences. *EST-1** and *EST-4** had two alleles each; and heterozygous condition was also recorded in some individuals in these loci. *EST-2**, *EST-3**, *EST-5**, *EST-6** and *EST-7** exhibited only one allele in all the populations.

4.1.2.4 *α-Glycerophosphate Dehydrogenase (αG_3PDH-1.1.1.8)*

α-Glycerophosphate dehydrogenase or αGlycerol-3-phosphate dehydrogenase was tested in all the stocks, two loci were found to be responsible for the enzyme activity

(**Figure 08**). The occurrence of the two different genotypes (*G_3PDH-2*AA* and *G_3PDH-2*AB*) and their band positions suggested that this *G_3PDH-2** locus had two alleles, *A* and *B*. But *G_3PDH-1** locus had only one allele or monomorphic.

4.1.2.5 *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 (**Figure 09**). 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 *G. curmuca*, 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 06**. During the present investigation, liver extracts showed the presence of a single polymorphic locus of *G_6PDH** having two alleles *A* and *B* (R_f values *100* and *120*) and it showed three types of genotypes *viz.*, slow homozygotes (*G_6PDH* AA*), heterozygotes (*G_6PDH* AB*) and fast homozygotes (G_6*PDH* BB*).

Table 06. Distribution of dimeric G_6PDH genotypes in male and female *G. curmuca* from different river systems

Locus	Genotypes (Alleles & R_f value)	No. of individuals					
		Periyar River		Chalakkudy River		Chaliyar River	
		Male	Female	Male	Female	Male	Female
G_6PDH	*AA* (100/100)	27	16	18	05	10	07
	AB (100/120)	04	16	05	07	06	06
	BB (120/120)	04	03	23	12	26	15

4.1.2.6 *Glucose Phosphate Isomerase (GPI-5.3.1.9)*

The spacing of the bands of this dimeric enzyme had suggested occurrence of two loci in *G. curmuca* (**Figure 10**). Agar overlay (2%) was used to prevent leaching out of end-products from the gel during staining. The locus *GPI-1** exhibited one

allele (A) and *GPI-2** exhibited two alleles (A and B) and presumed to be under the control of two independent loci.

4.1.2.7 *Lactate Dehydrogenase (LDH-1.1.1.27)*

Lactate dehydrogenase is tetrameric in structure in vertebrates with 5 bands in heterozygous individuals. The banding patterns of LDH enzyme system of *G. curmuca* are shown in **Figure 11.** The banding patterns showed three different zones (a fast moving zone, transient zone and slow moving zone) of enzyme activity, which were presumed to be under the control of three independent loci. They were designated as *LDH-1**, *LDH-2* and *LDH-3** according to their order of increasing mobility. The first locus (slow moving) and third locus (fast moving) were monomorphic and this locus exhibited only one genotype *LDH-1*AA* and *LDH-3*AA* respectively . The second locus - *LDH-2** - (transient moving zone) had three types of alleles *A, B* and *C.*

4.1.2.8 *Malate Dehydrogenase (MDH-1.1.1.37)*

Malate dehydrogenase is a dimeric allozyme. During the present study, **liver** extracts showed the presence of two loci of MDH. The Locus *MDH-1** is monomorphic and had only one allele *A*. The *MDH-2** locus stained intensely exhibiting polymorphic pattern (**Figure 12**) with two alleles *A* and *B* in *G. curmuca* showing three types of genotypes *viz*., fast homozygotes (*MDH-2* AA*), heterozygotes (*MDH-2* AB*) and slow homozygotes (*MDH-2* BB*).

4.1.2.9 *Phosphogluconate dehydrogenase (6PGDH-1.1.1.44)*

The spacing of the bands of this dimeric enzyme had suggested that only one locus in *G. curmuca* (**Figure 14**). The locus *6PGDH** exhibited two alleles *A* and *B* and produced 3 genotypes *viz*, *6PGDH*AA, 6PGDH*AB* and *6PGDH*BB.*

4.1.2.10 *Phosphoglucomutase (PGM-5.4.2.2)*

Phosphoglucomutase is monomeric in vertebrates and two zones/loci of enzyme activity were recorded in all the 3 populations of *G. curmuca* (**Figure 15**). Agar overlay was used in the staining protocol to avoid leaching out of end products.

Both the loci were polymorphic in nature with two alleles, *A* and *A'* (locus 2 – lower) and *B* and *B'* (locus 1 – upper). The pattern of homozygotes and heterozygotes of both the loci are depicted in **Figure 15**.

4.1.2.11 *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 (Richardson *et al.*, 1986). Two zones of enzyme activity are shown in **Figure 16**. The *SOD-1** had only one type of allele-*A* while *SOD-2** was polymorphic with two type of alleles-*A* (slow) and *B* (fast). The genotypes of *SOD** were represented by two homozygotes, *SOD-2*AA* and *SOD-2*BB* and a 3 banded heterozygote *SOD-2*AB*.

4.1.2.12 *Xanthine Dehydrogenase (XDH-1.1.1.204)*

The banding pattern of the dimeric xanthine dehydrogenase in *G. curmuca* shown in **Figure 17**. From the banding pattern, it was inferred that XDH is controlled by only one polymorphic locus that was designated as *XDH**. This locus was represented by two types of alleles-*A* and *B* that exhibited three types of genotypes; two homozygotes and a heterozygote *viz*, *XDH*AA*, *XDH*BB* and *XDH*AB* respectively.

4.1.3 Monomorphic enzymes

4.1.3.1 *Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH-1.2.1.12)*

Glyceraldehyde-3-Phosphate dehydrogenase is tetrameric in structure in vertebrates. The banding patterns of GAPDH enzyme in *G. curmuca* are shown in **Figure 07.** The banding pattern showed only one zone/locus. The monomorphic zone/locus is designated as *GAPDH** that exhibited only one allele-*A* in all the populations.

4.1.3.2 *Malic Enzyme (ME-1.1.1.40)*

Malic enzyme is tetrameric in structure. ME was tested in all the stocks (Periyar River, Chalakkudy River and Chaliyar River) of *G. curmuca* and an intensely staining single locus was found to be responsible for the enzyme activity (**Figure 13**). This locus was monomorphic in all the populations.

Table 07. The names of enzyme loci, number of loci and observed alleles for allozyme analysis in *Gonoproktopterus curmuca*. 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**	*100,108, 117*	Polymorphic
Creatine kinase	2	*CK-1**	*080,100*	Polymorphic
		*CK-2**	*100*	Monomorphic
Esterase	7	*EST-1**	*085, 100*	Polymorphic
		*EST-2**	*100*	Monomorphic
		*EST-3**	*100*	Monomorphic
		*EST-4**	*096, 100*	Polymorphic
		*EST-5**	*100*	Monomorphic
		*EST-6**	*100*	Monomorphic
		*EST-7**	*100*	Monomorphic
Fumarase	ns	*FUM**	ns	ns
Glutamate dehydrogenase	ns	*GDH**	ns	ns
Glucose dehydrogenase	ns	*GLDH**	ns	ns
Glucose phosphate isomerase	2	*GPI-1**	*100*	Monomorphic
		*GPI-2**	*088, 100, 118*	Polymorphic
Glucose-6-phosphate dehydrogenase	1	*G_6PDH**	*100, 120*	Polymorphic
α-Glycerophosphate dehydrogenase	2	*αG_3PDH-1**	*100*	Monomorphic
		*αG_3PDH-2**	*080, 100*	Polymorphic
Glyceraldehyde-3-Phosphate dehydrogenase	1	*GAPDH**	*100*	Monomorphic
Hexokinase	ns	*HK**	ns	ns
Isocitrate dehydrogenase	ns	*ICDH**	ns	ns
Lactate dehydrogenase	3	*LDH-1**	*100*	Monomorphic
		*LDH-2**	*078, 100,148*	Polymorphic
		*LDH-3**	*100*	Monomorphic
Malate dehydrogenase	2	*MDH-1**	*100*	Monomorphic
		*MDH-2**	*090, 100*	Polymorphic
Malic enzyme	1	*ME**	*100*	Monomorphic
Octonol dehydrogenase	ns	*ODH**	ns	ns
Phosphogluconate dehydrogenase	1	*6PGDH**	*083, 100*	Polymorphic
Phosphogluco mutase	2	*PGM-1**	*075,100*	Polymorphic
		*PGM-2**	*078, 100*	Polymorphic
Pyruvate kinase	ns	*PK**	ns	ns
Superoxide dismutase	2	*SOD-1**	*100*	Monomorphic
		*SOD-2**	*100,125*	Polymorphic
Xanthine dehydrogenase	1	*XDH**	*100, 108*	Polymorphic

Table 08. The distribution of allozyme genotypes and their R_f values in *G. cirrhosa* from three riverine systems.

Sl. No.	Enzymes	Locus	Genotypes (Alleles & Rf value)	No. of individuals		
				Periyar River	Chalakkudy River	Chaliyar River
1	AAT	*AAT-1**	*AA (100/100)*	70	70	70
		*AAT-2**	*AA (100/100)*	46	24	36
			BB (108/108)	06	02	13
			CC (117/117)	01	20	08
			AB (100/108)	10	06	09
			AC (100/117)	04	16	03
			BC (108/117)	03	02	01
2	CK	*CK-1**	*AA (100/100)*	51	46	44
			AB (080/100)	08	08	10
			BB (080/080)	11	16	16
		*CK-2**	*AA (100/100)*	70	70	70
3	EST	*EST-1**	*AA (100/100)*	12	28	09
			AB (085/100)	32	39	54
			BB (085/085)	26	03	07
		*EST-2**	*AA (100/100)*	70	70	70
		*EST-3**	*AA (100/100)*	70	70	70
		*EST-4**	*AA (100/100)*	21	46	17
			AB (096/100)	40	13	32
			BB (096/096)	09	11	21
		*EST-5**	*AA (100/100)*	70	70	70
		*EST-6**	*AA (100/100)*	70	70	70
		*EST-7**	*AA (100/100)*	70	70	70
4	GPI	*GPI-1**	*AA (100/100)*	70	70	70
		*GPI-2**	*AA (100/100)*	22	14	18
			BB (118/118)	14	28	10
			AB (100/118)	17	16	02
			CC (088/088)	09	08	34
			AC (100/088)	08	04	06
5	G_6PDH	*G_6PDH**	*AA (100/100)*	43	23	17
			AB (100/120)	20	12	12
			BB (120/120)	07	35	41
6	αG_3PDH	*αG_3PDH-1**	*AA (100/100)*	70	70	70
		*αG_3PDH-2**	*AA (100/100)*	47	28	18
			AB (080/100)	12	19	19
			BB (080/080)	11	23	33
7	GAPDH	*GAPDH-1**	*AA (100/100)*	70	70	70
8	LDH	*LDH-1**	*AA (100/100)*	70	70	70
		*LDH-2**	*AA (100/100)*	28	25	13
			AB (100/148)	26	18	02
			BB (148/148)	16	27	13
			CC (078/078)	00	00	24
			AC (078/100)	00	00	18
		*LDH-3**	*AA (100/100)*	70	70	70
9	MDH	*MDH-1**	*AA (100/100)*	70	70	70
		*MDH-2**	*AA (100/100)*	16	26	12
			AB (090/100)	32	20	28
			BB (090/090)	22	24	30
10	ME	*ME**	*AA (100/100)*	70	70	70
11	6PGDH	*6PGDH**	*AA (100/100)*	46	27	13
			AB (083/100)	12	16	18
			BB (083/083)	12	27	39
12	PGM	*PGM-1**	*BB (100/100)*	37	18	24
			B'B'(075/075)	19	28	36
			BB'(075/100)	14	24	10
		*PGM-2**	*AA (100/100)*	37	26	23
			A'A'(078/078)	27	23	40
			AA'(078/100)	06	21	07
13	SOD	*SOD-1**	*AA (100/100)*	70	70	70
		*SOD-2**	*AA (100/100)*	33	28	18
			AB (100/125)	23	21	22
			BB (125/125)	14	21	30
14	XDH	*XDH**	*AA (100/100)*	33	18	14
			AB (100/108)	17	25	10
			BB (108/108)	20	27	46

4.1.4 Genetic Variability

The allele frequencies of multiple collections of the same river (three years-details in **Table 01**) were tested for significant homogeneity using 'GENEPOP'. The genotype data from different collection sets exhibited allelic homogeneity and hence they were pooled. This yielded three combined data sets *viz.,* Periyar, Chalakkudy and Chaliyar and these were used for analysis of parameters of genetic variation and population structure of *G. curmuca.*

4.1.5 Number and percentage of polymorphic loci

Altogether, 14 polymorphic loci were obtained with 12 polymorphic allozymes (**Table 08**) across 3 populations (total number of allozymes including the monomorphic ones was 14 and total number of loci 29). In all the three populations, all the 14 loci were variable. The percentage of polymorphic loci for over all population was 48.28%.

4.1.6 Observed and effective number of alleles

The observed number of alleles ranged from 2 to 3 per locus in 14 loci of 14 allozymes among all the three populations studied. The highest numbers of alleles were found in Aspartate Amino Transferase (*AAT-2**) and Glucose phosphate isomerase (*GPI-2**), which had three alleles in all the populations. Rest of the polymorphic allozymes were with two alleles each. The *LDH-2** locus in Chaliyar River exhibited three alleles, in contrast to two alleles each in Periyar and Chalakkudy populations. The highest mean observed number of alleles (na) was in Chaliyar River population (1.6538) and whereas the mean observed number of alleles (na) for other two populations was 1.6154 (**Table 09**). The highest mean effective number of alleles (ne) was observed in Chaliyar (1.5347). The locus-wise highest ne value (2.7012) was exhibited by *LDH-2** in Chaliyar River population and while the lowest effective number of alleles was showed by *CK-1** (1.5077) in Periyar River population. In all the populations, AAT-2* and GPI-*2** expressed maximum number of alleles, *i.e.,* three. The mean effective number of alleles (ne) in Periyar River, Chalakkudy River and Chaliyar River

populations were 1.4609, 1.5274 and 1.5347 respectively. The locus-wise effective number of alleles (overall populations) ranged from 1.6423 (in *CK-1**) to 2.9576 (in *GPI-2**) with a mean value of 1.5849.

Table 09. Observed (na) and Effective (ne) number of allozyme alleles in three riverine populations of *G. curmuca*.

Locus	Periyar River		Chalakkudy River		Chaliyar River		Overall Populations	
	na	ne	na	ne	na	ne	na	ne
AAT-2	3	1.6413	3	2.3311	3	2.2395	3	2.1914
CK-1	2	1.5077	2	1.6897	2	1.7241	2	1.6423
EST-1	2	1.9231	2	1.7738	2	1.9984	2	1.9924
EST-4	2	1.9429	2	1.6000	2	1.9935	2	1.9195
GPI-2	3	2.6266	3	2.4848	3	2.4823	3	2.9576
G_6PDH	2	1.5817	2	1.9429	2	1.7896	2	2.0000
αG_3PDH-2	2	1.5817	2	1.9898	2	1.9122	2	1.9698
LDH-2	2	1.9429	2	1.9984	3	2.7012	3	2.6042
MDH-2	2	1.9854	2	1.9984	2	1.8760	2	1.9783
6PGDH	2	1.6182	2	2.0000	2	1.7575	2	1.9971
PGM-1	2	1.8760	2	1.9600	2	1.9429	2	1.9993
PGM-2	2	1.9600	2	1.9963	2	1.8886	2	1.9993
SOD-2	2	1.8628	2	1.9802	2	1.9429	2	1.9912
XDH	2	1.9333	2	1.9675	2	1.6543	2	1.9651
Total	30	---	30	---	31	---	31	---
Mean	1.6154	1.4609	1.6154	1.5274	1.6538	1.5347	1.6538	1.5849
± S.D.	0.6373	0.4798	0.6373	0.5235	0.6895	0.5475	0.6895	0.5992

4.1.7 Frequencies of alleles

The allelic frequencies of 14 polymorphic loci of 12 allozymes are given in **Table 10**. In Periyar River population, the allelic frequencies ranged from 0.0643 (in *AAT-2**) to 0.7857 (in *CK-1**). In Chalakkudy population, the allelic frequencies ranged from 0.0857 (in *AAT-2**) to 0.7500 (in *EST-4**). In Chaliyar population, the allelic frequencies ranged from 0.1429 (in *AAT--2**) to 0.7000 (in *CK-1**). The overall allele frequency value ranged from 0.1571 (in *LDH-2**) to 0.7333 (in *CK-1**).

Table 10. Allozyme alleles and allele frequencies in *G. curmuca* from three riverine populations and among populations.

Locus	Alleles	Periyar River	Chalakkudy River	Chaliyar River	Overall Populations
AAT-2	*100*	0.7571	0.5000	0.6000	0.6190
	108	0.1786	0.0857	0.2571	0.1738
	117	0.0643	0.4143	0.1429	0.2071
CK-1	*080*	0.7857	0.7143	0.7000	0.7333
	100	0.2143	0.2857	0.3000	0.2667
EST-1	*085*	0.4000	0.6786	0.5143	0.5310
	100	0.6000	0.3214	0.4857	0.4690
EST-4	*096*	0.5857	0.7500	0.4714	0.6024
	100	0.4143	0.2500	0.5286	0.3976
GPI-2	*088*	0.4929	0.3429	0.3143	0.3833
	100	0.3214	0.5143	0.1571	0.3310
	118	0.1857	0.1429	0.5286	0.2857
G_6PDH	*100*	0.7571	0.4143	0.3286	0.5000
	120	0.2429	0.5857	0.6714	0.5000
αG_3PDH-2	*080*	0.7571	0.5357	0.3929	0.5619
	100	0.2429	0.4643	0.6071	0.4381
LDH-2	*078*	---	---	**0.4714**[#]	0.4667
	100	0.4143	0.5143	0.2000	0.3762
	148	0.5857	0.4857	0.3286	0.1571
MDH-2	*090*	0.4571	0.5143	0.3714	0.4476
	100	0.5429	0.4857	0.6286	0.5524
6PGDH	*083*	0.7429	0.5000	0.3143	0.5190
	100	0.2571	0.5000	0.6857	0.4810
PGM-1	*075*	0.6286	0.4286	0.4143	0.4905
	100	0.3714	0.5714	0.5857	0.5095
PGM-2	*078*	0.5714	0.5214	0.3786	0.4905
	100	0.4286	0.4786	0.6214	0.5095
SOD-2	*100*	0.6357	0.5500	0.4143	0.5333
	125	0.3643	0.4500	0.5857	0.4667
XDH	*100*	0.5929	0.4357	0.2714	0.4333
	108	0.4071	0.5643	0.7286	0.5667

[#]Private allele

4.1.8 Stock-specific markers (private alleles)

In *LDH-2**, one of the allele (078) was present only in Chaliyar River population (allele frequency 0.4714) and this was treated as stock-specific / private allele (**Table 11**).

Table 11. Private allele in allozyme and its frequency

Locus	Private Allele (R_f value)	Allele frequency		
		Periyar River	Chalakkudy River	Chaliyar River
LDH-2	78	----	----	0.4714

4.1.9 Observed and expected heterozygosities

The range of observed heterozygosity was from 0.0797 (*EST-4**) to 0.4755 (*MDH-2**) in Periyar River population, where as the mean observed heterozygosity was 0.1560. The expected heterozygosity for this population ranged from 0.1868 (*EST-4**) to 0.4963 (*MDH-2**), with a mean of 0.2128 (**Table 12**). In Chalakkudy River population, the mean of observed heterozygosity was 0.1538, while the observed heterozygosity ranged from 0.1143 (*CK-1**) to 0.5272 (*EST-1**). The expected heterozygosity for this population ranged from 0.2996 (*LDH-2**) to 0.5710 (*AAT-2**), with a mean of 0.2233. In Chaliyar River population, the observed heterozygosity ranged from 0.1000 (*PGM-2**) to 0.7714 (*EST-1**). The mean observed heterozygosity was 0.1445. But, the expected heterozygosity for this population ranged from 0.1705 (*PGM-2**) to 0.4996 (*EST-1**) with a mean of 0.2035 (**Table 12**).

4.1.10 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 *EST-1**, *G_6PDH**, *LDH-2** and *MDH-2** in Periyar River population and *EST-4** in Chaliyar River population, after the sequential bonferroni correction was made to the probability levels (**Table 12**). Wright's fixation index (F_{IS}) (Wright, 1951) is a measure of heterozygote deficiency or excess and their significant values for each locus in each population are given in **Table 12**. The F_{IS} values for each locus ranged from –0.5390 for *EST-1** to +0.5500 for *EST-1**. In most of the loci, the value of F_{IS} significantly deviated from zero, indicating deficiency of heterozygotes.

Table 12. Summary of genetic variation and heterozygosity statistics of fourteen allozyme loci in *Gonoproktopterus curmuca.*

Locus	Populations (N=70 each)		
	Periyar River	Chalakkudy River	Chaliyar River
AAT-2			
H obs.	0.2429	0.3429	0.1857
H exp	0.3907	0.5710	0.2535
F_{IS}	+0.385	+0.406	+0.268
P_{HW}	<0.0001***	<0.0001***	<0.0001***
CK-1			
H obs.	0.1143	0.1143	0.1429
H exp	0.3367	0.3082	0.3024
F_{IS}	+0.265	+0.323	+.0664
P_{HW}	<0.0001***	<0.0001***	<0.0001***
EST-1			
H obs.	0.4571	0.5272	0.7714
H exp	0.4800	0.4362	0.4996
F_{IS}	+0.550	-0.271	-0.539
P_{HW}	0.8029	0.0285*	<0.0001***
EST-4			
H obs	0.0797	0.1856	0.4562
H exp	0.1868	0.3150	0.4984
F_{IS}	+0.284	+0.310	+0.090
P_{HW}	<0.0001***	<0.0001***	0.4799
GPI-2			
H obs.	0.3676	0.2855	0.1143
H exp	0.4193	0.3976	0.2971
F_{IS}	+0.149	+0.227	+0.311
P_{HW}	<0.0001***	<0.0001***	<0.0001***
G_6PDH			
H obs	0.2585	0.1714	0.1714
H exp	0.3678	0.3253	0.2412
F_{IS}	+0.230	+0.451	+0.316
P_{HW}	0.0970	<0.0001***	<0.0001***
αG_3PDH-2			
H obs	0.1712	0.2768	0.2714
H exp	0.2678	0.3974	0.3770
F_{IS}	+0.239	+0.360	+0.237
P_{HW}	<0.0001***	<0.0001***	<0.0001***
LDH-2			
H obs	0.3618	0.2571	0.2857
H exp	0.4853	0.2996	0.3298
F_{IS}	+0.241	+0.091	+0.151
P_{HW}	0.0513	<0.0001***	<0.0001***
MDH-2			
H obs	0.4755	0.2857	0.4213
H exp	0.4963	0.3696	0.4669
F_{IS}	+0.086	+0.234	+0.150
P_{HW}	0.0926	0.0036*	0.0036*

Table12 Continued

Locus	Populations (N=70 each)		
	Periyar River	Chalakkudy River	Chaliyar River
6PGDH			
H obs	0.1714	0.2286	0.2571
H exp	0.2220	0.3254	0.3310
F_{IS}	+0.156	+0.248	+0.149
P_{HW}	<0.0001***	<0.0001***	<0.0001***
PGM-1			
H obs	0.2000	0.3429	0.1625
H exp	0.2669	0.3898	0.2053
F_{IS}	+0.177	+0.177	+0.209
P_{HW}	<0.0001***	0.0143*	<0.0001***
PGM-2			
H obs.	0.0857	0.3000	0.1000
H exp.	0.1898	0.4291	0.1705
F_{IS}	+0.227	+0.105	+0.190
P_{HW}	<0.0001***	<0.0001***	<0.0001***
SOD-2			
H obs.	0.3286	0.3000	0.3143
H exp.	0.4632	0.4415	0.4553
F_{IS}	+0.297	+0.226	+0.329
P_{HW}	0.0192*	0.0014*	0.0033*
XDH			
H obs	0.2409	0.3515	0.1432
H exp	0.3228	0.4917	0.2955
F_{IS}	+0.102	+0.280	+0.243
P_{HW}	<0.0001***	0.0278*	<0.0001***
Mean overall loci			
H obs	0.1560	0.1538	0.1445
H exp	0.2128	0.2233	0.2035
F_{IS}	---	---	---
$P_{(0.95)}$	0.4385	0.4288	0.3482
$P_{(0.99)}$	0.4385	0.4288	0.3482
A_n	1.6154	1.6154	1.6542

H obs. = Observed heterozygosity
H exp. = Expected heterozygosity
F_{IS} = Inbreeding coefficient
P_{HW} = Probability value of significant deviation from HWE
$P_{(0.95)}$ = Polymorphism at 0.95 criteria
$P_{(0.99)}$ = Polymorphism at 0.99 criteria
A_n = Mean number of alleles per locus
* = Significant at P<0.05
*** = Significant after Bonferroni adjustment.

4.1.11 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.

Table 13. Fisher's exact test of allozyme allele homogeneity for all the population pairs of *Gonoproktopterus curmuca.*

Locus	Populations pairs	P-value (Exact test)	S.E.
AAT-2	PER & CHL	0.0000***	0.0000
	PER & CLR	0.0000***	0.0000
	CHL & CLR	0.0023*	0.0001
CK-1	PER & CHL	0.0041*	0.0003
	PER & CLR	0.0028*	0.0002
	CHL & CLR	0.0032*	0.0004
EST-1	PER & CHL	0.0000***	0.0000
	PER & CLR	0.0002*	0.0001
	CHL & CLR	0.0038*	0.0008
EST-4	PER & CHL	0.0000***	0.0000
	PER & CLR	0.0021*	0.0002
	CHL & CLR	0.0038*	0.0009
GPI-2	PER & CHL	0.0000***	0.0000
	PER & CLR	0.0036*	0.0006
	CHL & CLR	0.0048*	0.0012
G_6PDH	PER & CHL	0.0061*	0.0016
	PER & CLR	0.0000***	0.0000
	CHL & CLR	0.0000***	0.0000
αG_3PDH-2	PER & CHL	0.0030*	0.0003
	PER & CLR	0.0086*	0.0012
	CHL & CLR	0.0103*	0.0028
LDH-2	PER & CHL	0.0000***	0.0000
	PER & CLR	0.0000***	0.0000
	CHL & CLR	0.0000***	0.0000
MDH-2	PER & CHL	0.0165*	0.0022
	PER & CLR	0.0299*	0.0039
	CHL & CLR	0.0189*	0.0031
6PGDH	PER & CHL	0.0213*	0.0042
	PER & CLR	0.0049*	0.0016
	CHL & CLR	0.0021*	0.0004
PGM-1	PER & CHL	0.0223*	0.0046
	PER & CLR	0.0106*	0.0026
	CHL & CLR	0.0423*	0.0057
PGM-2	PER & CHL	0.0219*	0.0032
	PER & CLR	0.0164*	0.0017
	CHL & CLR	0.0300*	0.0036
SOD-2	PER & CHL	0.0086*	0.0012
	PER & CLR	0.0082*	0.0012
	CHL & CLR	0.0125*	0.0022
XDH	PER & CHL	0.0076*	0.0018
	PER & CLR	0.0062*	0.0006
	CHL & CLR	0.0136*	0.0030
Overall loci	**Overall population**	0.0000***	--

* Significant at P<0.05; *** significant at P<0.0001 after sequential Bonferroni adjustment;
PER - Periyar River; CHL – Chalakkudy River; CLR – Chaliyar River;
Markov chain parameters - dememorization: 1000, batches: 100 and iterations: 1000

4.1.12 Genetic differentiation

The co-efficient of genetic differentiation (F_{ST}) estimated through the estimator of Weir and Cockerham (1984) ranged from 0.0267 for *PGM-2** to 0.0704 for *6PGDH**, with a mean of 0.0510, indicating that 5.1% of the total genetic variation exists among populations (**Table 14**). The estimate of pair-wise F_{ST} differed significantly ($P<0.0001$) from zero for all pairs of riverine locations (**Table 15**). Locus-wise heterogeneity values between population pairs and for overall population (exact test) are depicted in **Table 13**. Out of the possible 42 tests, all population pairs exhibited genetic heterogeneity.

Table 14. F-statistics (F_{ST}) for overall populations of *Gonoproktopterus curmuca* using allozymes.

Locus	Sample Size	F_{ST}
AAT-2	210	0.0510
CK-1	210	0.0603
EST-1	210	0.0425
EST-4	210	0.0424
GPI-2	210	0.0601
G_6PDH	210	0.0631
αG_3PDH-2	210	0.0612
LDH-2	210	0.0603
MDH-2	210	0.0439
6PGDH	210	0.0704
PGM-1	210	0.0383
PGM-2	210	0.0267
SOD-2	210	0.0334
XDH	210	0.0601
Mean	**210**	**0.0510**

Table 15. Pair-wise Fisher's F_{ST} (θ) (above diagonal) and their significance levels (below diagonal) between riverine populations of *Gonoproktopterus curmuca* using allozyme markers.

Populations	Periyar	Chalakkudy	Chaliyar
Periyar	----	0.04018	0.05994
Chalakkudy	<0.0001***	-----	0.04995
Chaliyar	<0.0001***	<0.0001***	----

*** Significant after Bonferroni adjustment (P<0.0001)

4.1.13 Genetic relationship between populations

Nei's (1978) unbiased genetic identity and distance estimated between pairs of three populations of *G. curmuca* are presented in **Table 16**. Genetic distance values between the populations of Periyar River population and Chalakkudy River population was 0.0329; between Periyar River population and Chaliyar River population was 0.0702; and between Chalakkudy River population and Chaliyar River population was 0.0501. The Periyar River and Chalakkudy River populations are closer when compared to Chaliyar River population. The genetic distance values agree with the geographic distances between the populations (**Table 16**).

Table 16. Nei's (1978) genetic identity (above diagonal) and genetic distance (below diagonal) using allozyme markers in G. curmuca; geographical distances (in Km) are given in bracket

Populations	Periyar River	Chalakkudy River	Chaliyar River
Periyar River	****	0.9696	0.9326
Chalakkudy River	0.0329 (60)	****	0.9527
Chaliyar River	0.0702 (220)	0.0501 (160)	****

4.1.14 AMOVA

The analysis of molecular variance (AMOVA) using allozyme data indicated strongly significant genetic differentiation among *G. curmuca* populations (F_{ST}

0.0518; P<0.0001) with respect to the total population (**Table 17**). The F_{ST} value among population in the hierarchical analysis (5.18%) was similar to that obtained without using a hierarchical analysis (5.1%).

Table 17. Analysis of Molecular Variance (AMOVA) based on allozyme markers in three populations of *G. curmuca*.

Sources of Variation	Variance component	Percentage of Variation (%)	Fixation indices
Among populations (Among Rivers)	0.1423 (Va)	05.18	0.0518***
Within populations (Within River)	2.6070 (Vb)	94.82	---
Total	2.7493 (Vt)	---	---

***P<0.0001; Significance test after 1000 permutations

4.1.15 Dendrogram

On the basis of Nei's (1978) genetic distance values, the phylogenetic relationships between three populations of *Gonoproktopterus curmuca* were made through a dendrogram (**Figure 35**) following unweighted pair group method using arithmetic averages (UPGMA, Sneath and Sokal, 1973). The high bootstrap values suggested the populations have robust clusters.

4.1.16 Bottleneck analysis

The bottleneck results based on allozyme data indicated clear mode shift of allele diversity in all the populations in contrast to the expected L-shaped distribution, if the population followed mutation drift equilibrium (**Figure 17a**). The probability values (**Table 18**) also indicated significant genetic bottleneck in the populations of *G. curmuca*.

Table 18. Analysis of genetic bottleneck in G. curmuca with allozyme markers under infinite allele model (IAM) and two-phased model (TPM).

Periyar	He	IAM#		TPM	
		Heq	P	Heq	P
AAT-2	0.243	0.202	0.0486	0.219	0.0512
CK-1	0.116	0.109	0.0916	0.104	0.0866
EST-1	0.461	0.423	0.0504	0.410	0.0671
EST-4	0.000	0.000	---	0.000	---
GPI-2	0.371	0.261	0.0338	0.236	0.0428
G_6PDH	0.259	0.186	0.0426	0.195	0.0358
αG_3PDH-2	0.182	0.122	0.0113	0.136	0.0437
LDH-2	0.366	0.289	0.0268	0.269	0.0496
MDH-2	0.479	0.416	0.0402	0.446	0.0588
6PGDH	0.173	0.109	0.0434	0.122	0.0414
PGM-1	0.201	0.169	0.0485	0.152	0.0321
PGM-2	0.085	0.026	0.1365	0.043	0.1071
SOD-2	0.330	0.294	0.0547	0.288	0.0663
XDH	0.241	0.199	0.0512	0.173	0.0439
Wilcoxon Test (P)		0.00326*		0.00544*	
Chalakkudy	**He**	**IAM#**		**TPM**	
		Heq	**P**	**Heq**	**P**
AAT-2	0.345	0.234	0.0521	0.329	0.0678
CK-1	0.115	0.105	0.1318	0.109	0.1008
EST-1	0.531	0.499	0.0418	0.462	0.0501
EST-4	0.185	0.162	0.0488	0.159	0.0240
GPI-2	0.286	0.262	0.1464	0.213	0.1254
G_6PDH	0.172	0.113	0.0277	0.132	0.0195
αG_3PDH-2	0.277	0.270	0.3345	0.262	0.2116
LDH-2	0.257	0.192	0.0077	0.239	0.0086
MDH-2	0.285	0.203	0.0491	0.259	0.0445
6PGDH	0.230	0.201	0.0562	0.189	0.0414
PGM-1	0.343	0.313	0.0727	0.299	0.0849
PGM-2	0.300	0.289	0.1453	0.272	0.1218
SOD-2	0.301	0.286	0.0699	0.273	0.0442
XDH	0.353	0.326	0.0456	0.316	0.0532
Wilcoxon Test (P)		0.00341*		0.00396*	
Chaliyar	**He**	**IAM#**		**TPM**	
		Heq	**P**	**Heq**	**P**
AAT-2	0.186	0.171	0.2732	0.162	0.2964
CK-1	0.143	0.103	0.0374	0.109	0.0582
EST-1	0.772	0.619	0.0242	0.663	0.0394
EST-4	0.460	0.402	0.0427	0.399	0.0448
GPI-2	0.145	0.096	0.0335	0.093	0.0482
G_6PDH	0.172	0.142	0.0523	0.159	0.0619
αG_3PDH-2	0.271	0.252	0.0515	0.259	0.0660
LDH-2	0.286	0.203	0.0402	0.243	0.0488
MDH-2	0.423	0.400	0.1223	0.399	0.1497
6PGDH	0.260	0.211	0.0817	0.232	0.1103
PGM-1	0.164	0.132	0.0430	0.129	0.0391
PGM-2	0.100	0.092	0.1502	0.089	0.1655
SOD-2	0.315	0.299	0.3244	0.286	0.3466
XDH	0.142	0.077	0.0459	0.116	0.0520
Wilcoxon Test (P)		0.00385*		0.00404*	

Heq: Heterozygosity expected at mutation-drift equilibrium; **He**: measured/observed heterozygosity; * **Significant** value ($P<0.05$); # Infinite Allele Model (IAM) is widely used for allozymes.

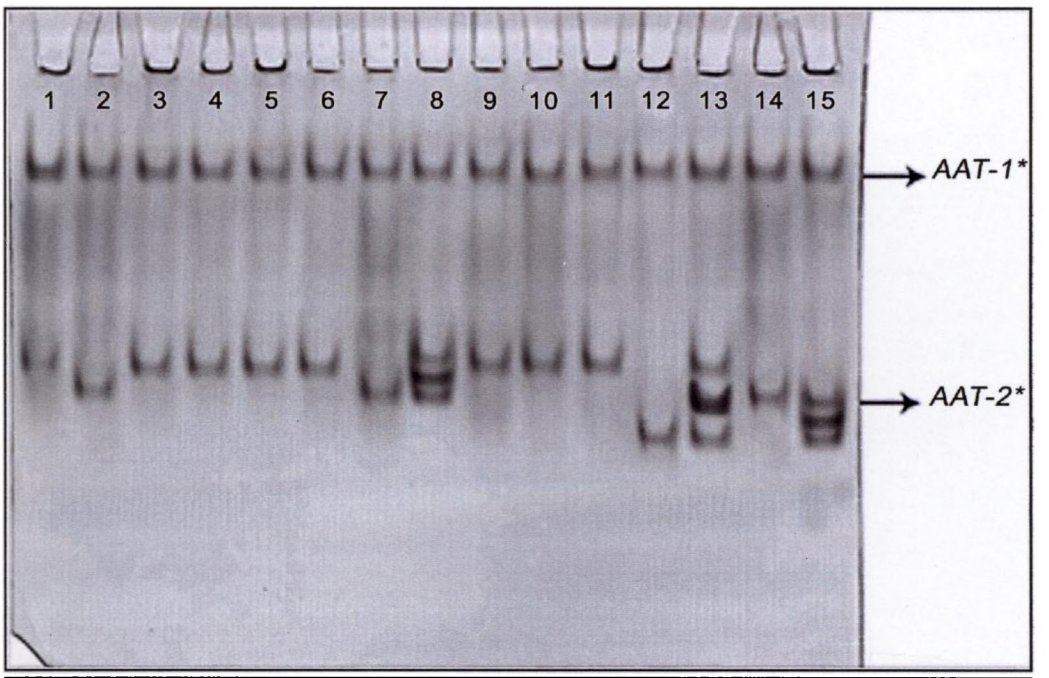

Fig. 4. Aspartate amino transferase (*AAT*) pattern in *G. curmuca*.
Lanes 1-5 samples from Periyar River,
6-10 Chalakkudy River and 11-15 Chaliyar River.

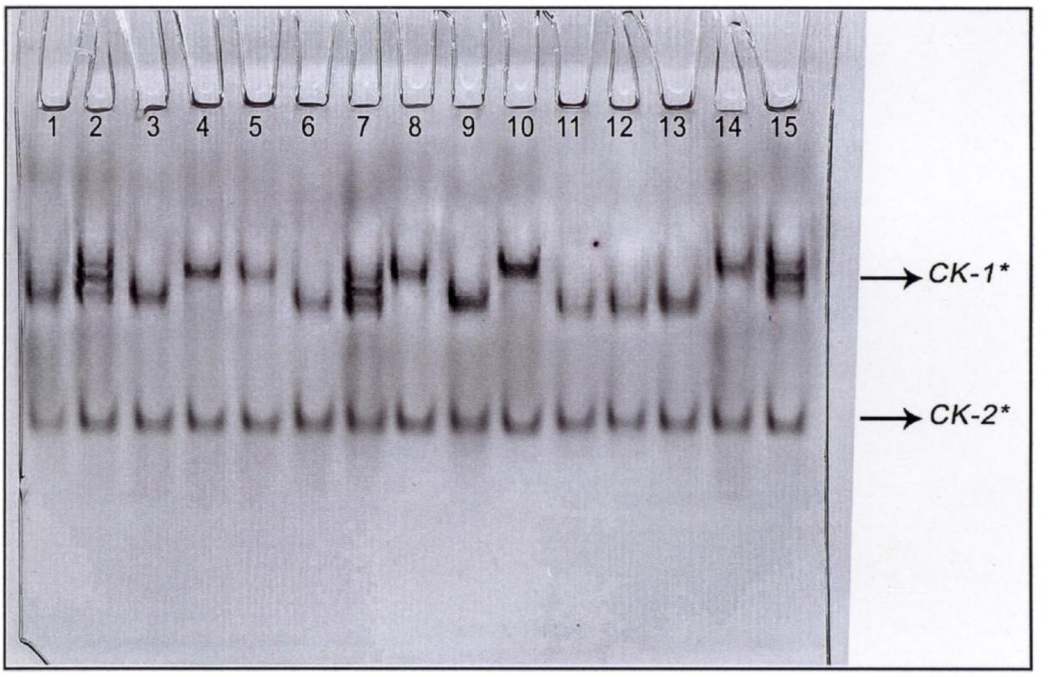

Fig. 5. Creatine kinase (*CK*) pattern in *G. curmuca*.
Lanes 1-5 samples from Periyar River,
6-10 Chalakkudy River and 11-15 Chaliyar River.

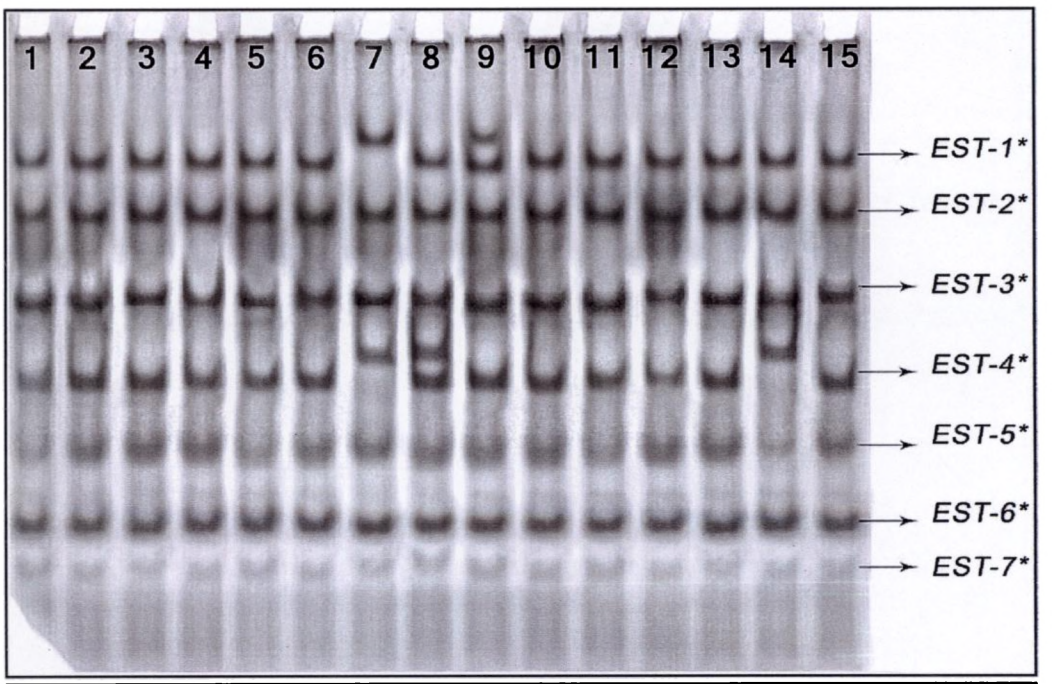

Fig. 6. Esterase (*EST*) pattern in *G. curmuca*.
Lanes 1-5 samples from Periyar River,
6-10 Chalakkudy River and 11-15 Chaliyar River.

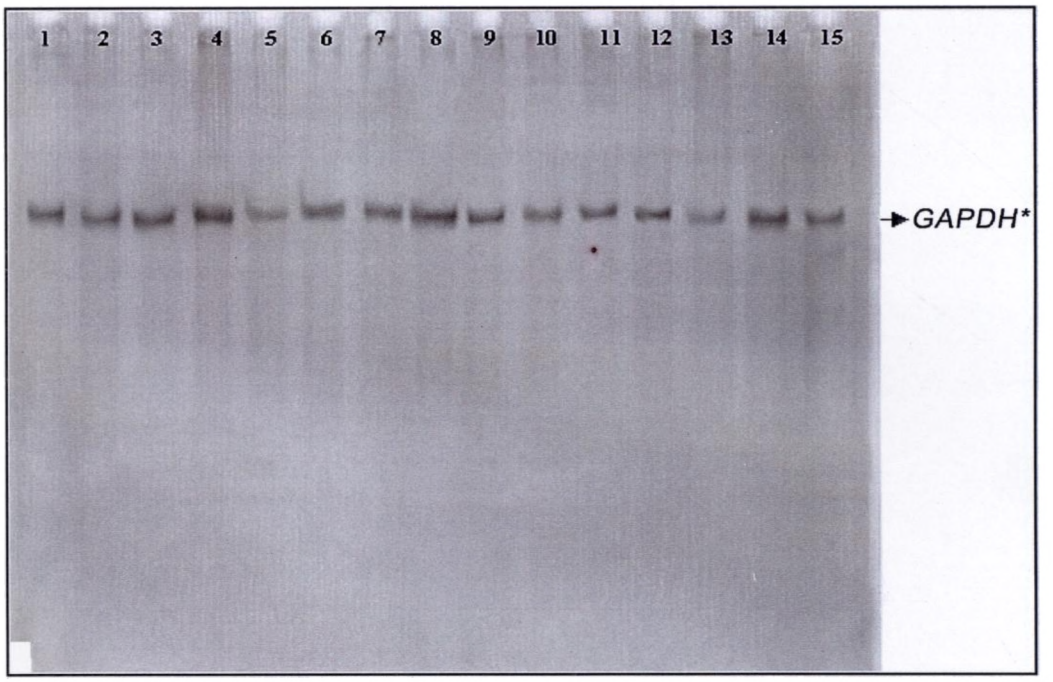

Fig. 7. Glyceraldehyde-3-Phosphate dehydrogenase (*GAPDH*) pattern in *G. curmuca*. Lanes 1-5 samples from Periyar River, 6-10 Chalakkudy River and 11-15 Chaliyar River.

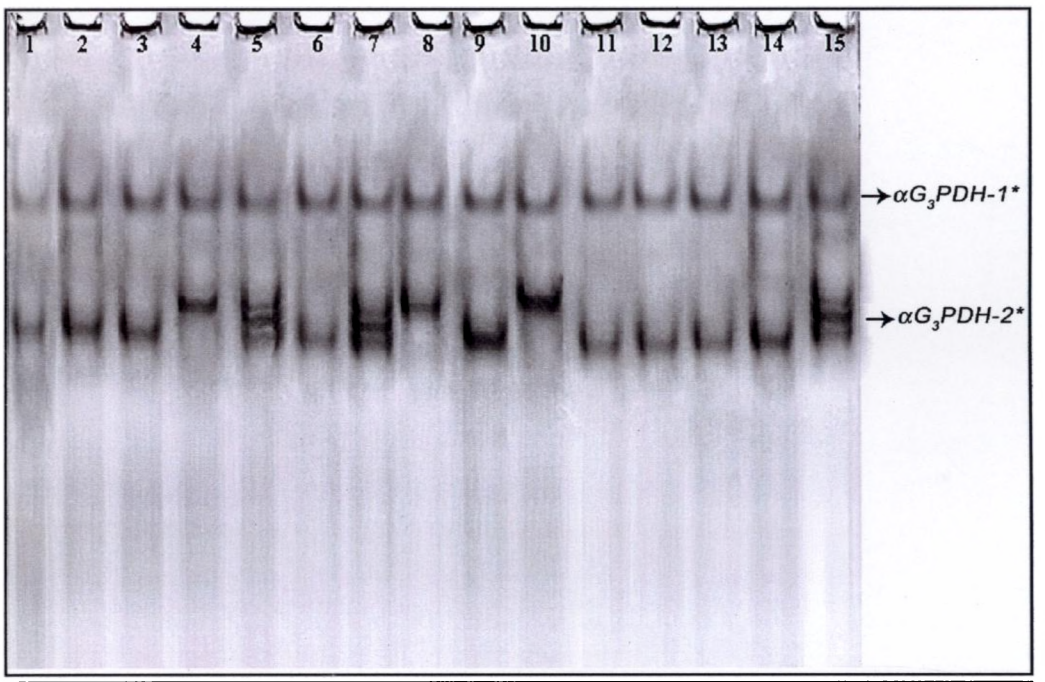

Fig. 8. α-Glycerophosphate (Glycerol 3-phosphate) dehydrogenase (αG_3PDH) pattern in *G. curmuca*. Lanes 1-5 samples from Periyar River, 6-10 Chalakkudy River and 11-15 Chaliyar River.

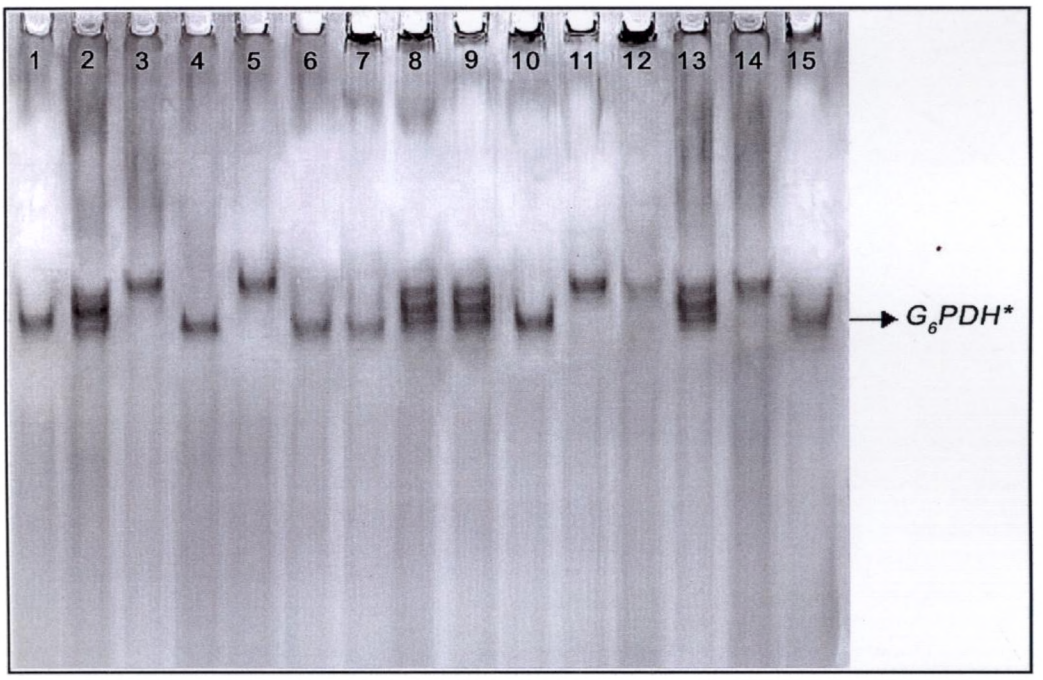

Fig. 9. Glucose-6-phosphate dehydrogenase (G_6PDH) pattern in *G. curmuca*. Lanes 1-5 samples from Periyar River, 6-10 Chalakkudy River and 11-15 Chaliyar River.

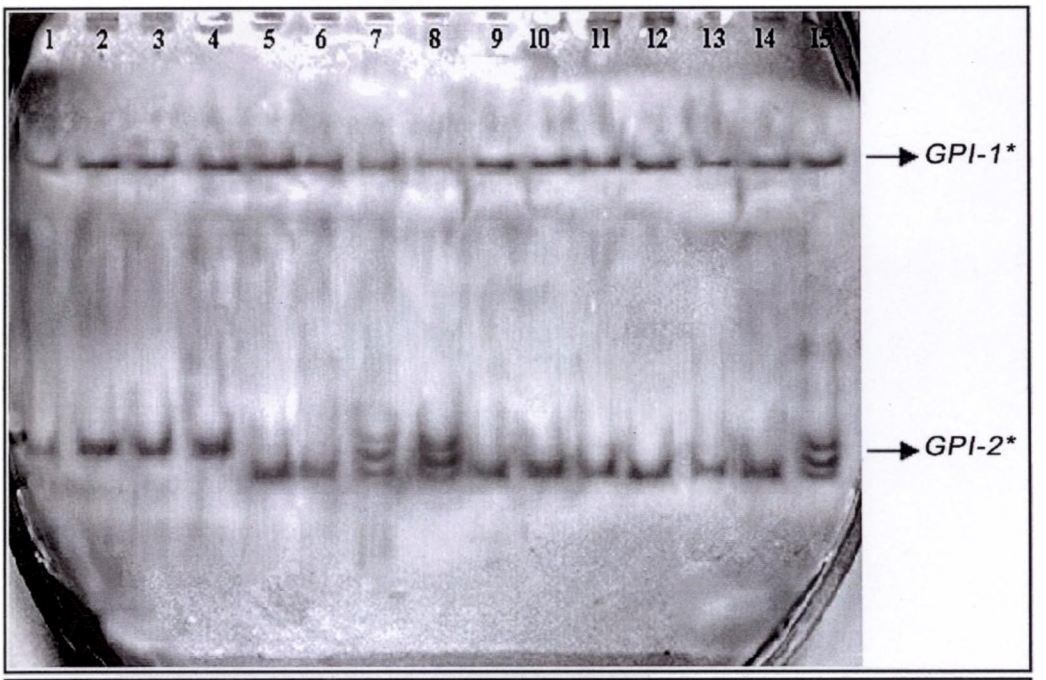

Fig. 10. Glucose phosphate isomerase (*GPI*) pattern in *G. curmuca*. Lanes 1-5 samples from Periyar River, 6-10 Chalakkudy River and 11-15 Chaliyar River.

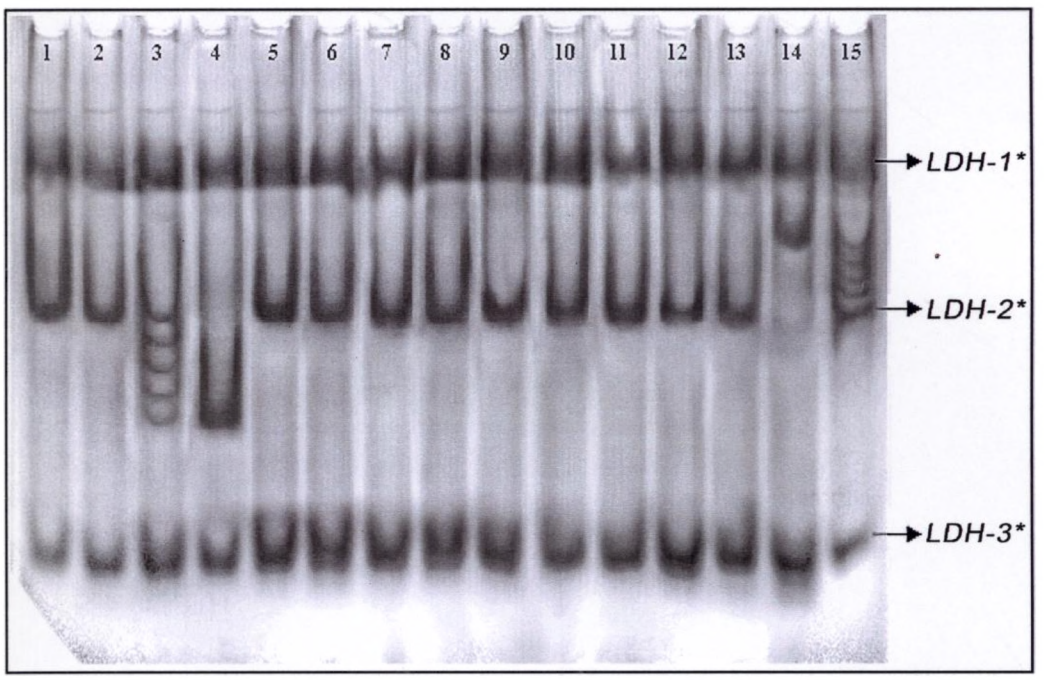

Fig. 11. Lactate dehydrogenase (*LDH*) pattern in *G. curmuca*. Lanes 1-5 samples from Periyar River, 6-10 Chalakkudy River and 11-15 Chaliyar River.

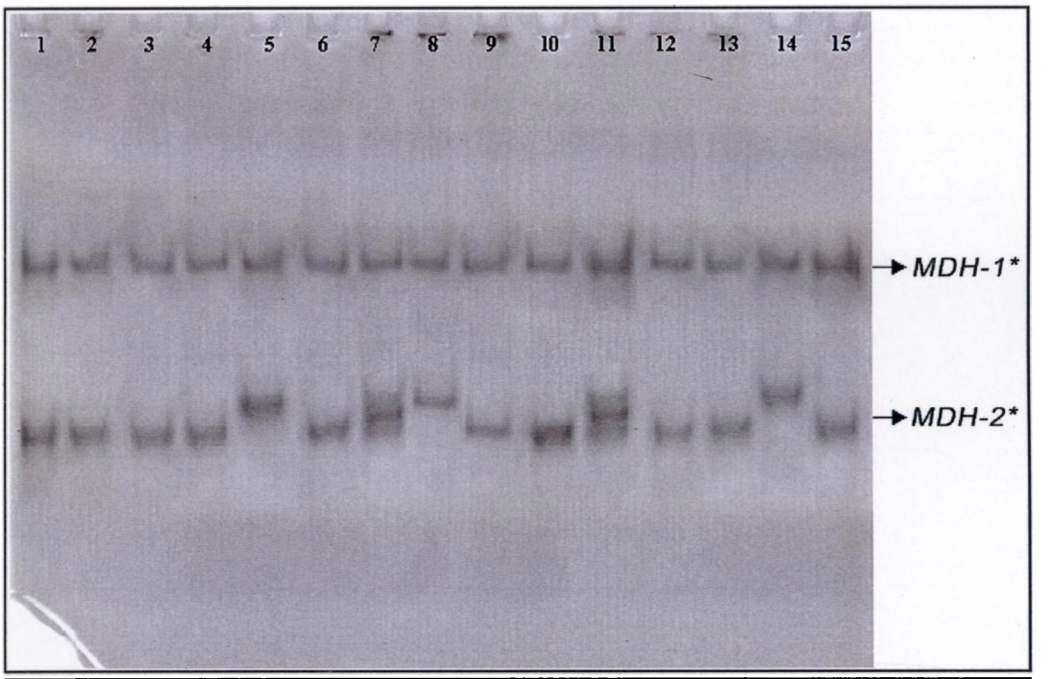

Fig. 12. Malate dehydrogenase (*MDH*) pattern in *G. curmuca.*
Lanes 1-5 samples from Periyar River,
6-10 Chalakkudy River and 11-15 Chaliyar River.

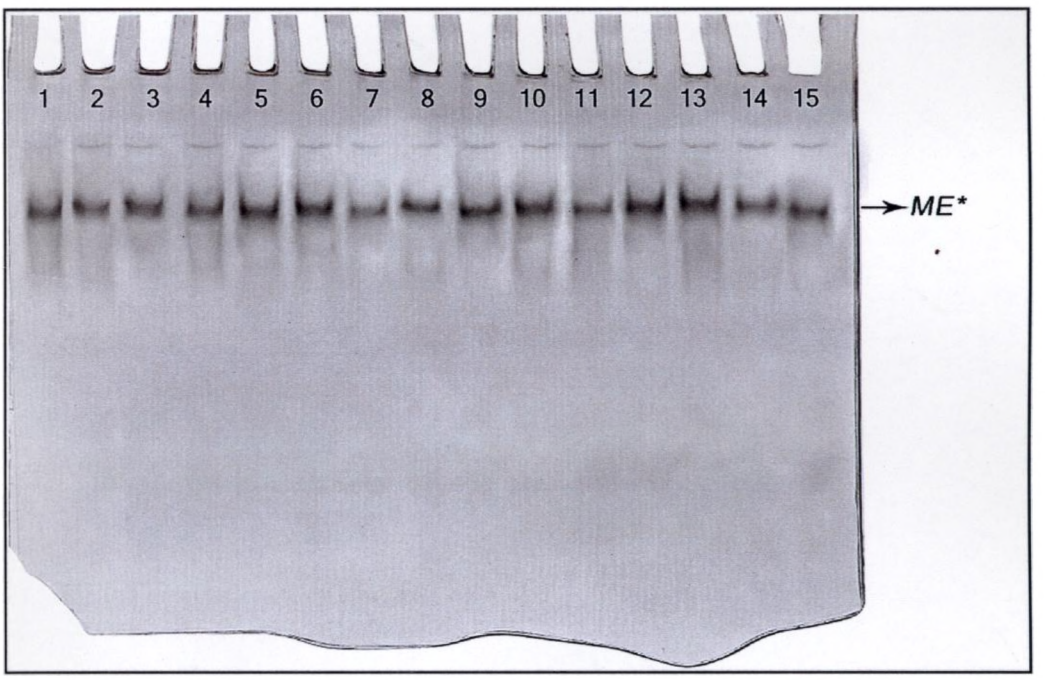

Fig. 13. Malic enzyme (*ME*) pattern in *G. curmuca*.
Lanes 1-5 samples from Periyar River,
6-10 Chalakkudy River and 11-15 Chaliyar River.

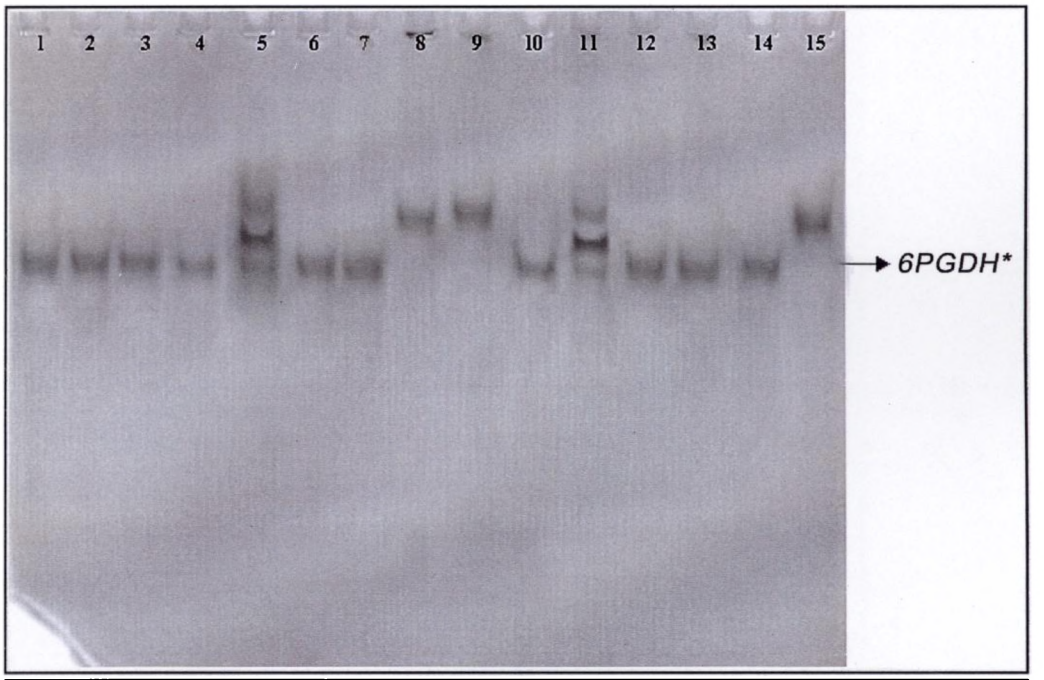

Fig. 14. **6-Phosphogluconate dehydrogenase (*6PGDH*) pattern in *G. curmuca.***
Lanes 1-5 samples from Periyar River,
6-10 Chalakkudy River and 11-15 Chaliyar River.

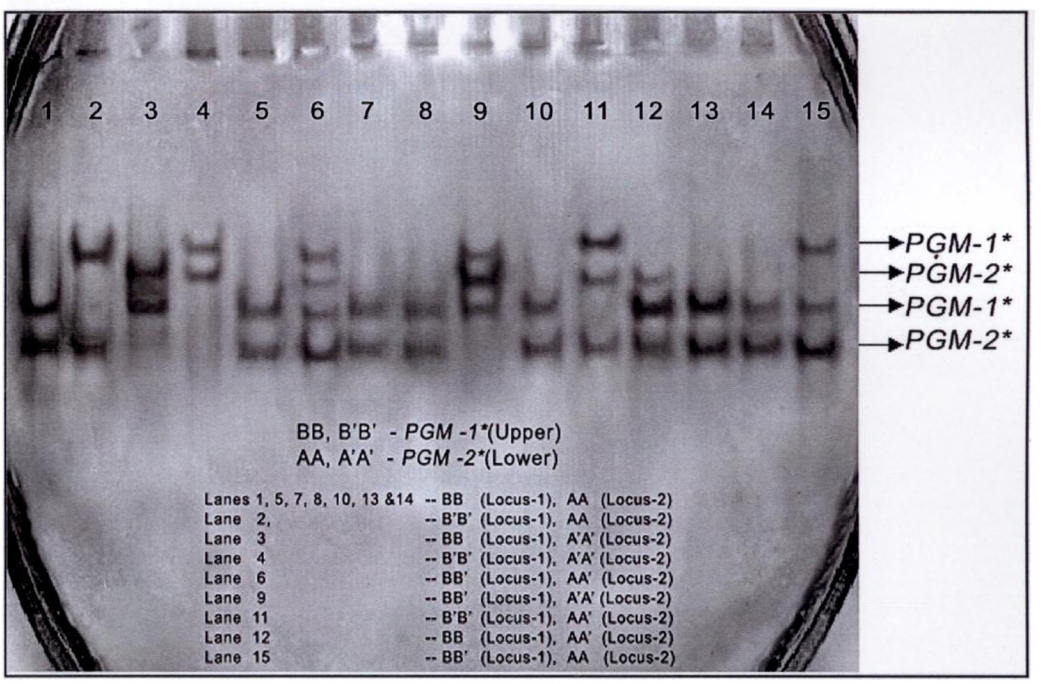

Fig. 15. Phosphoglucomutase (*PGM*) pattern in *G. curmuca.*
Lanes 1-5 samples from Periyar River,
6-10 Chalakkudy River and 11-15 Chaliyar River.

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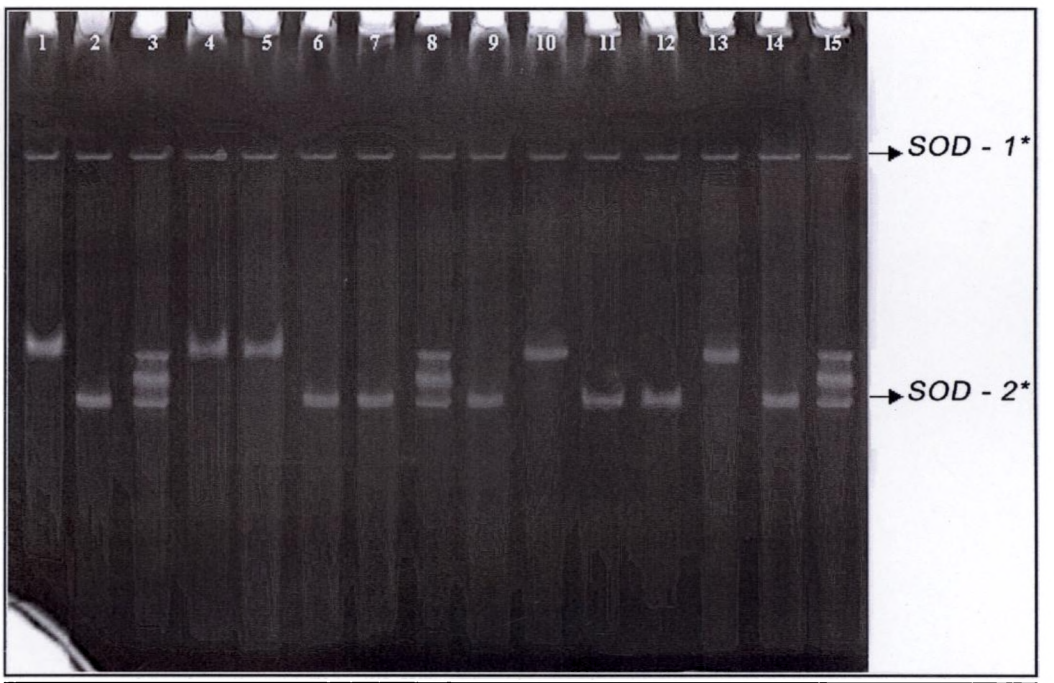

Fig. 16. Superoxide dismutase (*SOD*) pattern in *G. curmuca*.
Lanes 1-5 samples from Periyar River,
6-10 Chalakkudy River and 11-15 Chaliyar River.

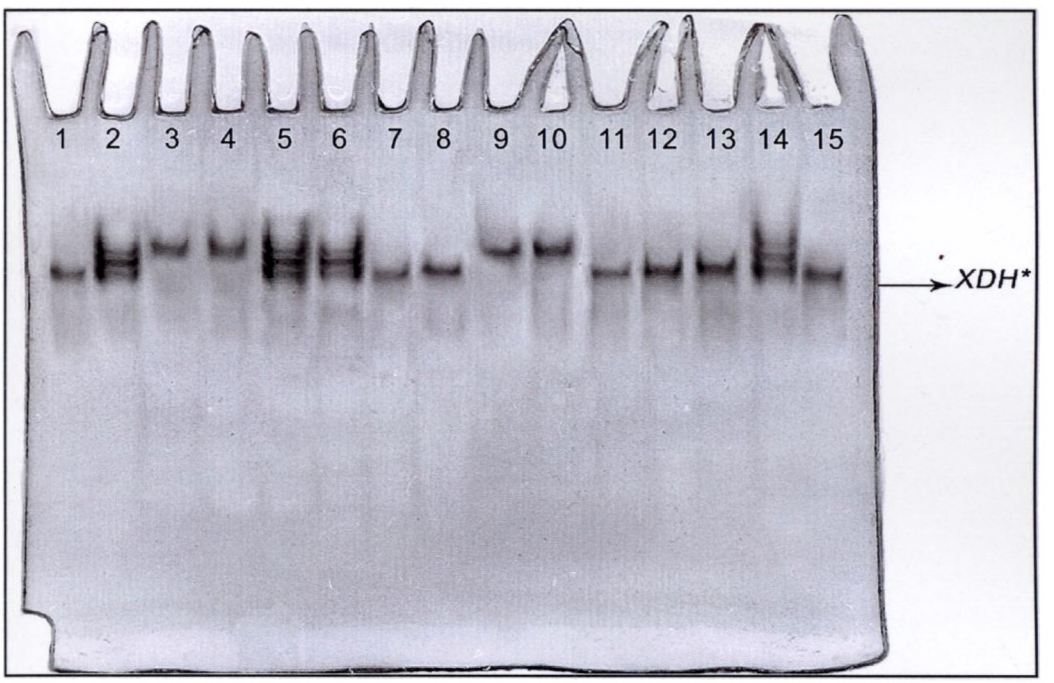

Fig. 17. Xanthine dehydrogenase (*XDH*) pattern in *G. curmuca*.
Lanes 1-5 samples from Periyar River,
6-10 Chalakkudy River and 11-15 Chaliyar River.

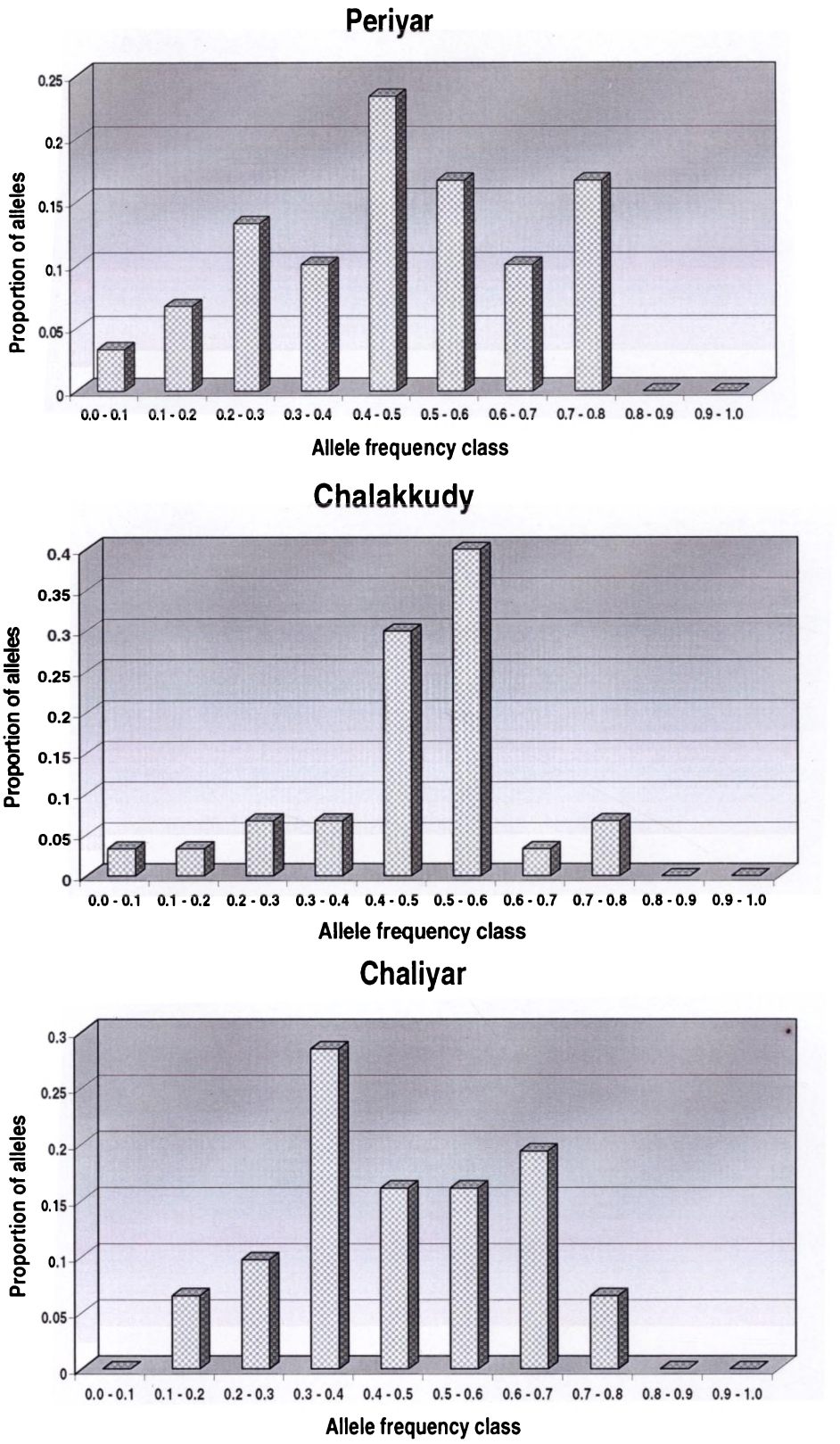

Fig.17 a. Qualitative "mode-shift" indicator test to discriminate bottlenecked populations of *Gonoproktopterus curmuca* from three rivers, based on allozyme allele frequency distribution.

4.2 Microsatellite 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. To analyse the yield and quality, the extracted DNA was electrophoresed through 0.7% agarose gel containing ethidium bromide (5µg/mL) and a single bright discrete band was observed near the well (high molecular weight DNA) without shearing and RNA contamination.

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 (25ng) 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

Forty primers (microsatellite flanking regions) from different cyprinid species collected from literature and NCBI GenBank accessions were used to study the **cross-species amplification** of microsatellites in *G. curmuca* **(Table 19)**. The annealing temperature (Ta) of these primers in the resource species and the same in *Gonoproktopterus curmuca* at which successful amplification occurred without stutter bands are given in **Table 19**. Only **eight** primers out of 40 gave scorable banding patterns (9 loci) after PCR amplification.

Table 19. Characteristics of polymorphic microsatellite loci in *G. curmuca*.

Sl. No.	Resource species					*Gonoproktopterus curmuca*			
	Species	Locus	Primer sequence (5'→3')	Repeat motif	Ta (°C)	Repeat motif	Ta (°C)	No. of alleles#	NCBI GenBank Accession Number
1	*Catla catla*	*CcatG1-1*	F: AGCAGGTTGAT CATTTCTCC R: TGCTGTGTTTCAAATGTTCC	(GATA)n - --(CCA)n	61	(GGA)n	51	7	DQ780015
2		*CcatG1-2*	F: AGCAGGTTGAT CATTTCTCC R: TGCTGTGTTTCAAATGTTCC	(GATA)n - --(CCA)n	61	XX	51	2	XX
3	*Cyprinus carpio*	*MFW1*	F: GTCCAGACTGTCATCAGGAG R: GAGGTGTACACTGAGTCACGC	(CA)n	55	(GT)n	59	8	DQ780014
4		*MFW11*	F: GCATTTGCCTTGATGGTTGTG R: TCGTCTGGTTTAGAGTGCTGC	(CA)n	55	(GT)n	58	8	EF582608
5		*MFW19*	F: GAATCCTCCATCATGCAAAC R: CAAACTCCACATTGTGCC	(CA)n	55	(CA)n	51	7	EF582609
6		*MFW26*	F: CCCTGAGATAGAAACCACTG R: CACCATGCTTGGATGCAAAAG	(CA)n	55	(CA)n	57	5	EF582610
7		*MFW72*	F: GCAGTGGCTGGCAAGTTAAT R: GCACTACATCCACTGCACACA	(GATA)n	55	(GATA)n	55	6	EF582611
8	*Pimephales promelas*	*Ppro48*	F: TGCTCTGCTCTCCTGCGTGTCATT R: CAGCCTCGGCGGTGTTGTTGC	(TG)n	60	(CA)n	65	5	EF582612
9		*Ppro126*	F: CTGCGTGTCTGATAACTGTGACTG R: GTCCCGGGACTTTAAGAAGGTC	(CA)n	60	(CA)n	63	7	EF582613

xx Due to lack of repeat sequences not considered for further analysis. # all loci are polymorphic

4.2.4 Confirmation of microsatellites

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

4.2.5 Confirmation of cloning

4.2.5.1 *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 microsatellite insert only was PCR amplified and visualized bands in 2% agarose gels.

4.2.6 Microsatellite loci confirmed after sequencing

After sequencing, the following eight loci were confirmed to contain microsatellites *viz,* ***CcatG1*-1, *MFW01*, *MFW11*, *MFW19*, *MFW26*, *MFW72*, *Ppro48*,** and ***Ppro126*.** All these loci were polymorphic and were further considered for population genetic analysis of *G. curmuca*. The repeat sequences of eight loci are given in **Figure 25b & c** and **Table 19**. The 9th locus (*CcatG1-2*) was discarded due to the lack of repeat regions in it.

4.2.7 Type and relative frequency of microsatellites

Of the 8 amplified loci, two were perfect (CA) *viz. MFW26* and *Ppro48* and their sequence information is presented in **Figure 25b & c**. Rest of the loci exhibited imperfect repeats. The length of the repeats (is equal to number of repeats) varied from 13 (*Ppro48*) to 25 (*MFW11*). The tandem repeats of seven microsatellite loci (CA) were same as that of the resource species, while repeat motifs of the locus *CcatG1-1* (GGA) differed from that of the resource species ((GATA)n ----(CCA)n) (**Table 19**).

The vertical non-denaturing polyacrylamide gel electrophoresis (PAGE) system (gel concentration 10%; gel size 100mm height X 100mm wide X 1mm thick; Amersham Biosciences, USA) with the silver staining protocol was comparatively inexpensive than the automated genotyping units and capable of separating DNA fragments that differed by as little as 2 base pairs (confirmed after sequencing the PCR products).

4.2.8 Variations in microsatellite band pattern

In ***CcatG1-1***, 7 alleles were observed (**Figure 18**). The size of the alleles ranged from 223 to 256bp (*i.e.*, 223, 229, 232, 238, 244, 250 and 256bp). Except the alleles 256bp in Chalakkudy River population and 223bp in Chaliyar River population, other alleles were common to all the populations (**Table 21**).

In ***MFW01***, a total of 8 alleles were observed (**Figure 19**). The sizes of the alleles were 153, 159, 163, 169, 175, 179, 183 and 187bp. The alleles having the size 183, 179 and 175bp were common in all the populations. The 169bp allele was absent in Periyar River population, the 187 and 163bp alleles were absent in Chalakkudy River population and the 159 and 153bp alleles were absent in Chaliyar River populations (**Table 21**).

In ***MFW11***, 8 alleles were observed (**Figure 20**). The size of the alleles were 162, 168, 172, 176, 180, 184, 190 and 196bp. The 162bp allele was absent in Periyar River population, where as 162 and 168bp alleles were absent in Chalakkudy River population and 168bp allele was absent in Chaliyar River population (**Table 21**). The allele 168bp is considered as a private allele of Periyar River population and the allele 162bp considered as private allele of Chaliyar River population (**Table 24**).

In ***MFW19,*** there were seven alleles and the size of the alleles were 189, 195, 201, 205, 211, 215 and 225bp (**Figure 21**). The allele 225bp was absent in Chaliyar River population, whereas all the alleles were present in both Periyar River and Chalakkudy River populations (**Table 21**).

In ***MFW26***, there were five alleles and their sizes were 145, 151, 157, 161 and 165bp (**Figure 22**). The allele 157bp was most common in all the populations. All five alleles were recorded in all the three populations (**Table 21**).

In ***MFW72***, six alleles were recorded (**Figure 23**). The size of the alleles ranged from 130 to 148bp (i.e., 130, 134, 138, 142, 146 and 148bp). In Periyar River population, the allele 148bp was not recorded; the alleles 134, 138 and 148bp were not observed in Chalakkudy River population and 134 and 138bp alleles absent in Chaliyar River population (**Table 21**). The alleles 138 and 134bp are considered as private alleles of Periyar River population and the allele 148bp considered as a private allele of Chaliyar River population (**Table 24**).

In ***Ppro48*** locus, only five alleles (*i.e.*, 216, 218, 224, 226 and 228bp) were observed (**Figure 24**). Out of these alleles, 218 and 226bp alleles were absent in Periyar River population; 226bp allele was absent in Chalakkudy River population and 218bp allele was absent in Chaliyar River population (**Table 21**). The allele 226bp is considered as a private allele of Chaliyar River population and the allele 218bp is considered as private allele of Chalakkudy River population (**Table 24**).

In ***Ppro126*** locus, there were only seven alleles (**Figure 25**). The size of the alleles ranged from 162 to 178bp (*i.e.*, 162, 168, 170, 172, 174, 176 and 178bp). The allele 178 and 176bp were most common in all populations. In Periyar River population, the alleles 174 and 170bp were not amplified and in Chalakkudy River population, the allele 162bp was absent and in Chaliyar River population, the alleles 170 and 162bp were not amplified (**Table 21**). The allele 170bp is considered as a private allele of Chalakkudy River population and the allele 162bp is considered as private allele of Periyar River population (**Table 24**)

Table 20. Observed (na) and effective (ne) number of microsatellite alleles in three riverine populations of *G. curmuca.*

Locus	Periyar River		Chalakkudy River		Chaliyar River		Overall Populations	
	na	ne	na	ne	na	ne	na	ne
CcatG1-1	7	3.2620	6	3.0948	6	2.3205	6	3.1024
MFW 01	7	3.7209	6	3.2356	6	3.7559	6	3.9269
MFW 11	7	3.6199	6	3.0918	7	4.6715	7	4.8056
MFW 19	7	4.1830	5	2.5417	7	3.1778	6	3.9895
MFW 26	5	3.9555	5	4.1078	4	4.0764	5	4.5312
MFW 72	5	2.2989	3	1.6385	4	1.9753	4	1.9679
Ppro 48	3	3.0418	3	2.2207	4	2.6080	3	2.8271
Ppro126	5	2.5662	6	4.7548	5	4.3537	5	4.3869
Total	46	26.6482	40	24.6857	43	26.9391	42	29.5375
Mean	**5.7500**	**3.3310**	**5.0000**	**3.0857**	**5.375**	**3.3674**	**5.250**	**3.6922**
± S.D.	1.4880	0.6644	1.3093	0.9994	1.3025	0.9981	1.2817	0.9739

Table 21. Microsatellite alleles and allele frequencies in *G. curmuca* from three riverine populations and overall populations.

Locus	Allele size (bp)	Periyar	Chalakkudy	Chaliyar	Overall Populations
CcatG1-1	256	0.0357	0.0000	0.3753	0.1370
	250	0.0323	0.3925	0.2250	0.2166
	244	0.1028	0.1106	0.1201	0.1112
	238	0.2463	0.2267	0.1948	0.2226
	232	0.4967	0.1952	0.0725	0.2548
	229	0.0237	0.0225	0.0123	0.0195
	223	0.0625	0.0525	0.0000	0.0383
MFW01	187	0.1818	0.0000	0.2365	0.1394
	183	0.2528	0.3249	0.3369	0.3049
	179	0.3275	0.2750	0.1714	0.2580
	175	0.1454	0.1479	0.1297	0.1410
	169	0.0000	0.1125	0.0828	0.0651
	163	0.0375	0.0000	0.0426	0.0267
	159	0.0425	0.0932	0.0000	0.0452
	153	0.0125	0.0465	0.0000	0.0197
MFW11	196	0.2053	0.2975	0.3021	0.2683
	190	0.2592	0.2863	0.2915	0.2790
	184	0.1967	0.1542	0.1553	0.1687
	180	0.1367	0.1121	0.0987	0.1158
	176	0.0964	0.0973	0.0825	0.0921
	172	0.0625	0.0526	0.0523	0.0558
	168	0.0432	0.0000	0.0000	0.0144
	162	0.0000	0.0000	0.0176	0.0059
MFW19	225	0.1236	0.1177	0.0000	0.0804
	215	0.1549	0.1613	0.0684	0.1282
	211	0.3152	0.3028	0.4102	0.3428
	205	0.1637	0.1589	0.2249	0.1825
	201	0.1246	0.1345	0.1717	0.1436
	195	0.0783	0.0867	0.1123	0.0924
	189	0.0397	0.0381	0.0125	0.0301
MFW26	165	0.2053	0.3250	0.0023	0.1775
	161	0.3613	0.1634	0.1247	0.2165
	157	0.2798	0.2855	0.3253	0.2969
	151	0.0885	0.1849	0.1824	0.1519
	145	0.0651	0.0412	0.3653	0.1572
MFW72	148	0.0000	0.0000	0.1287	0.0429
	146	0.0654	0.4517	0.4743	0.3305
	142	0.0981	0.3952	0.1694	0.2209
	138	0.2433	0.0000	0.0000	0.0811
	134	0.2278	0.0000	0.0000	0.0759
	130	0.3654	0.1531	0.2276	0.2487
Ppro48	228	0.4213	0.5612	0.4334	0.4719
	226	0.0000	0.0000	0.1332	0.0444
	224	0.3256	0.2350	0.2583	0.2730
	218	0.0000	0.0161	0.0000	0.0054
	216	0.2531	0.1877	0.1751	0.2053
Ppro126	178	0.2615	0.2752	0.2834	0.2734
	176	0.3434	0.3263	0.3598	0.3432
	174	0.0000	0.0104	0.0286	0.0130
	172	0.0594	0.0587	0.0688	0.0623
	170	0.0000	0.1138	0.0000	0.0379
	168	0.2232	0.2156	0.2594	0.2327
	162	0.1125	0.0000	0.0000	0.0375

4.2.9 Genetic variability

The allele frequencies of microsatellite loci from multiple collections of the same river (three years- details in **Table 01**) 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.*, Periyar, Chalakkudy, and Chaliyar River populations and they were used for analysis of parameters of genetic variation and population structure of *G. curmuca.*

4.2.10 Number and percentage of 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 21**.

4.2.11 Observed and effective number of alleles

Periyar River population: In this River, a total of 46 alleles were observed with 8 microsatellite loci. The maximum number of alleles (7) was exhibited by four loci *viz, CcatG1-1*, *MFW01*, *MFW11* and *MFW19*. Five alleles were present in the loci *MFW26*, *MFW72*and *Ppro126*. The locus *Ppro48* showed the minimum number of alleles (3). The mean observed number of alleles (na) in periyar pollution was 5.7500. River The highest effective number of alleles (ne) was exhibited by *MFW19* (4.1830) and lowest effective number was by *MFW72* (2.2989). The mean effective number of alleles in this population was 3.3310 (**Table 20**).

Chalakkudy River population: In this River, a total of 40 alleles were observed with 8 loci. The loci *CcatG1*-1, *MFW01*, *MFW11* and *Ppro126* had maximum six number of alleles; the loci *MFW19* and *MFW26* had five alleles each and the loci *MFW72* and *Ppro48-2* showed the minimum number of alleles ie., three. The mean observed number of alleles in this River population was 5.0000. In Chalakkudy River population, the highest effective number of alleles was 4.7548 (in *Ppro126)* and the lowest effective number of alleles was in *MFW72* (1.6385).

The mean effective number of alleles in the Chalakkudy River population was 3.0857 **(Table 20)**.

Chaliyar River population: In this River population, 43 alleles were observed with 8 loci. The loci *MFW11* and *MFW19* had maximum number of alleles, *i.e.*, seven and the loci *MFW26*, *MFW72* and *Ppro48* showed the minimum number of alleles (four). But the loci *CcatG1-1* and *MFW01* had six alleles and the locus *Ppro126* showed five alleles. The mean observed number of alleles in this population was 5.375. The highest effective number of alleles in this River population was 4.6715 with *MFW11* and lowest effective number with *MFW72* (1.9753). The mean effective number of alleles was 3.3674 in this River population **(Table 20)**.

Overall populations: Among the three riverine populations, a total of 42 alleles were observed in 8 loci. The maximum number of alleles (7) was recorded in the locus *MFW11* while, the locus *Ppro48* showed the minimum number (three) of alleles. The mean observed number of alleles for overall population was 5.250. The highest effective number of alleles was 4.8056 in *MFW11* and the lowest effective number (1.9679) of alleles was in *MFW72*. The mean effective number of alleles for overall River populations was 3.6922 **(Table 20)**.

4.2.12 Frequency of alleles

The allelic frequencies of 8 polymorphic microsatellite loci are given in **Table 21**. In Periyar River population, the allelic frequencies ranged from 0.0125 (*MFW01*) to 0.4967 (*CcatG1-1*). Seven alleles (two alleles in *Ppro48* and *Ppro126*; one allele each in *MFW01*, *MFW11* and *MFW72*) were totally absent in Periyar population. In Chalakkudy population, the allelic frequencies ranged from 0.0104 (*Ppro126*) to 0.5612 (*Ppro48*). Ten alleles (3 alleles in *MFW72*; 2 alleles in *MFW01* and *MFW11*; one allele each in *CcatG1-1*, *Ppro48* and *Ppro126*) were totally absent in this population. In Chaliyar River population, the allelic frequency ranged from 0.0023 (*MFW26*) to 0.4743 (*MFW72*). In this population also, ten alleles (two alleles each in *MFW01*, *MFW72* and *Ppro126* and one allele each in *MFW01*, *MFW11*, *MFW19* and

Ppro48) were totally absent. The overall allele frequency value ranged from 0.0054 (*Ppro48*) to 0.4719 (*Ppro48*) (Table 21).

4.2.13 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 , *MFW11*, *MFW19*, *MFW72* and *Ppro48* in Periyar River population; *MFW01*, *MFW19*, *MFW72* and *Ppro126* in Chalakkudy River population; and *CcatG1-1*, *MFW11*. *MFW19*, *MFW72* and *Ppro126* in Chaliyar population after sequential Bonferroni correction was made to the probability levels (**Table 23**). 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 three populations are given in **Table 23**. The values ranged from –0.0834 for the locus *MFW72* to +0.4552 for locus *MFW11* in Chaliyar and Chalakkudy River populations respectively. In most of the loci, the value of F_{IS} was found to deviate significantly from zero, indicating a deficiency of heterozygotes.

4.2.14 Frequency of null alleles

Seven of the 8 primer pairs in *G. curmuca* indicated positive F_{IS} values in different populations (**Table 23**). 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 (**Table 22**) There was also the absence of 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 22. Summary statistics of null allele frequencies in *G. curmuca*

Locus	Populations showing positive F_{IS} values	Null allele frequency* (from MICRO-CHECKER)			
		Van Oosterhout	Chakraborty	Brooksfield 1	Brooksfield 2
CcatG1-1	Periyar	0.0116	0.0095	0.0108	0.0108
	Chalakkudy	0.0293	0.0278	0.0281	0.0281
MFW01	Periyar	0.0043	0.0038	0.0048	0.0048
	Chaliyar	0.0163	0.0171	0.0188	0.0188
MFW11	Chalakkudy	0.0013	0.0014	0.0017	0.0017
	Chaliyar	0.0025	0.0027	0.0032	0.0032
MFW19	Chaliyar	0.0096	0.0077	0.0081	0.0081
MFW26	Periyar	0.0380	0.0414	0.0373	0.0373
	Chalakkudy	0.0313	0.0402	0.0411	0.0411
	Chaliyar	0.0253	0.0189	0.0218	0.0218
Ppro48	Chalakkudy	0.0153	0.0145	0.0102	0.0102
	Chaliyar	0.0104	0.0104	0.0201	0.0201
Ppro126	Periyar	0.0063	0.0058	0.0064	0.0064
	Chalakkudy	0.0025	0.0023	0.0033	0.0033

(* P< 0.05)

4.2.15 Observed (H_{obs}) and expected (H_{exp}) heterozygosities

Periyar River population: In this population, the range of observed heterozygosity (H_{obs}) was from 0.3336 (*MFW11*) to 0.7429 (*MFW72*) and the mean was 0.5148. The expected heterozygosity (H_{exp}) for this population ranged from 0.3261 (*MFW11*) to 0.7976 (*CcatG1-1*), with a mean of 0.6067 (**Table 23**).

Chalakkudy River population: In this population, the range of observed heterozygosity (H_{obs}) was from 0.1143 (*MFW11*) to 0.7857 (*MFW19*) with a mean value of 0.5360. The expected heterozygosity (H_{exp}) for this population ranged from 0.2767 (*MFW11*) to 0.78112 (*CcatG1-1*) with a mean value of 0.5996 (**Table 23**).

Chaliyar River population: In this population, the observed heterozygosity ranged from 0.2571 (*MFW11*) to 0.7571 (*MFW19*) with a mean value of 0.5239. The expected heterozygosity for this population ranged from 0.2743 (*MFW11*) to 0.8037 (*MFW19*) with a mean value of 0.5619 (**Table 23**)

Table 23. Summary of genetic variation and heterozygosity statistics of eight microsatellite loci in *Gonoproktopterus curmuca.*

Locus	Populations (N=70 each)		
	Periyar	**Chalakkudy**	**Chaliyar**
CcatG1-1			
H obs.	0.3571	0.5310	0.5501
H exp.	0.7976	0.7811	0.5308
F_{IS}	+0.3292	+0.2741	-0.0370
P_{HW}	<0. 0001***	<0. 0001***	0.7863
MFW01			
H obs	0.5943	0.6927	0.5571
H exp	0.7652	0.6479	0.6604
F_{IS}	+0.3344	-0.0470	+0.1631
P_{HW}	<0. 0001***	1.0000	<0. 0001***
MFW11			
H obs	0.3336	0.1143	0.2571
H exp	0.3261	0.2767	0.2743
F_{IS}	-0. 0123	+0.4552	+0.0931
P_{HW}	0.9867	<0. 0001***	0.0842
MFW19			
H obs	0.7098	0.7857	0.7571
H exp	0.6894	0.7539	0.8037
Fis	-0.0193	-0.0225	+0.0676
P_{HW}	0.9862	1.0000	0.0741
MFW26			
H obs	0.3857	0.3180	0.3286
H exp	0.5291	0.4168	0.4321
F_{IS}	+0.3748	+0.2143	+0.2827
P_{HW}	<0. 0001***	0.0122*	<0.0001***
MFW72			
H obs	0.7429	0.7387	0.6014
H exp	0.6871	0.7236	0.5644
F_{IS}	-0.0743	-0.0254	-0.0834
P_{HW}	0.8072	1.0000	0.7828
Ppro48			
H obs	0.6143	0.4802	0.5071
H exp	0.5949	0.5674	0.6098
F_{IS}	-0.0318	+0.3022	+0.4316
P_{HW}	0.9517	<0.0001***	<0.0001***
Ppro126			
H obs	0.3714	0.5857	0.6271
H exp	0.4636	0.6282	0.6200
F_{IS}	+0.1982	+0.0685	-0.0104
P_{HW}	0.0497*	0.8652	1.0000
Mean Overall Loci			
H obs	0.5148	0.5360	0.5239
H exp	0.6067	0.5996	0.5619
F_{IS}	---	---	---
$P_{(0.95)}$	1.0000	1.0000	1.0000
$P_{(0.99)}$	1.0000	1.0000	1.0000
A_n	5.7500	5.0000	5.3750

H obs = Observed heterozygosity
H exp = Expected heterozygosity
F_{IS} = Inbreeding coefficient
P_{HW} = Probability value of significant deviation from HWE
$P_{(0.95)}$ Polymorphism at 0.95 criteria
$P_{(0.99)}$ Polymorphism at 0.99 criteria
A_n = Mean number of alleles per locus
* = Significant at P<0.05
*** Significant after Bonferroni adjustment.

4.2.16 Private alleles (Stock-specific markers)

There were nine private alleles - **four** private alleles in Periyar River population, **two** in Chalakkudy River population and **three** in Chaliyar River population (**Table 24**). The private alleles in **Periyar River population** include the 168bp fragment in *MFW11* locus; 138 & 134bp fragments in *MFW72* locus and 162bp fragment in *Ppro126* locus. The frequencies of these alleles were 0.0432, 0.2433, 0.2278 and 0.1125 respectively. The two private alleles in **Chalakkudy River population** are the 218bp fragment in *Ppro48* and the 170bp allele in *Ppro126* locus. The frequencies of these alleles were 0.0161 and 0.1138 respectively. In Chaliyar River population, the allele 162bp in *MFW11* locus, 148bp in MFW72 locus and the allele 226bp in Ppro48 locus formed the stock-specific markers. The frequencies of these alleles were 0.0176, 0.1287 and 0.1332 respectively.

Table 24. Private alleles in microsatellite and their frequencies

Locus	Private allele size (bp)	Allele frequency		
		Periyar	Chalakkudy	Chaliyar
MFW11	*168*	0.0432	----	----
	162	----	----	0.0176
MFW72	*148*	----	----	0.1287
	138	0.2433	----	----
	134	0.2278	----	----
Ppro48	*226*	----	----	0.1332
	218	----	0.0161	----
Ppro126	*170*	----	0.1138	----
	162	0.1125	----	----

4.2.17 Linkage disequilibrium

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

Table 25. Fisher's Exact test of microsatellite allele homogeneity for all the population pairs of *G. curmuca.*

Locus	Population paris	P-value (Exact test)	S.E.
CcatlaG1-1	CHL & PER	0.0061*	0.0002
	CLR & PER	0.0152*	0.0009
	CLR & CHL	0.0010*	0.0001
MFW01	CHL & PER	0.0000***	0.0000
	CLR & PER	0.0000***	0.0000
	CLR & CHL	0.0000***	0.0000
MFW11	CHL & PER	0.0022*	0.0003
	CLR & PER	0.0036*	0.0003
	CLR & CHL	0.0596	0.0012
MFW19	CHL & PER	0.4604	0.0023
	CLR & PER	0.0261*	0.0000
	CLR & CHL	0.0030*	0.0003
MFW26	CHL & PER	0.0092*	0.0006
	CLR & PER	0.0366*	0.0028
	CLR & CHL	0.0221*	0.0061
MFW72	CHL & PER	0.0000***	0.0000
	CLR & PER	0.0000***	0.0000
	CLR & CHL	0.0000***	0.0000
Ppro48	CHL & PER	0.0324*	0.0031
	CLR & PER	0.0000***	0.0000
	CLR & CHL	0.0000***	0.0000
Ppro126	CHL & PER	0.0014*	0.0003
	CLR & PER	0.0000***	0.0000
	CLR & CHL	0.0364*	0.0024
Overall loci	Overall population	0.0000***	--

* Significant at P<0.05; *** Significant at P<0.0001 after sequential Bonferroni adjustment;
PER - Periyar River; CHL – Chalakkudy River; CLR – Chaliyar River;
Markov chain parameters - dememorization: 1000, batches: 100 and iterations: 1000

4.2.18 Genetic differentiation

The coefficient of genetic differentiation, F_{ST} ranged from 0.0490 for the locus *CcatG1-1* to 0.1114 for the locus *MFW72*, with a mean of 0.0689, indicating that 6.89% of the total genetic variation exists among 3 populations (**Table 26**). Pair-wise F_{ST} estimates between populations differed significantly ($P < 0.0001$) from zero for all the pairs of riverine locations (**Table 27**). The loci exhibiting significant heterogeneity in genotype proportions between population pairs and

for overall population employing Exact test are depicted in **Table 25**. Out of the possible 24 comparisons, 22 pairs exhibited significant genotypic heterogeneity.

In addition to F_{ST}, population differentiation was measured also using pair-wise and overall R_{ST} (Slatkin, 1995) based on the differences in repeat numbers (allele sizes) in microsatellite loci. The R_{ST} values were similar to that of F_{ST} in *G. curmuca*. Locus-wise R_{ST} varied from 0.0510 (*MFW26*) to 0.1090 (*MFW72*) with an overall value of 0.0729 (**Table 26**). Pair-wise R_{ST} values between populations differed significantly ($P<0.0001$) for all the pairs of riverine locations (**Table 27**).

Table 26. F-statistics (F_{ST}) and Rho-statistics (R_{ST}) for overall populations in *G. curmuca*

Microsatellite Locus	Sample Size	F_{ST}	R_{ST}
CcatG1-1	210	0.0490	0.0593
MFW01	210	0.0575	0.0612
MFW11	210	0.0940	0.1043
MFW19	210	0.0512	0.0608
MFW26	210	0.0496	0.0510
MFW72	210	0.1114	0.1090
Ppro48	210	0.0770	0.0840
Ppro126	210	0.0615	0.0532
Mean	**210**	**0.0689**	**0.0729**

Table 27. Pair-wise Fisher's F_{ST} (θ) (above diagonal) and R_{ST} (below diagonal) between riverine samples of *Gonoproktopterus curmuca* using microsatellite markers.

Populations	Periyar	Chalakkudy	Chaliyar
Periyar	----	0.04723***	0.06381***
Chalakkudy	0.05472***	-----	0.05202***
Chaliyar	0.08911***	0.06121***	----

*** Significant after Bonferroni adjustment ($P<0.0001$)

4.2.19 AMOVA

The AMOVA based on microsatellite data indicated significant genetic differentiation among *G. curmuca* populations (F_{ST} 0.0673; $P<0.0001$) (**Table 28**). The value (6.73%) was congruent to that obtained without the hierarchical analysis (6.89%).

for overall population employing Exact test are depicted in **Table 25**. Out of the possible 24 comparisons, 22 pairs exhibited significant genotypic heterogeneity.

In addition to F_{ST}, population differentiation was measured also using pair-wise and overall R_{ST} (Slatkin, 1995) based on the differences in repeat numbers (allele sizes) in microsatellite loci. The R_{ST} values were similar to that of F_{ST} in *G. curmuca*. Locus-wise R_{ST} varied from 0.0510 (*MFW26*) to 0.1090 (*MFW72*) with an overall value of 0.0729 (**Table 26**). Pair-wise R_{ST} values between populations differed significantly ($P<0.0001$) for all the pairs of riverine locations (**Table 27**).

Table 26. F-statistics (F_{ST}) and Rho-statistics (R_{ST}) for overall populations in *G. curmuca*

Microsatellite Locus	Sample Size	F_{ST}	R_{ST}
CcatG1-1	210	0.0490	0.0593
MFW01	210	0.0575	0.0612
MFW11	210	0.0940	0.1043
MFW19	210	0.0512	0.0608
MFW26	210	0.0496	0.0510
MFW72	210	0.1114	0.1090
Ppro48	210	0.0770	0.0840
Ppro126	210	0.0615	0.0532
Mean	**210**	**0.0689**	**0.0729**

Table 27. Pair-wise Fisher's F_{ST} (θ) (above diagonal) and R_{ST} (below diagonal) between riverine samples of *Gonoproktopterus curmuca* using microsatellite markers.

Populations	Periyar	Chalakkudy	Chaliyar
Periyar	----	0.04723***	0.06381***
Chalakkudy	0.05472***	-----	0.05202***
Chaliyar	0.08911***	0.06121***	----

*** Significant after Bonferroni adjustment ($P<0.0001$)

4.2.19 AMOVA

The AMOVA based on microsatellite data indicated significant genetic differentiation among *G. curmuca* populations (F_{ST} 0.0673; $P<0.0001$) (**Table 28**). The value (6.73%) was congruent to that obtained without the hierarchical analysis (6.89%).

Table 28. Analysis of Molecular Variance (AMOVA) based on microsatellite markers in three populations of *G. curmuca.*

Sources of Variation	Variance component	Percentage of Variation (%)	Fixation indices
Among populations (Among Rivers)	0.1954 (Va)	06.73	0.0673***
Within populations (Within River)	2.7097 (Vb)	93.27	---
Total	2.9051 (Vt)	---	---

***P<0.0001; Significance test after 1000 permutations

4.2.20 Genetic distance and similarity

Nei's (1978) unbiased genetic identity and distance estimated between pairs of three populations of *G. curmuca* are presented in **Table 29**. The genetic distance between Periyar and Chalakkudy River populations was 0.0739; between Chalakkudy and Chaliyar River populations 0.1085; and between Periyar and Chaliyar River populations was 0.1764. The results agreed with the geographic distances between the populations (**Table 29**).

Table 29. Nei's (1978) genetic identity (above diagonal) and genetic distance (below diagonal) using microsatellite markers in *G. curmuca*: geographical distances (in Km) are given in bracket

Populations	Periyar	Chalakkudy	Chaliyar
Periyar	----	0.9298	0.8285
Chalakkudy	0.0739 (60)	----	0.9003
Chaliyar	0.1764 (220)	0.1085 (160)	----

4.2.21 Dendrogram

On the basis of Nei's genetic distance values an UPGMA dendrogram was constructed. The cluster values indicated distinct relationship between the 3 populations of *G. curmuca* (**Figure 35**). The high bootstrap values suggested, the populations have a robust cluster.

4.2.22 Bottleneck analysis

The bottleneck results based on microsatellite data indicated clear mode shift of allele diversity in all the populations in contrast to the expected L-shaped

distribution, if the population followed mutation drift equilibrium (**Figure 25a**). The probability values (**Table 30**) also indicated significant genetic bottleneck in *G. curmuca* populations.

Table 30. Analysis of genetic bottleneck in *G. curmuca* with microsatellite markers under infinite allele model (IAM) and two-phased model (TPM).

Periyar	**He**	**IAM**		**TPM#**	
		Heq	**P**	**Heq**	**P**
CcatG1-1	0.357	0.300	0.0495	0.344	0.1201
MFW01	0.597	0.569	0.0678	0.545	0.0792
MFW11	0.332	0.209	0.0408	0.244	0.0488
MFW19	0.709	0.602	0.0301	0.644	0.0451
MFW26	0.395	0.264	0.0254	0.252	0.0420
MFW72	0.692	0.586	0.0484	0.603	0.0401
Ppro48	0.607	0.508	0.0096	0.534	0.0142
Ppro126	0.368	0.307	0.0445	0.292	0.0474
Wilcoxon Test (P)		**0.00392***		**0.00998***	
Chalakkudy	**He**	**IAM**		**TPM#**	
		Heq	**P**	**Heq**	**P**
CcatG1-1	0.588	0.547	0.0866	0.542	0.0842
MFW01	0.693	0.574	0.0465	0.555	0.0279
MFW11	0.128	0.112	0.0916	0.117	0.1210
MFW19	0.759	0.585	0.0074	0.641	0.0173
MFW26	0.328	0.316	0.1038	0.314	0.1407
MFW72	0.727	0.607	0.0345	0.600	0.0427
Ppro48	0.472	0.324	0.0221	0.234	0.0118
Ppro126	0.592	0.518	0.0477	0.503	0.0349
Wilcoxon Test (P)		**0.00622***		**0.01141***	
Chaliyar	**He**	**IAM**		**TPM#**	
		Heq	**P**	**Heq**	**P**
CcatG1-1	0.539	0.499	0.0735	0.524	0.0841
MFW01	0.565	0.548	0.2732	0.510	0.2502
MFW11	0.282	0.241	0.0874	0.214	0.0804
MFW19	0.755	0.602	0.0342	0.681	0.0415
MFW26	0.327	0.206	0.0327	0.212	0.0202
MFW72	0.608	0.407	0.0392	0.502	0.0499
Ppro48	0.507	0.315	0.0365	0.359	0.0469
Ppro126	0.624	0.507	0.0266	0.415	0.0448
Wilcoxon Test (P)		**0.00795***		**0.01293***	

Heq: Heterozygosity expected at mutation-drift equilibrium; He: measured/observed heterozygosity; * Significant value ($P<0.05$); # two-phased model (TPM) is more suitable for microsatellites according to Cornuet & Luikart (1996).

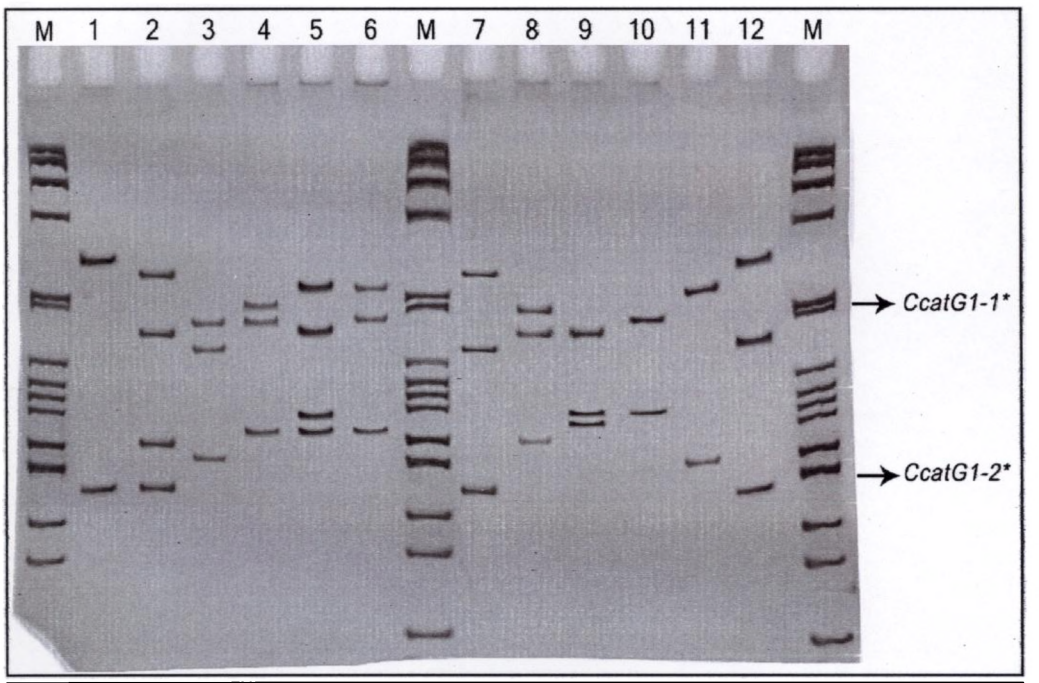

Fig. 18. Microsatellite pattern of locus *CcatG1-1* in *G. curmuca*.
Lanes 1-4 samples from Periyar, 5-8 Chalakkudy and 9-12 Chaliyar Rivers.
M - molecular weight marker (pBR322 with *Msp*I cut)

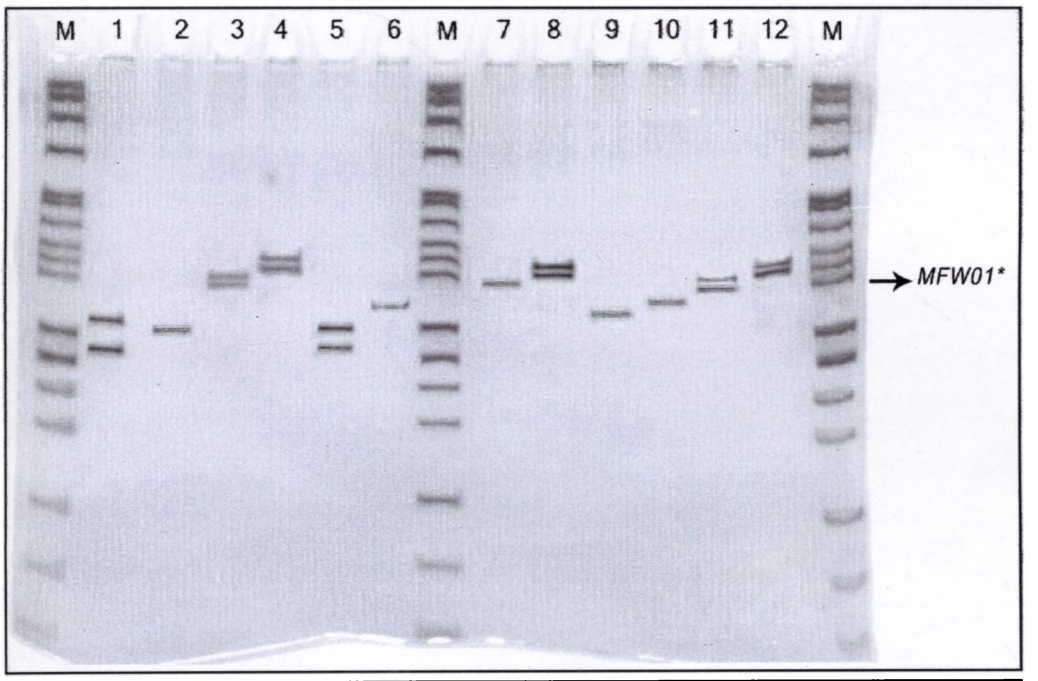

Fig. 19. Microsatellite pattern of locus *MFW01* in *G. curmuca*.
Lanes 1-4 samples from Periyar, 5-8 Chalakkudy and 9-12 Chaliyar Rivers.
M - molecular weight marker (pBR322 with *Msp*I cut)

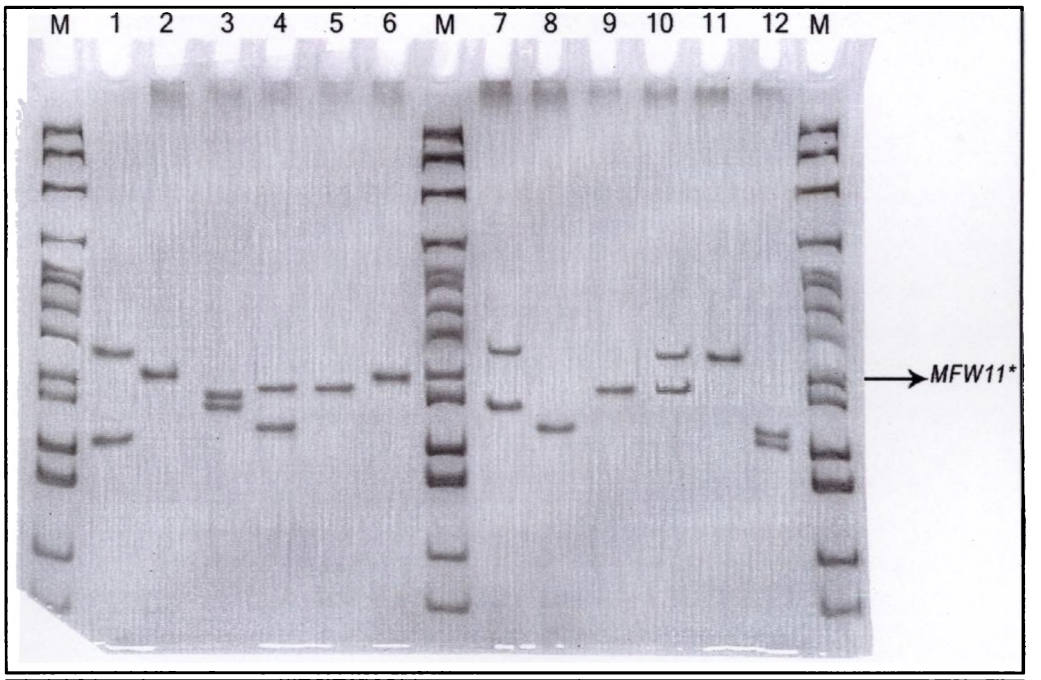

Fig. 20. Microsatellite pattern of locus *MFW11* in *G. curmuca*.
Lanes 1-4 samples from Periyar, 5-8 Chalakkudy and 9-12 Chaliyar Rivers.
M - molecular weight marker (pBR322 with *Msp*I cut)

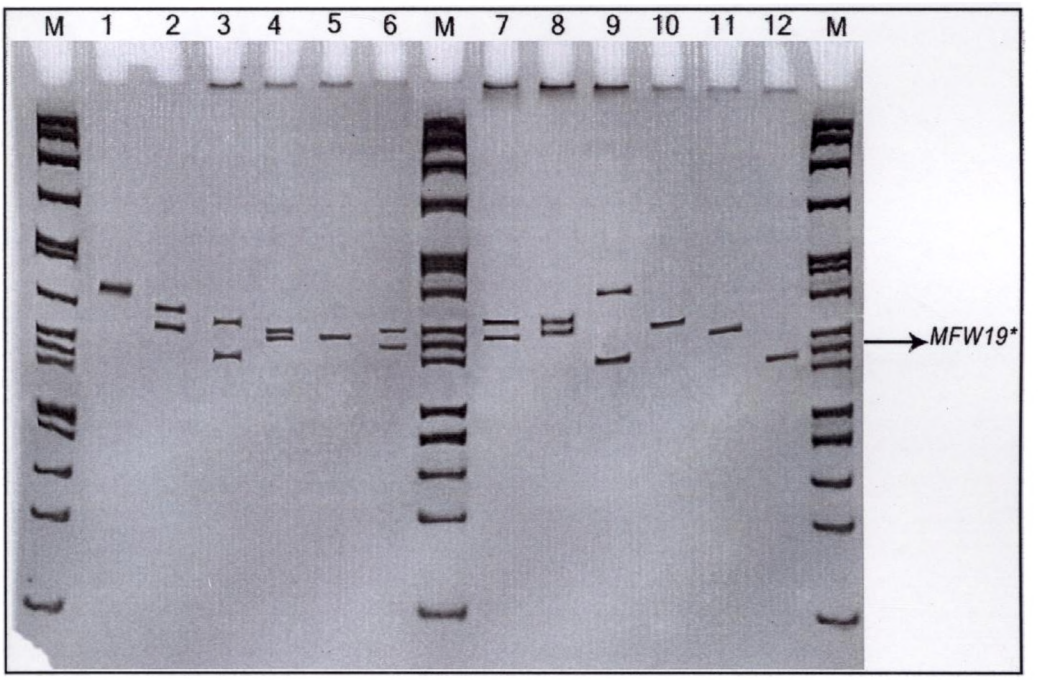

Fig. 21. Microsatellite pattern of locus *MFW19* in *G. curmuca*.
Lanes 1-4 samples from Periyar, 5-8 Chalakkudy and 9-12 Chaliyar Rivers.
M - molecular weight marker (pBR322 with *Msp*I cut)

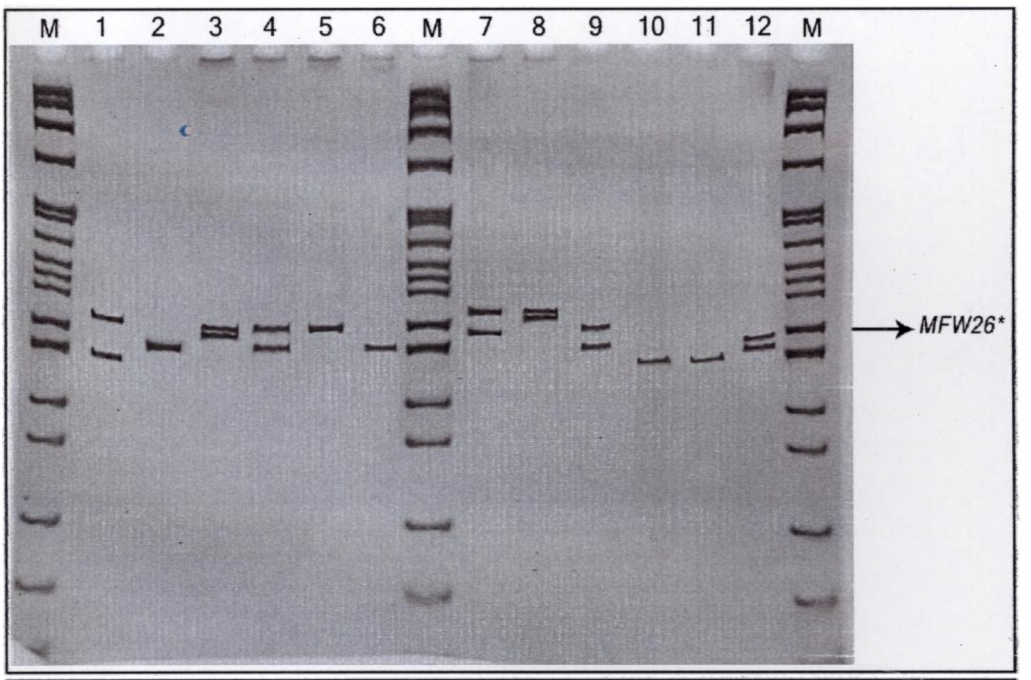

Fig. 22. Microsatellite pattern of locus *MFW26* in *G. curmuca*.
Lanes 1-4 samples from Periyar, 5-8 Chalakkudy and 9-12 Chaliyar Rivers.
M - molecular weight marker (pBR322 with *Msp*I cut)

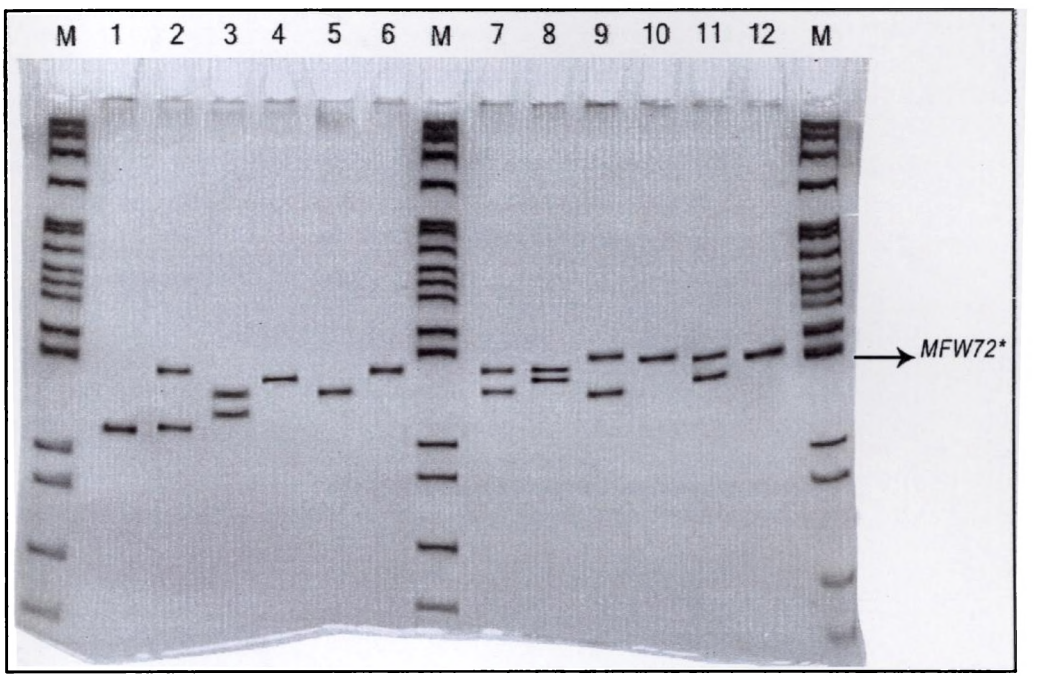

Fig. 23. Microsatellite pattern of locus *MFW72* in *G. curmuca*.
Lanes 1-4 samples from Periyar, 5-8 Chalakkudy and 9-12 Chaliyar Rivers.
M - molecular weight marker (pBR322 with *Msp*I cut)

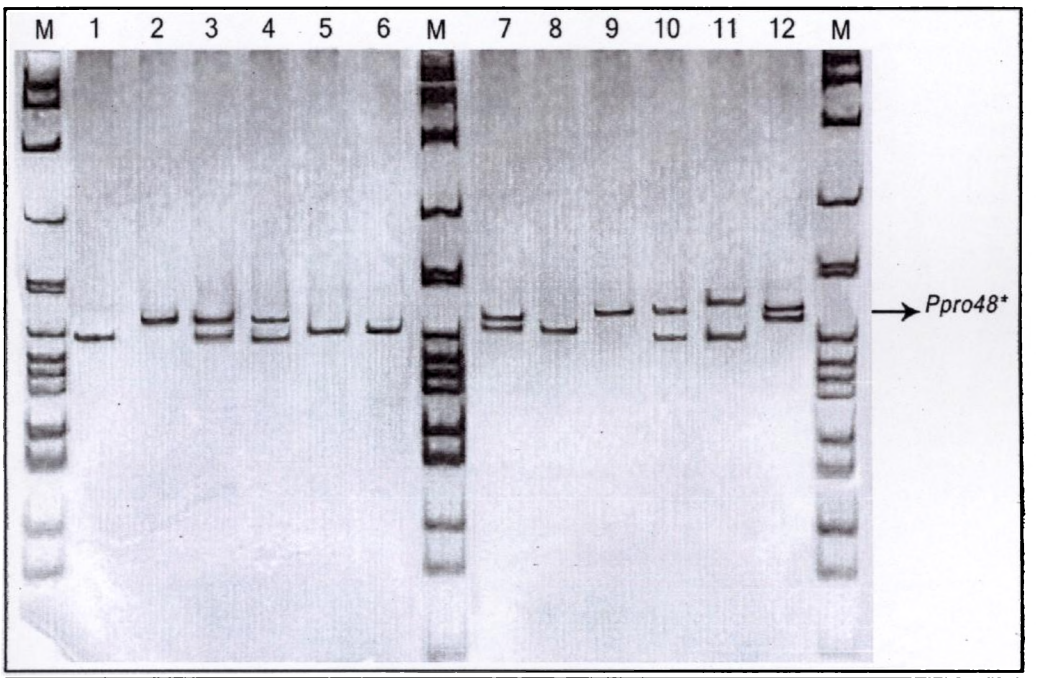

Fig. 24. Microsatellite pattern of locus *Ppro48* in *G. curmuca*.
Lanes 1-4 samples from Periyar, 5-8 Chalakkudy and 9-12 Chaliyar Rivers.
M - molecular weight marker (pBR322 with *Msp*I cut)

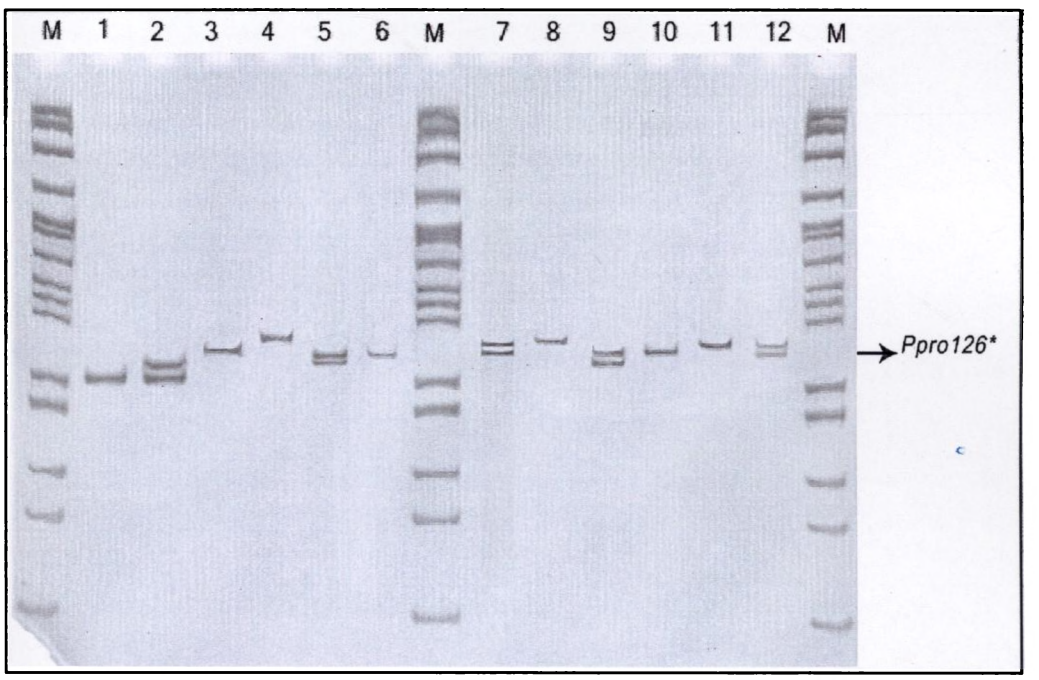

Fig. 25. Microsatellite pattern of locus *Ppro126* in *G. curmuca*.
Lanes 1-4 samples from Periyar, 5-8 Chalakkudy and 9-12 Chaliyar Rivers.
M - molecular weight marker (pBR322 with *Msp*I cut)

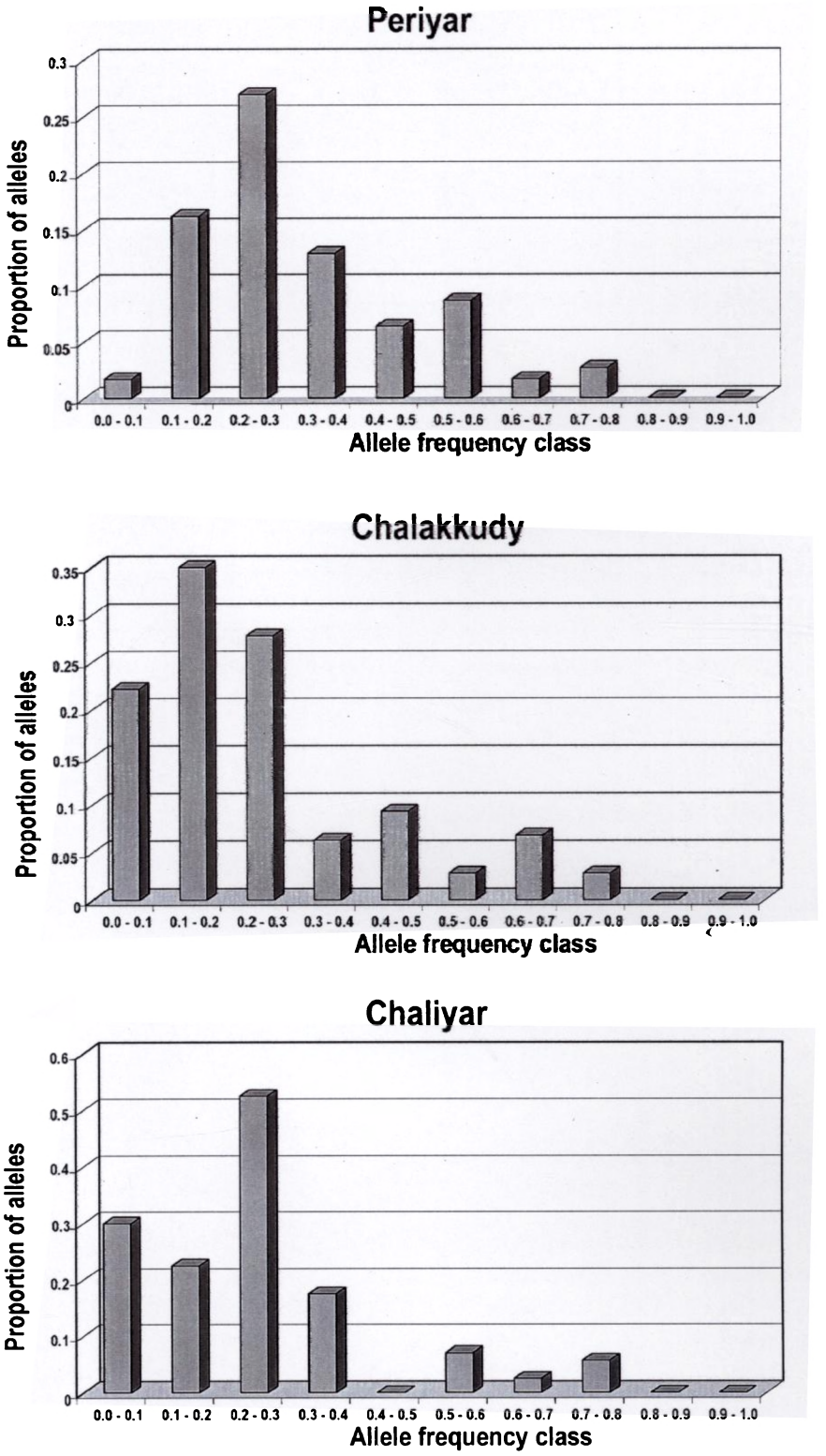

Fig 25a Qualitative "mode-shift" indicator test to discriminate bottlenecked populations of *Gonoproktopterus curmuca* from three rivers, based on microsatellite allele frequency distribution.

CcatG1-1 (NCBI GenBank Accession # DQ780015)

agcaggttga tcatttctcc agctcagtga gatcacaagc
acttggactt cattctattt ctggtctggc tgccatttat
agaacattca gtactttaaa ggaggaggac ggaggaggag
gaggaggagt ggaggaatct ggaggaggag gaggaggagg
gaggactgca ggtcaaactc aaaaaactaa tcttgacacc
ctttcaatta attaagggaa ggaacatttg aaacacagca

MFW01 (NCBI GenBank Accession # DQ780014)

gtccagactg tcatcaggag cttctgcgct tgaacctgat
gaaatgcagc ctgctctgct gtgaaagaat gttggtgtgt
gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt tgggagagag
aaatgtaaga caggtacttt acagaggctg cattgtcatg
agcgtgactc agtgtacacc tc

MFW11 (NCBI GenBank Accession # EF582608)

gcatttgcct tgatggttgt gacatttttt gatattcaaa
gtctaacctt tctgtgggtc tgttgtaagg aaatgtgtgg
gaggattttc cgtgtgttgt tgtgtgtgtg atgtgtgtgt
gtgtgtgtgt gtgtgtgtgt gtgtgtctat atgaagcagc
actctaaacc agacga

MFW19 (NCBI GenBank Accession # EF582609)

gaatcctcca tcatgcaaac agggtaggct cgaccaagca
gtcgacgaat tcagattaca gatggggaat gagagcatcc
gagcgtgact gtacaatgag tctgctaaca cacatcacaa
cacacacaca cacacacaca cacagcacac acacaggtgt
atcaacaaga aatcacgaat attggcacaa tgtggagttt
g

Fig. 25b. The nucleotide sequence of each microsatellite locus in *G. curmuca*.
Repeat sequences are given in red colour & primer sequences are in blue colour.

MFW26 (NCBI GenBank Accession # EF582610)

ccctgagata gaaaccactg gacattaatt ttaatattta
aactaggttt tgttactaaa acaaactgga tagtttttac
acacacacac acacacacac acacacaaaa tgtggaaatt
tccacgtttc gacacaacag ttttcttttg catccaagca
tggtg

MFW 72 (NCBI GenBank Accession # EF582611)

gcagtggctg gcaagttaat aaacttttt ttttatctgc
tcttttctat acaacagtga ctgcatgata gatagataga
tacatagata tgatagatag atagatagat agatagatag
atagatagat atttttaaag attttaaaaa tgtgtgcagt
ggatgtagtg c

Ppro48 (NCBI GenBank Accession # EF582612)

tgctctgctc tcctgcgtgt cattctagca gaagcttttc
tgtctacaag ctgtccagaa tgagagacgg caatgctctg
ctctcctgcg tgtcattacg gtgtcagcac taacgtctct
ctgcttcaca cacacacaca cacacacaca catctgaact
catcgcctgc aacaacaccg ccgaggctgc tcaacgaaaa
gtgtgcttct ggtagagcgc ctccaccgcg gcaacaacac
cgccgaggct g

Ppro126 (NCBI GenBank Accession # EF582613)

ctgcgtgtct gataactgtg actggtgact gcagacggcg
gttctctctg gacatctcct caaacaactg aaacacaccc
acacacacac atcacacaca cacacacaca cacacactgt
tgctgactca accagtttaa ccacaaaccc ctcagacctt
cttaaagtcc cgggactccg atcctctcgt gacgatatag
tcgttcgaga ccgaatcaaa cctggacctt cttaaagtcc
cgggac

Fig. 25c. **The nucleotide sequence of each microsatellite locus in *G. curmuca*.** Repeat sequences are given in red colour & primer sequences are in blue colour.

4.3 RAPD Analysis

4.3.1 Selection of primers

Thirty one oligonucleotide primers were selected from 80 primers (4 kits- 20 primers each from kit OPA, OPAA, OPAC & OPAH) on primary screening; however, only 09 primers were selected *viz.* OPA-15, OPA-16, OPAA-07, OPAA-08, OPAC-05, OPAC-06, OPAH-03, OPAH-17 and OPAH-19 for population genetic analysis (**Table 31**).

Table 31. Number of RAPD fragments and their size range for each Operon primer

Sl. No.	Primer Code	Sequence	No. of fragments	Size range (~bp)
1	OPA -15	ttccgaaccc	06-11	1250 - 5000
2	OPA -16	agccagcgaa	10-14	**0800 - 6500**
3	OPAA-07	ctacgctcac	08-12	1100 - 3550
4	OPAA-08	tccgcagtag	**04**-08	1000 - 3500
5	OPAC-05	gttagtgcgg	07-16	0850 - 3400
6	OPAC-06	ccagaacgga	06-10	1350 - 3400
7	OPAH-03	ggttactgcc	05-12	1450 - 3250
8	OPAH-17	cagtggggag	06-14	1100 - 2250
9	OPAH-19	ggcagttctc	08-**20**	0750 - 2250

4.3.2 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 -20^0C for more than 6 months, demonstrating the robustness of the technique.

4.3.3 Genetic variability

4.3.3.1 *Number of amplified fragments*

A total of 117 different randomly amplified DNA fragments from specimens of *G. curmuca* were detected consistently with all 09 decamer primers in three populations. The size of the fragments ranged from 800bp to 6500bp. The number of fragments generated per primer varied from 04 to 20 (**Table 32**).

Table 32. The total number of RAPD fragments; number & % of polymorphic bands and average gene diversity for each and overall populations of *Gonoproktopterus curmuca*

Primer Code	Periyar River				Chalakkudy River				Chaliyar River				Overall populations			
	Total no. of bands	No. of poly-morphic bands	% of poly-morphic bands (%p)	Average Gene diversity (H)	Total no. of bands	No. of poly-morphic bands	% of poly-morphic bands (%p)	Average Gene diversity (H)	Total no. of bands	No. of poly-morphic bands	% of poly-morphic bands (%p)	Average Gene diversity (H)	Total no. of bands	No. of poly-morphic bands	% of poly-morphic bands (%p)	Average Gene diversity (H)
OPA -15	08	**0**	**00.00**	**0.0000**	08	1	12.50	0.0437	06	1	16.67	0.1666	11	06	54.55	0.2146
OPA -16	12	3	25.00	0.0778	13	3	23.08	0.3356	10	3	30.00	0.1762	14	05	35.71	0.1330
OPAA-07	10	2	20.00	0.0378	10	3	30.00	0.0424	09	3	33.33	0.1648	12	05	41.67	0.1584
OPAA-08	06	1	16.67	0.0315	06	**0**	**00.00**	**0.0000**	05	1	20.00	0.0989	08	04	50.00	0.1370
OPAC-05	13	3	23.08	0.1051	08	2	25.00	0.3380	08	2	25.00	0.0873	16	10	62.50	0.2256
OPAC-06	09	2	22.22	0.0598	08	1	12.50	0.1241	06	**0**	**00.00**	**0.0000**	10	05	50.00	0.1540
OPAH-03	07	2	28.57	0.0892	06	1	16.67	0.3492	10	3	30.00	0.0567	12	09	75.00	0.2510
OPAH-17	07	2	28.57	0.0540	09	3	33.33	0.1889	09	3	33.33	0.1147	14	09	64.29	0.1998
OPAH-19	16	4	25.00	0.0473	14	4	28.57	0.0538	09	2	22.22	0.0420	20	12	60.00	0.1901
Total	**88**	**19**	--	--	**82**	**18**	--	--	**72**	**18**	--	--	**117**	**65**	--	--
Mean Primers	--	--	**21.59**	**0.0558**	--	--	**21.95**	**0.1640**	--	--	**25.00**	**0.1008**	--	--	**55.56**	**0.1848**

Chaliyar River population: A total of 72 amplified DNA fragments were detected consistently with nine Operon primers in Chaliyar River population, and out of these, 18 (25.00%) fragments were polymorphic. The remaining 54 bands were monomorphic (75.00%). The primer-wise percentage of polymorphic bands ranged from 0% (OPAC-06) to 33.33% (OPAA-07 & OPAH-17). The number of fragments and polymorphic bands for each primer are given in **Table 32**.

4.3.3.2 *Linkage disequilibrium*

Pairs of RAPD loci did not show any significant linkage disequilibrium (P>0.05) in all the populations of red tailed barb. It was therefore assumed that allelic variation at RAPD loci could be considered independent.

4.3.3.3 *Genetic differentiation*

The value of coefficient of differentiation (G_{ST}) was estimated for each primer across all populations (**Table 33**). The maximum value of G_{ST} (0.2924) was shown by the primer OPAC-05, while the minimum value (0.1365) was shown by the primer OPAH-17. The G_{ST} for overall populations was 0.2286.

Table 33. Co-efficient of genetic differentiation (G_{ST}) for overall populations

Primer code	G_{ST}
OPA -15	0.2716
OPA -16	0.2173
OPAA-07	0.1679
OPAA-08	0.2416
OPAC-05	0.2924
OPAC-06	0.2735
OPAH-03	0.1572
OPAH-17	0.1365
OPAH-19	0.1598
Mean	**0.2131**
Overall populations	**0.2286**

4.3.3.4 *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. Overall 41 RAPD fragments were detected as stock-specific markers with 9

primers (**Table 34**). Eighteen fragments were exclusive to Periyar population. In Chalakkudy population, 15 fragments were specific, while eight stock-specific RAPD bands were detected in Chaliyar population (**Table 34**).

Table 34. Stock- specific RAPD markers with size for each population

Primer code	Fragment No.	Size (~bp)	PER	CHL	CLR
OPA -15	6th	1800	+	-	-
	7th	1700	+	-	-
	8th	1650	-	+	-
	9th	1550	-	+	-
	11th	1250	-	+	-
OPA -16	5th	2000	-	-	+
OPAA-07	6th	1700	+	-	-
	8th	1350	-	+	-
OPAA-08	3rd	1900	-	+	-
	4th	1450	-	-	+
	6th	1300	+	-	-
OPAC-05	2nd	3300	-	+	-
	3rd	2200	+	-	-
	4th	2000	+	-	-
	12th	1450	+	-	-
	15th	1150	-	+	-
	16th	1000	+	-	-
OPAC-06	3th	3150	+	-	-
	4th	2300	+	-	-
OPAH-03	2nd	3400	+	-	-
	3rd	2750	-	-	+
	5th	1600	-	-	+
	11th	1400	-	+	-
OPAH-17	1st	2200	-	+	-
	2nd	2000	-	+	-
	5th	1850	-	-	+
	6th	1650	-	-	+
	8th	1400	+	-	-
	12th	1250	-	+	-
	13th	1150	-	-	+
	14th	1050	-	-	+
OPAH-19	4th	1800	+	-	-
	5th	1750	+	-	-
	9th	1300	-	+	-
	10th	1200	+	-	-
	13th	0930	-	+	-
	15th	0900	-	+	-
	16th	0870	+	-	-
	17th	0850	+	-	-
	18th	0840	+	-	-
	19th	830	-	+	-

4.3.3.5 *Genetic distance and similarity index*

Nei's (1978) unbiased genetic identity and distance estimated between pairs of three populations of *G.curmuca* are presented in **Table 35**. The genetic distance between Periyar and Chalakkudy River populations was 0.1013; between Periyar and Chaliyar River populations 0.1903; and between Chalakkudy and Chaliyar River populations was 0.1217. This indicates Periyar River populations and Chalakkudy River populations are genetically closer than Chaliyar River population. These results are in agreement with the geographic distances between pairs of the populations.

Table 35. Nei's genetic identity (above diagonal) and genetic distance (below diagonal), using RAPD markers in *G.* curmuca; geographical distances (in Km) are given in bracket

Populations	Periyar River	Chalakkudy River	Chaliyar River
Periyar River	****	0.9014	0.8156
Chalakkudy River	0.1013 (60)	****	0.8735
Chaliyar River	0.1903 (220)	0.1217 (160)	****

4.3.3.6 *Dendrogram*

Phylogenetic relationships among three riverine populations of *G. curmuca* were made based on RAPD data following the Un-weighted Pair Group Method using Arithmetic average (UPGMA) method (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 (Winboot) and the high bootstrap values (above 90) suggested the populations had a robust cluster (**Figure 35**).

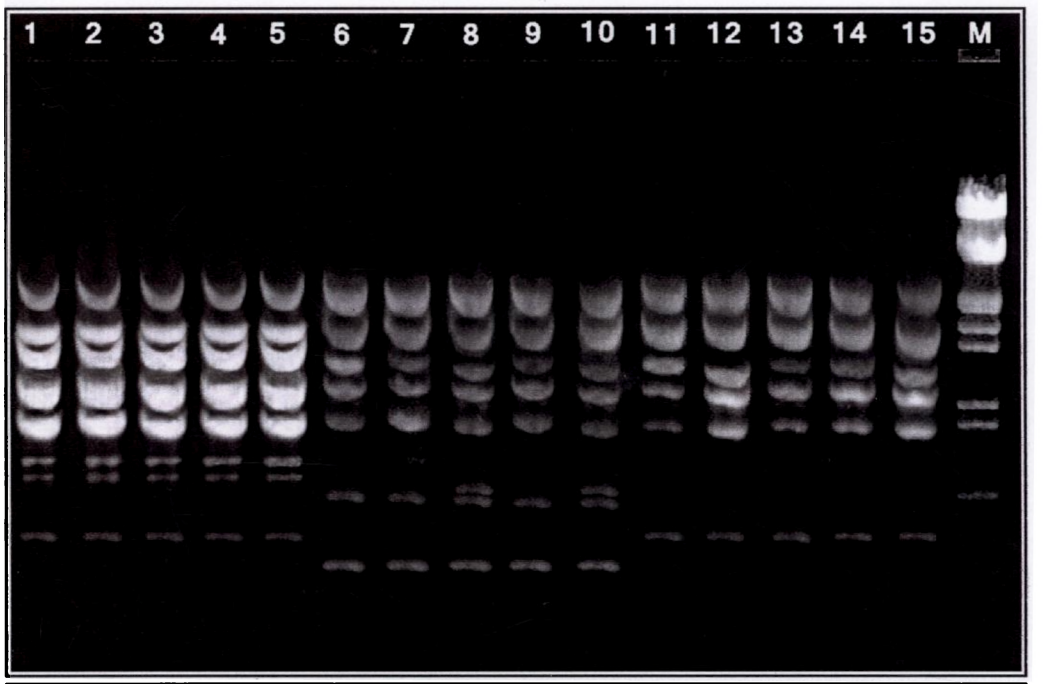

Fig. 26. RAPD pattern of *G. curmuca* with primer OPA-15.
Lanes 1-5 samples from Periyar, 6-10 Chalakkudy and 11-15 Chaliyar Rivers.
M- molecular weight marker (λDNA with *Eco*R1 & *Hin*dIII double digest)

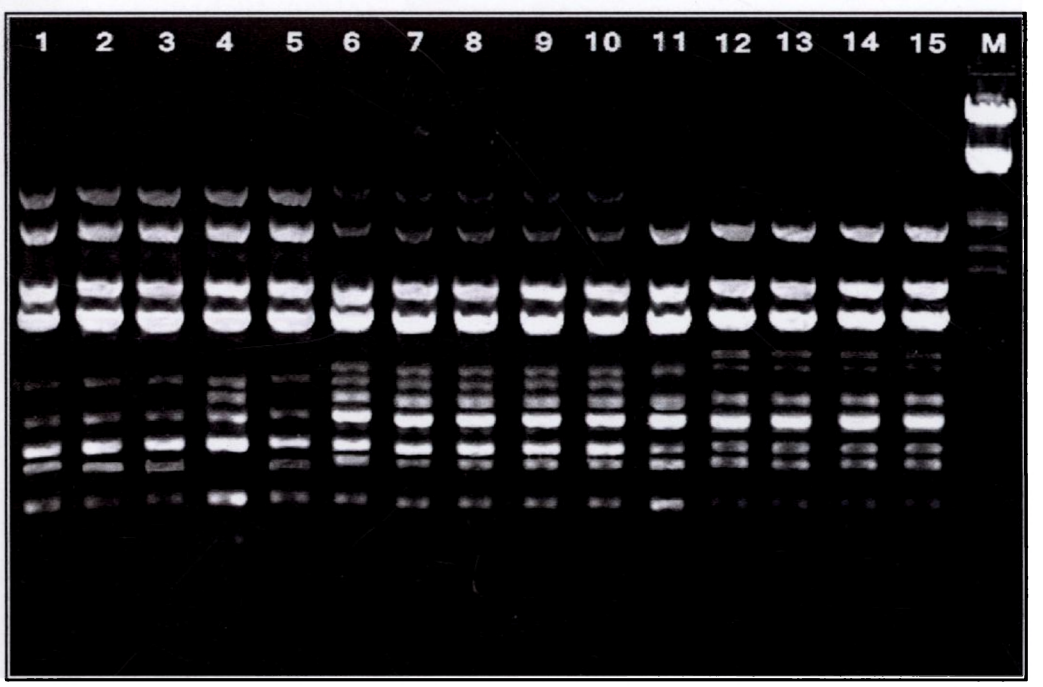

Fig. 27. RAPD pattern of *G. curmuca* with primer OPA-16.
Lanes 1-5 samples from Periyar, 6-10 Chalakkudy and 11-15 Chaliyar Rivers.
M- molecular weight marker (λDNA with *Eco*R1 & *Hin*dIII double digest)

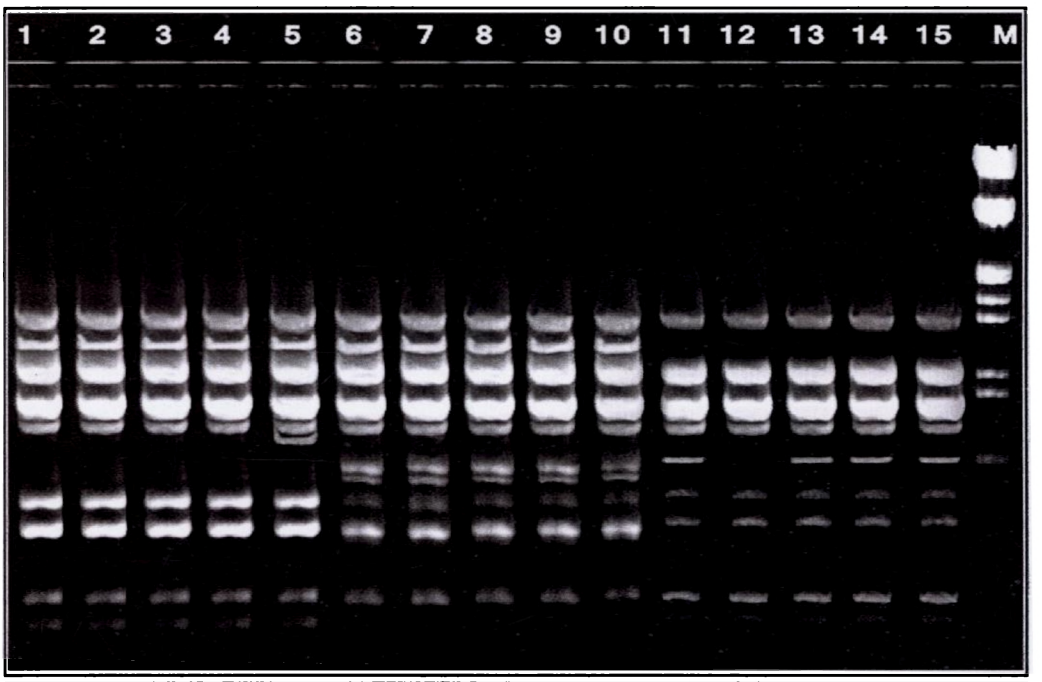

Fig. 28. RAPD pattern of *G. curmuca* with primer OPAA-07.
Lanes 1-5 samples from Periyar, 6-10 Chalakkudy and 11-15 Chaliyar Rivers.
M- molecular weight marker (λDNA with *Eco*R1 & *Hin*dIII double digest)

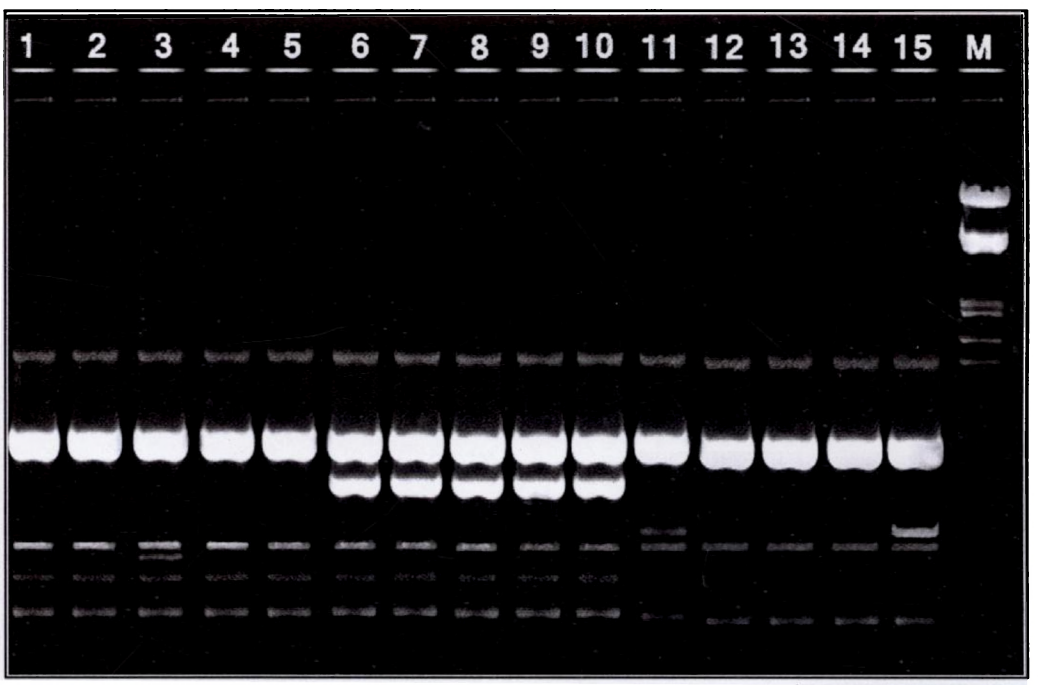

Fig. 29. RAPD pattern of *G. curmuca* with primer OPAA-08.
Lanes 1-5 samples from Periyar, 6-10 Chalakkudy and 11-15 Chaliyar Rivers.
M- molecular weight marker (λDNA with *Eco*R1 & *Hin*dIII double digest)

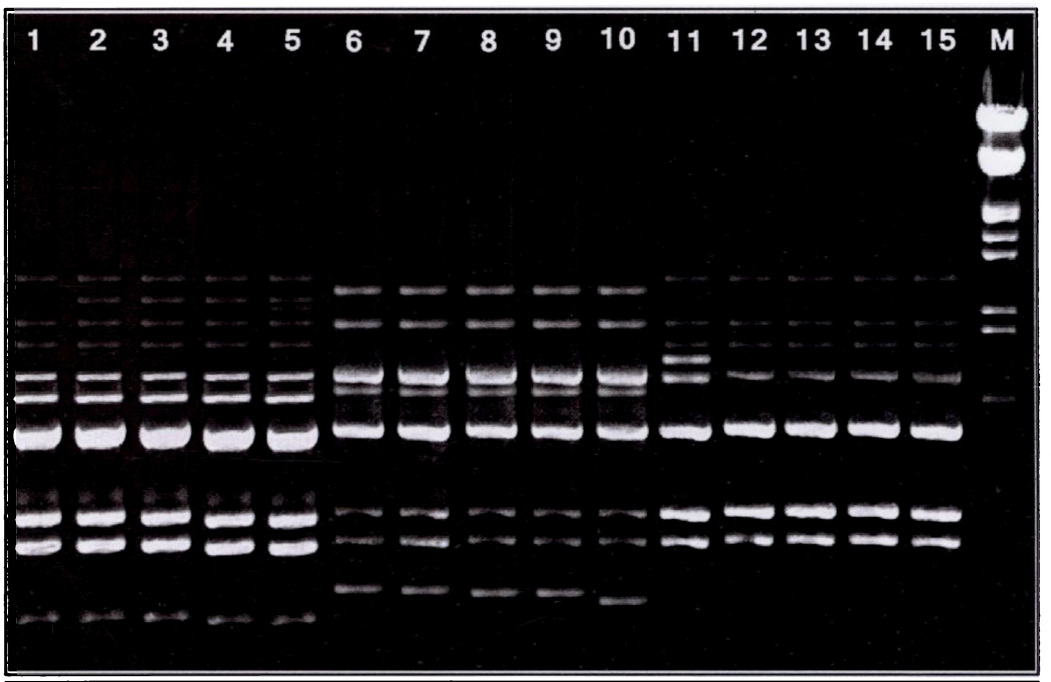

Fig. 30. RAPD pattern of *G. curmuca* with primer OPAC-05.
Lanes 1-5 samples from Periyar, 6-10 Chalakkudy and 11-15 Chaliyar Rivers.
M- molecular weight marker (λDNA with *Eco*R1 & *Hin*dIII double digest)

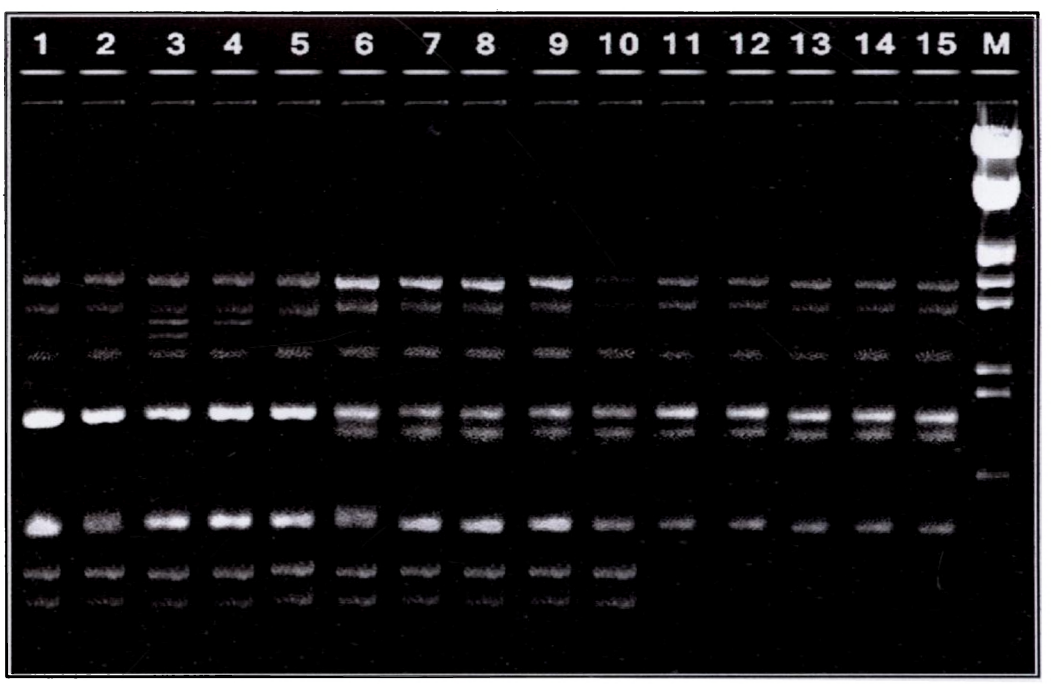

Fig. 31. RAPD pattern of *G. curmuca* with primer OPAC-06.
Lanes 1-5 samples from Periyar, 6-10 Chalakkudy and 11-15 Chaliyar Rivers.
M- molecular weight marker (λDNA with *Eco*R1 & *Hin*dIII double digest)

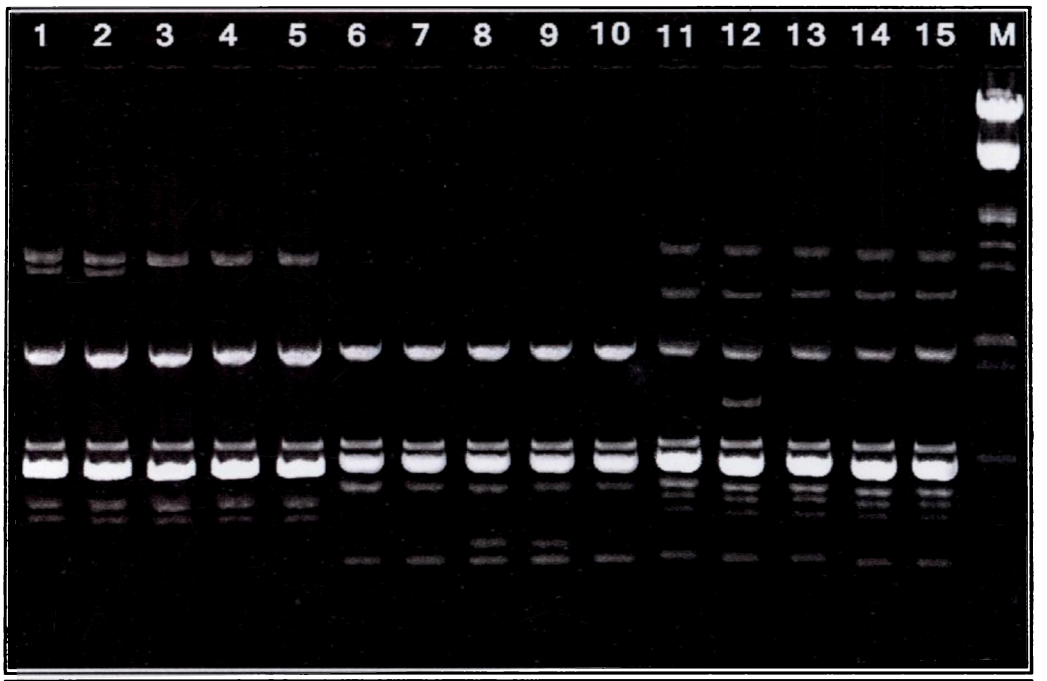

Fig. 32. RAPD pattern of *G. curmuca* with primer OPAH-03.
Lanes 1-5 samples from Periyar, 6-10 Chalakkudy and 11-15 Chaliyar Rivers.
M- molecular weight marker (λDNA with *Eco*R1 & *Hin*dIII double digest)

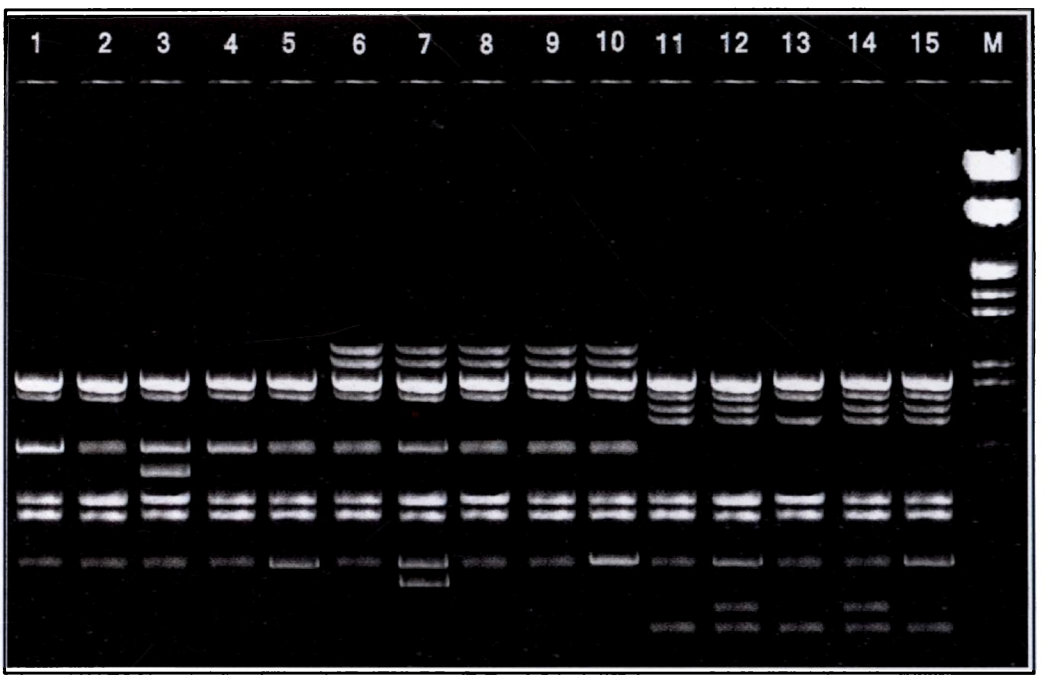

Fig. 33. RAPD pattern of *G. curmuca* with primer OPAH-17.
Lanes 1-5 samples from Periyar, 6-10 Chalakkudy and 11-15 Chaliyar Rivers.
M- molecular weight marker (λDNA with *Eco*R1 & *Hin*dIII double digest)

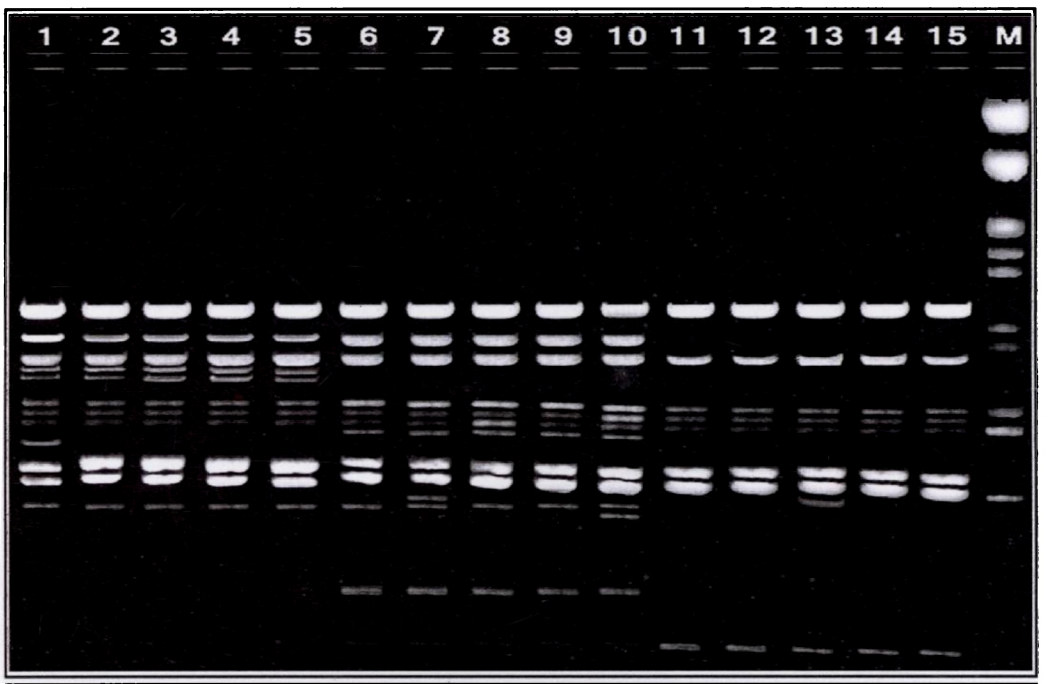

Fig. 34. RAPD pattern of *G. curmuca* with primer OPAH-19.
Lanes 1-5 samples from Periyar, 6-10 Chalakkudy and 11-15 Chaliyar Rivers.
M- molecular weight marker (λDNA with *Eco*R1 & *Hin*dIII double digest)

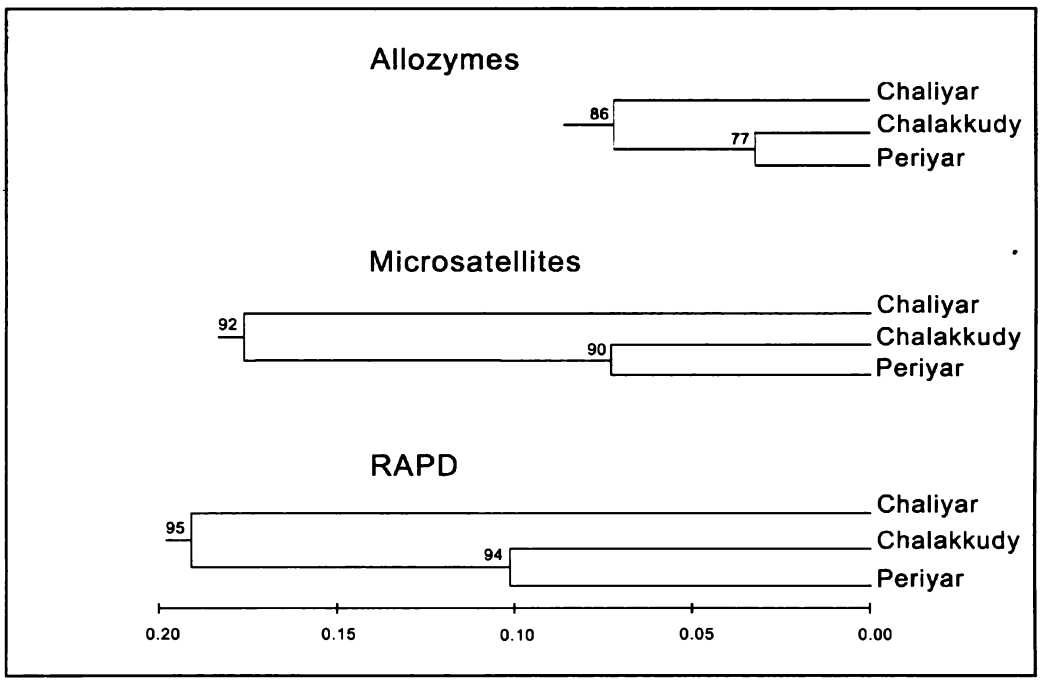

Fig. 35. UPGMA dendrograms of three riverine populations of *G.curmuca* based on Nei's (1978) pair wise genetic distance using Allozyme, Microsatellite and RAPD markers.
The values at nodes represent bootstrap confidence levels (1000 replications).

4.4 Comparative assessment of results of three markers

The results derived by using allozymes, microsatellites and RAPDs in *Gonoproktopterus curmuca* are compared as follows:

4.4.1 Number of loci and alleles

The number of loci obtained was 29 with fourteen allozymes; 8 loci with microsatellites and 117 with nine RAPD primers. Altogether 31 alleles were obtained in allozymes and 46 alleles in microsatellites (**Table 36**).

4.4.2 Percentage of polymorphic loci

In allozymes, 14 loci out of the 29 were polymorphic (48.28%); in microsatellites, all the 8 loci were polymorphic (100%) and in RAPD analysis, 65 (55.56%) were polymorphic (**Table 36**).

4.4.3 Observed and expected heterozygosities

In allozymes, the observed heterozygosity (H_{obs}) was 0.1514 and expected heterozygosity (H_{exp}) was 0.2132. In RAPD, the average heterozygosity or gene diversity (H) was 0.1848 and for microsatellites, H_{obs} and H_{exp} were 0.5249 and 0.5894 respectively (**Table 36**).

4.4.4 Genetic differentiation

The co-efficient of genetic differentiation (F_{ST} or G_{ST} or R_{ST}) values for overall population with allozymes, microsatellites and RAPDs were 0.0510, 0.0689 and 0.2286 respectively (**Table 36**). The pair-wise F_{ST} values between Periyar and Chalakkudy populations of *G. curmuca* using allozymes and microsatellites were 0.04018 and 0.04723 respectively; between Chalakkudy and Chaliyar 0.04995 0.05202 respectively and between Periyar and Chaliyar 0.05994 and 0.06381 respectively. The pair-wise R_{ST} values with microsatellite markers between Periyar and Chalakkudy, Chalakkudy and Chaliyar and Periyar and Chaliyar were 0.05472, 0.06121 and 0.08911 respectively (**Table 37**).

Table 36. Comparative assessment of results of three markers

Markers	No. of enzymes / primers checked	No. of enzymes / primers used	No. of allele / loci	No of poly-morphic loci	Heterozygosity		Overall F_{ST}/ G_{ST}/R_{ST}
					Hob	Hex	
Allozymes	14	14	31/29	14	0.1514	0.2132	0.0510
RAPD	80	9	117/--	65	0.1848		0.2286
Microsatellites	40	8	46/08	8	0.5249	0.5894	0.0689 (F_{ST}) 0.0729 (R_{ST})

Table 37. The comparison of genetic distance, genetic differentiation estimates between pair-wise populations using three markers

Population pairs	Allozymes		RAPDs	Microsatellites		
	Nei's Genetic Distance	Pairwise F_{ST}	Nei's Genetic Distance	Nei's Genetic Distance	Pairwise F_{ST}	Pairwise R_{ST}
Periyar & Chalakkudy	0.0329	0.04018***	0.1013	0.0739	0.04723***	0.05472 ***
Chalakkudy & Chaliyar	0.0501	0.04995***	0.1217	0.1085	0.05202***	0.06121***
Periyar & Chaliyar	0.0702	0.05994***	0.1903	0.1764	0.06381***	0.08911***

*** P<0.0001; significant after sequential Bonferroni adjustment

4.4.5 Private alleles

In allozyme analysis, only one private allele was identified in *LDH-2** (in Chaliyar) (**Table 11**). In microsatellites total nine private alleles (four in Periyar, 2 in Chalakkudy and three in Chaliyar (**Table 24**) were obtained. With RAPD markers, altogether 41 alleles were specific to different populations (**Table 34**).

4.4.6 Genetic distance and similarity

The comparison of pair-wise Nei's genetic distance values for allozymes, microsatellites and RAPDs are given in **Table 37**. The pair-wise genetic distance values using allozymes, microsatellites and RAPDs between Periyar and Chalakkudy were 0.0329, 0.1013 and 0.0739 respectively; between Chalakkudy and Chaliyar 0.0501, 0.1217 and 0.1085 respectively; and between Periyar and Chaliyar were 0.0702, 0.1903 and 0.1764 respectively.

4.4.7 The UPGMA based dendrogram

The UPGMA based dendrogram constructed using the three different genetic markers are compared in **Figure 35**. Irrespective of the markers used, topologies of the dendrogram exhibited similar pattern of genetic divergence in *G. curmuca*.

Chapter 5

DISCUSSION

Contents

Genetic markers can be used to address questions of relevance to the management and conservation of fauna and flora. Particularly in fisheries science, these genetic markers have been applied to three areas - stock structure analysis, selective breeding programmes in aquaculture and taxonomy/systematics (Ward and Grewe, 1994a), 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 at intra-specific level. 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 (Waldman and Wirgin, 1993; 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 (MUs) (Moritz, 1994). In general, the objectives of the electrophoretic analysis of proteins and enzymes in different commercially important fin fish and shellfish are to answer the basic fisheries management questions such as (1) whether the allelic frequencies in the sample populations are similar or different? (2) whether the observed or expected genotype frequencies are in Hardy-Weinberg equilibrium? (3) what is the level of the genetic variation in the species and its different populations? and (4) if the populations are genetically homogenous or heterogeneous, then what are the implications of the findings with reference to their exploitation, conservation and management?

In the present study, the genetic characteristics of *Gonoproktopterus curmuca*, a cyprinid, endemic to the Western Ghats region were analysed for discriminating the natural populations by applying biochemical and molecular techniques *viz.*, (1)

electrophoretic allozymes analysis, (2) analysis of microsatellite markers and, (3) analysis of patterns of random amplified polymorphic DNA (RAPD).

5.1 Allozymes

In the present study, the analysis of 14 allozymes with 29 loci gave sharp zones of enzyme activity, enabling proper interpretation of results, thus discriminating three geographically isolated populations of *G. curmuca.* In population genetic studies based on 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.

5.1.1 Polymorphic allozyme markers

In *G. curmuca*, out of the 14 enzymes studied, 12 enzymes (14 loci) were polymorphic 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 allozymes. In the population genetic analysis of *Barbus callensis*, Berrebi *et al.* (1995) used 10 polymorphic allozyme markers and a polymorphic general protein. In *Cobitis sp.*, Perdices *et al.* (1995) reported variations in 15 allozymes helpful in identifying the stocks of this species. In *Tenualosa ilisha*, Salini *et al.* (2004) used 13 polymorphic enzymes (5 loci) to detect genetic variation in Bangladesh populations, while Lal *et al.* (2004a) reported polymorphism in 13 out of 26 scorable loci in the same species from the 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. 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 for 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 Indian mackerel, *Rastrelliger kanagurta*, Menezes *et al.* (1990) 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 compared to that of freshwater finfish species. Migration, egg and larval dispersal through water 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.*, 1984; Menezes *et al.*, 1993) compared to that of freshwater fish species.

In all the above examples and in the present study, several polymorphic allozymes were common *viz*, AAT, EST, G_6PDH, GPI, LDH, PGM, SOD etc indicating their usefulness in delineating intra-specific differences. In *G. curmuca*, these allozymes were found to be helpful in estimating the degree of divergence. Allozymes such as MDH, GAPDH, ME and XDH *etc.* are rarely used in stock structure studies in freshwater fishes. G_6PDH allozyme pattern did not exhibit sex-linked inheritance in *G. curmuca,* unlike in human beings (Richardson *et al.*, 1986). Both male and female individuals 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 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 belonging to genetically different stocks do not interbreed (Allendorf *et al.*, 1987; Ayala and Keiger, 1980; Bye, 1983; Altukov, 1981).

Genetic diversity expressed in terms of mean 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 *G. curmuca* (1.6538) collected from 3 geographically distinct rivers 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). Chauhan *et al.* (2007) observed a mean number of alleles per locus ranging from 1.32 to 1.41 for allozyme loci in Indian Major Carp, *Cirrhinus mrigala*. In common carp, mean number of 1.06 to 1.81 alleles per locus was reported by Kohlmann *et al.* (2003). In another cyprinid belonging to the Genus *Leuciscus*, at allozyme loci, a mean number of 1.0 to 1.3 alleles per loci were observed (Coelho *et al.*, 1995). Also, these results are comparable with those reported for four 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). Appleyard and Mather (2002) also reported a value of na (1.1665) for *Oreochromis niloticus*, *O. mossambicus* and their red hybrid. Slightly lower values of na (1.3475) were reported in catfish species like *Clarias gariepinus*, *C. anguillaris* and *C. albopunctatus* (Rognon *et al.*, 1998) and in pangasiid catfishes (1.305) (Pouyaud *et al.*, 2000).

The mean value of polymorphic loci ($P_{0.95}$) across populations was 0.43 (43%) in *G. curmuca*. The percentage of polymorphic loci ranged from 3.7% to 18.5% in

different populations of the cyprinid, *Leuciscus sp.* (Coelho *et al.*, 1995); whereas 6.2% to 43.8% of polymorphic loci were observed by Kohlmann *et al.* (2003) in different allozymes in *Cyprinus carpio* and 22% to 27% polymorphic loci in *Cirrhinus mrigala* by Chauhan *et al.* (2007). 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). The value is more over similar in fishes like *Clarias gariepinus* ($P_{0.95}$ = 48%) and greater than *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% polymorphic loci with the criterion $P_{0.95}$ which is comparable with that of *G. curmuca*. In some marine species, lower values of polymorphic loci were reported (Menezes *et al.*, 1993; 1994; Begg *et al.*, 1998).

The best estimate of genetic variation in natural population is the mean observed heterozygosity (H_{obs}) per locus (Allendorf and Utter, 1979). The values of H_{obs} vary non-randomly between loci, populations and species. To avoid serious error in the estimation of H_{obs}, 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 0.1560 for Periyar River population, 0.1538 for Chalakkudy River population; 0.1445 for Chaliyar River population and the mean value for overall population was 0.1500. The H_{obs} value falls within the range reported for 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; 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 *G. curmuca* are lower than that of the expected values (H_{exp}), indicating the deficiency of heterozygotes except in one locus (*EST-1**) in Chalakkudy and Chaliyar populations. All the loci (except *EST-1** in Chalakkudy and Chaliyar Rivers) deviated significantly from Hardy-Weinberg equilibrium (HWE) after Bonferroni 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 *G. curmuca*. This could be due to non-random mating or effect of other evolutionary forces like selection/migration or reduction in effective breeding population. Mixing of non-native genetic stocks can also be another reason. *G. curmuca* fetches a high price as ornamental and food fish and there has been a massive exploitation for the species for aquarium trade over the last several 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 red-tailed barb 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 *G. curmuca* as samples were collected from geographically isolated river systems (minimum distance between Periyar River and Chalakkudy River 60Km and between Chalakkudy River and Chaliyar River 160Km; 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 *G. curmuca* 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" indicated the populations of *G.curmuca* had undergone demographic bottleneck in recent times (discussed in detail in pages 148-149).

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 un-interpretable variations. However, in the present study, using polyacrylamide electrophoresis (PAGE), the EST and MEP bands obtained were always sharp (ALDH not tried) and scoring difficulties were not encountered.

5.1.3 Linkage disequilibrium

No allozyme loci showed linkage disequilibrium (after Bonferroni correction) in any of the three populations of *G.curmuca*. It is therefore assumed that allelic variation at allozyme loci could be independent as observed in many species of fishes (Weir, 1979; Rognon *et al.*, 1998; Sapna, 1998; Pouyaud *et al.*, 2000; Cook *et al.*, 2002; McGlashan and Hughes, 2000; Rebello, 2002; Appleyard and Mather 2002).

5.1.4 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, 1975). One (*LDH-2**) private allele was observed in *G. curmuca*, in Chaliyar River population in the present study (**Table 11**). Many authors showed that the private alleles can be used to distinguish stocks or to discriminate species. Peres *et al.* (2002) reported the two private alleles, one in G_6PDH-*1* specific to Parana River population and the other in $MDH\text{-}A_2$ 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 by Muneer (2005) indicated physical isolation and genetic differentiation and usefulness of these alleles in identifying distinct populations of the 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. The private allele (at *LDH-2** locus, R_f value 78) obtained in the present study can be used to distinguish *G. curmuca* stock from Chaliyar River.

5.1.5 Population genetic structure

Pair-wise comparisons between different riverine locations for allelic homogeneity in *G. curmuca* yielded significant deviations at all loci in their frequencies after 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 statistics F_{ST} refers to the genetic differentiation of sub-populations within the total population and it describes how much variation in allele frequencies is present between the local populations. F_{ST} values vary between 0 and 1; with 0 indicating no differentiation (random mating between individuals of the populations) and 1 complete differentiation between populations (Beaumont and Hoare, 2003). Negative F_{ST} values are also reported sometimes due to the computation methods used by the estimator, but treated equal to zero (Balloux and Lugon-Moulin, 2002; Hardy *et al.*, 2003). The overall value (0.0510) of the coefficient of genetic differentiation (F_{ST}) among samples also indicates that there is genetic differentiation into local populations in the species (**Table 14**). There was considerable heterogeneity between loci, with estimates of overall population F_{ST} ranging from 0.0267 to 0.0704 due to population differences. Rognon *et al.* (1998) reported a lower yet significant F_{ST} value (= 0.044) for populations of

Clarias gariepinus with allozymes. 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.* (1995) in the populations on the genus *Cobitis*. Coelho *et al.* (1995) reported the range of overall population F_{ST} values of 0.044 to 0.863 in *Leuciscus pyrenaicus* and *L. carolitertii*. Genetic relatedness of *G. curmuca* populations derived from pair-wise F_{ST} (θ) between populations using allozyme data 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 were moderately low but were highly significant ($P<0.0001$) and fell within the range reported for freshwater fishes (Ward *et al.*, 1994b). Similar overall and pair-wise F_{ST} values and significant levels of genetic differentiation among populations were also reported in Australian freshwater fish (*Craterocephalus stercusmuscarum*) using allozyme markers (McGlashan and Hughes, 2000) and stocks of freshwater prawn, *Macrobrachium australiense* between river catchments in Australia (Cook *et al.*, 2002).

The significant F_{ST} estimation (θ) based on allozymes in the present study also comparable to that obtained using microsatellite markers in *G. curmuca* (discussed in microsatellite section in this thesis). AMOVA analysis of the data also indicated significant genetic differentiation among sampled population (**Table 17**). These values of total genetic differentiation (from F-statistics and AMOVA) were close to those reported in other fishes (McGlashan and Hughes, 2000; Cook *et al.*, 2002). Genetic differentiation can be influenced by a number of evolutionary forces and their interaction that act on natural populations including migration, random genetic drift and mutation (Hartl and Clark, 1997). Random genetic drift will tend to cause genetic differentiation, after sub-populations are fragmented and gene flow between them in either reduced or absent (Slatkin and Barton, 1989). The geographical barriers (land mass) between different river basins selected in the present study do not permit mixing of fish

populations even during flood. Absence of gene flow among populations could be the major reason for the random genetic drift and subsequent genetic heterogeneity among *G. curmuca* populations in the river systems sampled here. The occurrence of private or locality specific alleles also argues in favour of absence of gene flow among populations.

5.1.6 Genetic distance values

The genetic relationship among populations in *G. curmuca* was determined using Nei's genetic distance measure. The genetic distance values ranged from 0.0329 to 0.0702, and the values were close to the average obtained by Shaklee *et al.* (1985) for con-specific populations of marine and freshwater fish (D = 0.05 and I=0.977). Based on the genetic distance, a UPGMA dendrogram was constructed for the *G. curmuca* that showed three populations as three distinct groups with the Chaliyar stock farther from Chalakkudy and Periyar River populations. In Clariid catfishes, Nei's genetic distance at intraspecific level ranged from 0.008 to 0.29 in *Clarias gariepinus;* and 0.005 to 0.043 in *C. anguillaris* (Rognon *et al.*, 1998) with a mean genetic distance of 0.207±0.081 between the species (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*). In marine species due to the exchange of individuals low genetic heterogeneity is generally recorded. This is supported by low values of genetic distances. Benzie *et al.* (1992) reported a very low value of genetic distance in populations of *Penaeus monodon* in Australia ranging from 0.000 to 0.015.

5.1.7 Bottleneck Analysis

Populations that have experienced a recent reduction of their effective population size exhibit a correlative reduction of the allele numbers (k) and gene diversity (He or Hardy – Weinberg heterozygosity) at polymorphic loci. But the allele numbers are reduced faster than the gene diversity. Thus in a recently

bottlenecked population, the observed/measured gene diversity is higher than the expected equilibrium gene diversity (Heq), which is computed from the observed number of alleles (k) under the assumption of constant- size (mutation-drift equilibrium) population. The programme BOTTLENECK (Cornuet and Luikart, 1996) was used to detect past population reduction by testing for a transient (~0.2 to 4.0 Ne generations) excess in measured heterozygosity compared with the expected heterozygosity at mutation drift equilibrium. This excess in heterozygosity is generated because rare alleles are quickly lost due to drift during a bottleneck, but they contribute little to the expected heterozygosity (Pearse *et al.*, 2004; Slatkin, 1985). It is crucial to identify populations that have undergone ancient and recent bottlenecks, because they may have been affected by the small population size through demographic stochasticity, inbreeding or fixation of deleterious alleles, possibly leading to a reduced evolutionary potential and increased probability of extinction. The distribution profile of the allozyme alleles, heterozygosities, heterozygosity excess [where the infinite allele model(IAM)] indicated clear mode shift of allele diversity in all the populations of *G. curmuca* in contrast to the expected L-shaped distribution. The probability values also indicated evidence for recent declines in population size. The results are in concordance with the observations based on microsatellite markers in the present study (discussed in microsatellites section). Although the Kerala State Fisheries Department do not have the data to judge any historical evolution in inland fisheries mortality, the Conservation Assessment Management Plan (CAMP) workshop held at NBFGR in 1997 (Anon., 1998) had clearly indicated depletion in the population size of *G. curmuca* in the Western Ghats Rivers as a result of over-exploitation, destructive fishing practices and habitat alterations. Over-fishing in vulnerable areas, especially at the spawning sites, destruction of spawning habitats will lead to a reduction in effective population size and yield (So *et al.*, 2006). Probably these anthropogenic influences would have led to the loss of genetic diversity, genetic bottleneck and population viability of *G. curmuca*, as reported in sutchi catfish, *Pangasianodon hypopthalmus* (So *et al.*, 2006) and in Atlantic salmon (*Salmo salar*) by Ayllon *et al.* (2004).

In **conclusion**, the allozyme studies alone provide positive proof for the existence of genetically different stocks of *G. curmuca* in the 3 rivers along the Western Ghats region, India. Occurrence of distinct stocks of red-tailed barb 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. In addition, it is estimated that only less than 25% of amino acid substitutions are detectable by conventional gel electrophoresis (Bindhu Paul, 2000). 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 microsatellites are better for population genetic studies. Nevertheless, 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, significant genetic differentiation among populations of *G. curmuca* was obtained in the present study with allozymes that are the products of functional genes. This finding is of considerable importance because, (1) the extent of differentiation has reached upto the level of slow – evolving, functional nuclear genes, and (2) the slow evolving nuclear genes have reached equilibrium between gene flow and genetic drift as the river basins were last colonized by *G. curmuca* (Slatkin and

Barton, 1989; Slatkin, 1985 & 1993). There are similar reports of significant stock differences detected using only allozymes in fishes and shellfishes (Utter, 1989; Ihssen *et al.,* 1981b; 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 microsatellites, RAPDs, mtDNA 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.*, 2002; 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 red-tailed barb using allozymes were low as compared to that of RAPDs and microsatellites. But overall broad overlap of divergence levels from allozymes to molecular markers (RAPD and microsatellites) in this study suggests that all 3 sets of allelic frequency distributions represent neutral markers in *G. curmuca,* as reported in above mentioned studies. Therefore, the present work on red-tailed barb 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 numbers of polymorphic and heterogenic loci were detected.

5.2 Microsatellites

Microsatellites are co-dominant markers and inherited in Mendelian fashion, revealing polymorphic amplification products helping in characterization of individuals in a population. Many features of microsatellites render them invaluable for examining fish population structure. 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 forensic science (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).

Understanding the mutation model underlying microsatellite evolution is of great importance for the development of statistics accurately reflecting genetic structuring. Two models of mutation generally proposed to account for variation at microsatellite loci are the stepwise mutation model (SMM) (Rousset, 1996) 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. According to SMM, alleles of very different sizes will be more distantly related than alleles of similar sizes. 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). IAM does not allow for homoplasy and identical alleles share the same ancestry and are identical by descent (IBD). Even though SMM is thought to reflect more accurately the mutation pattern of microsatellites (Balloux and Lougon-Moulin, 2002), it is considered rather to be an unrealistic model as the microsatellite mutation process is often known to deviate from a strict SMM (Cornuet and Luikart, 1996). Hence, the two-phase mutation model (TPM) was followed for analysis such as bottleneck in the present study as it is intermediate to the SMM and IAM and most microsatellite loci better fit in this model (Cornuet and Luikart, 1996).

Many microsatellite loci despite their extremely fast rates of repeat 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 (Galbusera *et al.*, 2000). Generally, the development of new species-specific microsatellite primers is by constructing microsatellite enriched genomic libraries and subsequent screening of the colonies that is expensive, time-consuming and laborious, but the above mentioned

attractive alternative option is cheap and fast. Primers developed for a species by this method have been successfully tested for "cross-species amplification" or "cross-priming" in its related species in several teleosts, including Asian cyprinids (Zardoya *et al.*, 1996; Scribner *et al.*, 1996; Galbusera *et al.*, 2000; Mohindra *et al.*, 2001a, 2004, 2005; Lal *et al.*, 2004b; Gopalakrishnan *et al.*, 2004; Chauhan *et al.*, 2007) and it was possible to obtain a set of useful markers for each study species by cross-priming.

In the present study, altogether 40 primer pairs developed for six (resource) homologous fish species belonging to the Order Cypriniformes were evaluated for cross-species amplification of microsatellite loci in *Gonoproktopterus curmuca*. 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 *G. curmuca* 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 Cypriniformes in *G. curmuca* 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, 2004, 2005; Lal *et al.*, 2004b; Das and Barat, 2002 a, b, c; Gopalakrishnan *et al.*, 2002, 2004; Das *et al.*, 2005; Chauhan *et al.*, 2007). Zardoya *et al.* (1996) also reported that homologous microsatellite loci 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 *G. curmuca* supports this view.

The present study demonstrated successful cross-priming of microsatellite loci, between the fish species of the same family. Also, in some other works, certain sequences flanking the tandem repeats could be conserved even between the different families as reported by Scribner *et al.* (1996), Zardoya *et al.* (1996) and

Muneer (2005). 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 Cypriniform species found in India.

There are several separation methods currently employed to determine the length of the PCR product in microsatellite analyses among which, native or denaturing Polyacrylamide gel electrophoresis (PAGE) is commonly used. The amplification products in PAGE are typically visualized with radioactive labeling, fluorescent dye labeling or silver staining. Visualization methods with radioactive / fluorescent dye labeling are either hazardous or expensive. Highly sensitive capillary electrophoresis and automated genotyping units also have been used to accurately determine length polymorphism of microsatellite makers (Wang *et al.*, 2003); but these methods require sophisticated and costly instruments and fluorescently tagged primers, which are expensive. The vertical native PAGE (slab gel) system (100mm height X 100mm wide X 1mm thick; Amersham Biosciences, USA) with silver staining protocol used in the present study was inexpensive and capable of separating DNA fragments that differed by as little as 2 base pairs (confirmed after sequencing the PCR products) as reported by Wang *et al.* (2003). The cost per gel excluding the PCR cost is currently estimated at about Rs.60/- ($1.50). This system has been successfully used for genotyping microsatellite markers in many teleost (Cypriniform, Siluriform and Osteoglossiform) species (Chauhan *et al.*, 2007; Gopalakrishnan *et al.*, 2004, 2006a; Lal *et al.*, 2004a, 2006; Mohindra *et al.*, 2001a, 2005, 2008; Muneer, 2005; Punia, *et al.*, 2006) and could be a valuable and cost effective tool for researchers employing microsatellite markers in other species.

5.2.1 Type and relative frequency of microsatellite arrays observed

In *G. curmuca*, 9 amplified presumptive microsatellite loci were cloned and sequenced and among these, 8 loci were confirmed to contain microsatellites. The tandem repeats of the microsatellite loci observed in the present study are comparable to that of the resource species. The GATA and CA repeats (*MFW72; MFW01, MFW11, MFW19, MFW26, Ppro48* and *Ppro126* primers) of the resource species, *Cyprinus carpio* and *Pimephales promelas* are exactly similar in *G. curmuca*, though the numbers of repeats varied. But, the GATA repeat of the microsatellite locus in *CcatG1-1* (resource species, *Catla catla*) differed in *G. curmuca* and replaced by GGA repeats. 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 present study found GT and CA rich microsatellites abundant in *G. curmuca* 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 *G. curmuca* 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.

5.2.2 Genetic variability and Hardy-Weinberg Equilibrium

The number of alleles at different microsatellite loci in *G. curmuca* varied from 3 to 7 with an average value of 5. Primers CcatG1, MFW01, MFW11, and MFW19 exhibited maximum allele number (7) compared to other primers (three to six alleles). High microsatellite allele variation was recorded in Thai silver barb (*Puntius gonionotus*) in four microsatellite loci with an 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 seabream (16-32 alleles/locus) (Takagi *et al.*,

1999a); Atlantic cod (8-46 alleles/locus) (Bentzen *et al.*, 1996) and in seabass (4-11 alleles/locus) (Garcia de Leon *et al.*, 1995). However, a low variation was observed among microsatellite loci of some other fishes such as brown trout (5-6 alleles /locus) (Estoup *et al.*, 1993) and northern pike (3-5 alleles/locus (Miller and Kapuscinski, 1996). Neff and Gross (2001) reported mean number of alleles at different microsatellite loci of 27 species of marine and freshwater finfishes 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 *G. curmuca.* 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 *G. curmuca* (which is purely a freshwater fish), can be due to differences in biology and life history traits compared to that of the marine species (having higher number of microsatellite allele variation) as suggested by Neff and Gross (2001).

In the present study, variations of allele sizes were quite low for *MFW26, MFW72,* and *Ppro48* loci which might be due to their small number of repeat units and similar level of allele size variation is reported in many other freshwater

teleosts and higher vertebrates (Carvalho and Hauser, 1994; Liu and Cordes, 2004). The same trend of relationship was observed in *Clarias macrocephalus* (Na-Nakorn *et al.*, 1999) and Thai silver barb (Kamonrat, 1996). The mean observed number of alleles (na) at each locus (5.25) in red-tailed barb was closer to the value obtained in Asian cyprinids *viz. Cirrhinus mrigala* (4.5), *Tor putitora* (4.42) and *Labeo dyocheilus* (4.42) (Chauhan *et al.*, 2007; Mohindra *et al.*, 2004 & 2005 respectively). Similar results were also reported by Liao *et al.* (2006) in *Cyprinus carpio* from China; by Han *et al.* (2000) in striped bass (*Morone saxatilis*); by Watanabe *et al.* (2001) in *Pseudobagrus ichikawai* (na = 4.75) 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). But the value was lower than that observed in *G. curmuca* in *Mystus nemurus* (na=3.2) (Usmani *et al.*, 2003); in *Ictalurus punctatus* (na=3.9) (Tan *et al.*, 1999) and in *Pangasius hypophthalmus* (Volckaert *et al.*, 1999). However, a higher value of na was reported in some fishes - by Na-Nakorn *et al.* (1999) in *Clarias macrocephalus* (na=12.0) and Volckaert *et al.* (1999) in *Clarias batrachus* (na=5.8). DeWoody and Avise (2000) pointed out that the value of na fluctuates widely in many teleosts with a mean of 9.1±6.1.

In *G. curmuca,* the mean observed heterozygosity (H_{obs}) per locus per population was 0.5249 and the mean expected heterozygosity (H_{exp}) per locus per population was 0.5894. The H_{obs} in the present study is congruent with value reported for most of the freshwater species (0.54±0.25) as reported by DeWoody and Avise (2000). In other Asian cyprinids the observed heterozygosity values using microsatellites ranged from 0.26 to 0.82 in *Catla catla* population (McConnel *et al.*, 2001); 0.28 to 0.39 in *Tor putitora* (Mohindra *et al.*, 2004); and 0.33 to 0.42 in *Cirrhinus mrigala* population (Lal *et al.*, 2004; Chauhan *et al.*, 2007). Usmani *et al.* (2003) in *Mystus nemurus* reported a value of mean observed heterozygosity (H_{obs} = 0.4986), however, the mean expected heterozygosity was lower than that of the present study. In *G. curmuca,* a significant overall deficiency of heterozygotes was revealed in all the populations with exception in some loci

(*MFW11*, *MFW19*, *MFW72* and *Ppro48* loci in Periyar River, *MFW01*, *MFW19* and *MFW72* loci in Chalakkudy River and *CcatG1-1*, *MFW72* and *Ppro126* in Chaliyar River). In *Clarias macrocephalus*, Na-Nakorn *et al.* (1999) and in *Tor putitora* Mohindra *et al.* (2004) reported the deficiency of heterozygotes (H_{obs}=0.67 and 0.28; and H_{exp}=0.76 and 0.42 respectively). Small sample size can be a reason for inability to detect all the alleles in the population and to conclude the occurrence of heterozygote deficiency (Na-Nakorn *et al.*, 1999). But the sample size of 70 for each population of *G. curmuca* 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 *G. curmuca*. Seven of the eight-microsatellite loci (except *MFW26*) showed significant deviations ($P<0.05$) from Hardy-Weinberg Equilibrium (HWE). Deviations from HWE is usually attributed to null alleles (Gopalakrishnan *et al.*, 2004; 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 red-tailed barb has been noticed in rivers of Kerala for last 25 years 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 (Robertson and Hill, 1984; 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.*, 1996; Yue *et al.*, 2000; Gopalakrishnan *et al.*, 2004a).

5.2.3 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 red-tailed barb 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 red-tailed barb, 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 *G. curmuca*. Van Oosterhout *et al.* (2004) suggested that in such a situation, the overall homozygosity can be due to deviations from HWE such as 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 ruled 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, most of the amplified products were di-nucleotide repeats and allele size variation ranged between 2-36 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 our constant interaction with the fishermen, local people and aquarium traders during the study period.

5.2.4 Linkage disequilibrium

There were no significant associations indicative of linkage disequilibrium between any pair-wise combinations of microsatellite alleles in *Gonoproktopterus curmuca* (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; Weir, 1979 & 1990; Muneer, 2005).

5.2.5 Stock-specific markers

Four microsatellite alleles (*MFW11*-168, *MFW72*-138 & 134 and *Ppro126*-162) found in Periyar River samples were not found in Chalakkudy and Chaliyar Rivers and hence they were treated as private alleles of Periyar River. Two microsatellite alleles (*Ppro48*-218 and *Ppro126*-170) found in Chalakkudy River samples were not found in Periyar and Chaliyar Rivers and hence they were treated as private alleles of Chalakkudy River population. Similarly, the three microsatellite alleles (*MFW11*-162, *MFW72*-148 and *Ppro48*-226) were exclusive to the Chaliyar River population. The detection of significant private alleles in Periyar, Chalakkudy and Chaliyar populations are the clear-cut evidence for no mixing of the gene pools between these populations in *Gonoproktopterus curmuca*. 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.* (1999b) reported the stock specific markers in the populations of tuna species of the genus *Thunnus*.

Similarly, 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/supportive breeding programmes and also for mixed stock analysis (MSA).

5.2.6 Genetic differentiation

Pair-wise comparison between different riverine locations for microsatellite allelic homogeneity in *G. curmuca* 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 (0.0689) of microsatellite loci in *G. curmuca* 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 more polymorphism) in microsatellites result in greater power for population differentiation (Rousset, 1997 & 2000; Rousset and Raymond, 1995; Goudet *et al.*, 1996). Levels of genetic differentiation recorded for red-tailed barb (overall $F_{ST} = 0.0689$) are comparable to those significant values seen in Pacific herring ($F_{ST}=0.043$), Atlantic herring ($F_{ST} = 0.045$), Atlantic salmon ($F_{ST} = 0.064$) (McConnell *et al.*, 1995) and yellow catfish (Muneer, 2005). The genetic relatedness of *G.curmuca* 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 result.

In addition to the allelic frequency, microsatellites provide additional information – *i.e.*, difference in number of repeats – that will be helpful in measuring the population

sub-division. But F_{ST} takes care of only allelic frequencies; hence in addition to F_{ST}, the population differentiation was also estimated by pair-wise and overall R_{ST} (Slatkin, 1995) based on the variance in microsatellite allele sizes in *G. curmuca* populations. The pair-wise and overall R_{ST} values exhibited significant genetic differentiation ($P<0.0001$) among the populations of *G. curmuca*. The R_{ST} values were very similar to that of F_{ST} estimates in the present study as reported in many teleosts (Hardy *et al.*, 2004; Chauhan *et al.*, 2007). Comparing F_{ST} and R_{ST} values computed on the same data can provide valuable insights into the main causes of population differentiation, *i.e.*, genetic drift or mutation, because these statistics share equal expectations, when differentiation is caused solely by drift ($R_{ST} = F_{ST}$); where as R_{ST} is expected to be larger than F_{ST} under a contribution of step-wise mutation (Pearse and Crandall, 2004). Hardy *et al.* (2003) developed a test, based on a randomization procedure of allele sizes to analyse whether step-wise mutations contributed to genetic differentiation – *i.e.*, whether R_{ST} was superior to F_{ST} in case of microsatellites. They observed that (1) in a small population sampled at a micro-geographic scale, R_{ST} and F_{ST} estimates were similar, where as population sampled at a macro/mega-geographic scale showed significantly higher R_{ST} compared to F_{ST} indicating that mutation becomes important relative to migration at this scale; and (2) the R_{ST} and F_{ST} are generally similar when the level of significant genetic differentiation is comparatively low, where as R_{ST} is often superior to F_{ST} when differentiation is high. The results in *G. curmuca* ($R_{ST} = F_{ST}$) can be explained based on the light of above observations – populations were sampled from a small geographic area; and moderate, yet significant levels of genetic differentiation existing among these populations.

AMOVA analysis of the microsatellite data also indicated significant genetic differentiation among sampled *G. curmuca* populations (**Table 28**) and the same trend was observed in the data sets based on allozyme analysis in the present study. The values (F_{ST}) are similar to those recorded in other species (Liao *et al.*, 2006; Cook *et al.* 2002). The geographically isolated river basins selected in the present study do not permit the mixing of populations of *G. curmuca* and the

absence of gene flow among the populations could be the prime factor for the significant genetic heterogeneity and population structuring of the species. The occurrence of nine (9) private alleles in three populations further supports the hypothesis of absence of gene flow among the populations of *G. curmuca*.

5.2.7 Genetic relationships among populations

The genetic relationships among populations would be explained largely through the geographic distance or isolation by distance between sampling locations (Rousset, 1997; Slatkin, 1993). The two populations, Periyar and Chalakkudy Rivers always clustered closely than the Chaliyar River population as revealed in allozymes and RAPD analysis using the same fish samples. The Chalakkudy River population was always intermediate in position between Periyar River and Chaliyar River populations and their genetic distances calculated from microsatellite data agreed with geographic distance. The UPGMA dendrogram of *G. curmuca* using microsatellite data also indicated similar topology as observed with allozymes and RAPD markers of this species.

5.2.8 Bottleneck analysis

An increased number of alleles per locus and heterozygosity at microsatellite loci provide higher statistical power to detect historical bottlenecks and to monitor genetic variation for detecting potential future bottlenecks (Luikart and Cornuet, 1998). The microsatellite data was analysed under the more suitable two phase model (TPM) (Cornuet and Luikart, 1996) in addition to the IAM. In this work, both the Wilcoxon test and the mode shift test detected evidence that the population of *G. curmuca* has a recent bottleneck. The results are congruent with the allozyme data analysis in the present study. Drastic decline in the population of *G. curmuca* due to over-exploitation and habitat alteration was pointed out by the CAMP workshop in 1997 (Anon., 1998). Our continuous interaction with the fisher folk and the local people for more than four years (2002 to 2006) during field surveys also revealed the decline *G. curmuca* catches as a result of over-fishing in these river systems, even though no records of its past abundance in

terms of number and weight were available. So *et al.* (2006) had cautioned destruction of spawning grounds and excessive fishing during breeding season would lead to genetic bottleneck in fishes. Most likely, the anthropogenic factors (massive hunt by the ornamental traders and habitat alteration) could be the reasons for the genetic bottleneck and inbreeding in *G. curmuca*, as reported in other commercially important endemic species of the Western Ghats (Gopalakrishnan and Ponniah, 2000).

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

In **conclusion**, the analysis using novel hypervariable microsatellite loci in *G. curmuca* revealed significant results: First, the potential use of heterologous PCR primers was explored and many of them appeared to be conserved in this cyprinid. 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 red-tailed barb populations between different river systems. Third, the study suggested that the three allopatric populations of this species *viz.*, Periyar, Chalakkudy and Chaliyar that are divergent in their genetic characteristics can be identified through microsatellite loci. Four, recent reduction in effective population size could be detected which is mostly due to heavy fishing pressure and habitat destruction. The information generated will be helpful to design strategic plans for rehabilitation of declining stocks of *G. curmuca* 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.

5.3 Random amplified polymorphic DNA (RAPDs)

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, b; 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 (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 *Gonoproktopterus curmuca* from three river systems.

5.3.1 Reproducibility of RAPD markers

Hadrys *et al.* (1992); Schierwater and Ender (1993); Lynch and Milligam (1994); Allegrucci *et al.* (1995); Naish *et al.* (1995) and Ali *et al.* (2004) reported several technical problems associated with application of the RAPD technique in the field of genetic population studies. A disadvantage of this technique is reproducibility of the results (Liu *et al.*, 1999b; Dinesh *et al.*, 1995; Penner *et al.*, 1993). RAPDs can generate unreliable products through PCR or the same pattern will not be obtained again even under identical screening conditions, unless the technique is well standardized. To get the reproducible results for RAPD, the quality and quantity of the template DNA used is a major key factor (Dinesh *et al.*, 1995).

To standardize the experimental conditions Mamuris *et al.* (1998) used two different DNA extraction methods, two different polymerases and two thermal cyclers. *Taq* polymerase purchased from different manufacturers produced similar results when applied on DNA from the same individual in the same thermal cycler. On the contrary, within the same laboratory, different polymerases as well as different thermal cyclers 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

(Genei Ltd, Bangalore, India) and PCR and electrophoresis were carried out at different intervals. The template DNA quantity (1μL per single reaction mix) and concentration were kept uniform across samples (circa 25ng). This resulted in high level of reproducibility and sharpness of RAPD profiles in *G.curmuca* 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) and in the review by Liu and Cordes (2004).

5.3.2 Genetic variability in RAPD analysis

The RAPD method was applied to identify genetic similarity and diversity in red-tailed barb, *G. curmuca* using 9 polymorphic Operon primers. The number of fragments generated per primer varied from 04 to 20. 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 *G.curmuca* ranged from 0800-6000bp and this confirms with the range of fragment sizes observed in other teleosts (Ali *et al.*, 2004).

In *G. curmuca,* 09 primers generated a total of 117 fragments, producing an average of 13 bands per primer. Among these fragments, 65 (55.55%) were found to be polymorphic as summarized in **Table 32**. In Periyar population, a total 19 bands out of 88 amplicons (21.59%) were polymorphic; in Chalakkudy population, 18 out of 82 (25.00%); and in Chaliyar population 18 out of 72 (25.00%) were polymorphic. The percentage of polymorphism at intra-population level in *G, curmuca* was relatively low, but in overall population the percentage was high (55.55%) compared to many 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 Yesan population. Chong *et al.* (2000) reported 42 polymorphic RAPD markers in *Mystus nemurus*. Liu *et al.* (1998a) reported the production of 462 polymorphic bands, an average of 6.1 bands per primer in *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 *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; Ali *et al.*, 2004).

Average gene diversity, also known as average heterozygosity (H) is a measure of genetic variation for randomly mating population and it is analogous to Wright's fixation index (Silas *et al.*, 2004 & 2005). Many authors had estimated this parameter 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 Talley, 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). The average gene diversity (H) in *G. curmuca* ranged from 0.0558 (Periyar population) to 0.1640 (Chalakkudy population), with a value of 0.1848 for overall population (**Table 32**). 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 as reported by Silas *et al.* (2004 & 2005) in mahseers. The values of H and %P were lower than those reported for populations of other fishes (Khoo *et al.*, 2002; Chen, 1999).

5.3.3 The size and number of the RAPD amplicons

The molecular weight of 117 RAPD-PCR fragments in *G. curmuca* ranged from 0800 to 6000 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. 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.

5.3.4 Linkage disequilibrium

Pairs of RAPD loci did not show any significant linkage disequilibrium ($P>0.05$) in all the populations of red-tailed barb. It was therefore assumed that allelic variation at RAPD loci could be considered independent as reported in other teleosts (Muneer, 2005; Silas *et al.*, 2005).

5.3.5 Population specific RAPD markers

Using RAPD analysis, the present study observed 41 population-specific bands in three natural populations of *G. curmuca*. Among these, 18 specific bands were found in Periyar River population, 15 specific bands in Chalakkudy population and only 8 specific bands in Chaliyar population (**Table 34**). Population specific RAPD markers are reported in other fishes also by Yoon and Kim (2001), in *Silurus asotus*; Cagigas *et al.* (1999) in brown trout, *Salmo trutta;* Klinbunga *et al.* (2000b) in mud crabs; Govindaraju and Jayasankar, (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 developed in tropical oyster (*Crassostrea belcheri*) in Thailand

(Klinbunga *et al.*, 2000a) to generate profiles at the intra-specific level. Zhou *et al.* (2001) used specific RAPD fragments to develop SCAR markers for identifying crucian carp clones. Similarly, Araneda *et al.* (2005) developed SCAR markers associated with colour traits in Coho salmon from RAPD bands. The population-specific RAPD markers in *G. curmuca* could be useful as genetic tags or to generate SCAR markers in future that would be helpful in aquaculture and selective and supportive breeding programmes of this species.

5.3.6 Genetic differentiation

The coefficient of gene differentiation (G_{ST}) is a measure based on allele frequencies to investigate how the genetic variation is partitioned among populations (Nei, 1987). A relatively high overall population genetic differentiation among riverine populations were observed in *G. curmuca* in the present study (G_{ST} = 0.2286). This clearly indicates that the wild *G. curmuca* populations are strongly sub-structured. Gomes *et al.* (1998) reported a similar value of Q_{ST}, (an analogue of G_{ST} or F_{ST} (Excoffier *et al.* (1992)) in the stock discrimination of four-wing flying fish, *Hirundichthys affinis* 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 distinct tilapia stocks. 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) and Benzie and Stoddart (1992). Similarly, Silberman *et al.* (1994) suggested that an overall Q_{ST} value of 0.032 indicated little evidence of genetic sub-division in the spiny lobster, *Panulirus argus*. As in the present study, a high overall G_{ST} value using RAPD markers was recorded in yellow catfish suggesting that there was little gene exchange between stocks (Muneer, 2005).

5.3.7 Genetic relationship between populations

Results of RAPD analysis indicate a more distant relationship between Periyar population and Chaliyar populations (genetic distance, D = 0.1903) of *G. curmuca*. The populations between Periyar and Chalakkudy Rivers are genetically

closer (D = 0.1013). The genetic distance between Chalakkudy River population and Chaliyar River population was 0.1217 (**Table 35**). 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 0.2150 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). In marine teleosts, the genetic distance values appear low and the populations are weakly sub-structured compared to the freshwater counterparts (Govindaraju and Jayasankar, 2004). For example, 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.

UPGMA dendrogram of *G. curmuca* (**Figure 35**) using RAPD markers exhibited distinct clustering suggesting 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 drawback of RAPD marker are (1) these are dominant (*i.e.*, it is not possible to determine if an individual is a homozygote or heterozygote) at a locus and, (2) 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). Also in RAPD analysis we are assuming that the populations are in HWE, that may not true often. 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). Thus, one must be cautious about systematic conclusions based on RAPD analysis alone.

The RAPD profiles in the present study displayed a high degree of polymorphism, which indicated a population structure for red-tailed barb entirely consistent with that obtained from analysis of allozymes and microsatellites. This confirms suitability of RAPD markers for discrimination of this red-tailed barb stocks. In brief, the study yielded highly reproducible RAPD fingerprints, which were used as reliable and useful tool for discrimination of population structure in *G. curmuca* from three geographically separated river systems, *viz.* Periyar, Chalakkudy and Chaliyar Rivers of the Western Ghat region, Kerala, India.

5.4 Comparative analysis of results with three markers in *G. curmuca.*

Allozyme, RAPD and microsatellite markers could be considered as random indicators to discriminate the three populations of the red-tailed barb, *G. curmuca.* 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; Muneer, 2005). 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 sacrificing the specimens.

The percentage of polymorphism obtained using these three markers varied in *G. curmuca*. Several factors contribute to the differences observed in the results produced by the three methods. Some are due to the dominant nature of RAPDs. 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 (Cockerham, 1973; Lynch and Milligan, 1994). In allozymes, only 52.6% of loci were polymorphic, which was less compared with RAPDs and microsatellites (86.0% and 100% respectively). This result can be explained by the fact that the mutation rate of allozymes (functional protein) 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 *G. curmuca* samples than allozyme markers.

Compared with allozymes and RAPDs, microsatellites exhibited a large number of alleles in *G. curmuca* (46 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 (1.66 and 5.25 in allozymes and microsatellites respectively).

In the present study, the heterozygosity value was higher for microsatellites (H_{obs} = 0.5249) than the allozymes (H_{obs} = 0.1514). RAPD showed the value of average gene diversity or heterozygosity (H), in between that of microsatellites and allozymes *i.e.*, 0.1848. Both, allozymes and microsatellites 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) and Muneer (2005), in the populations of brown trout and yellow catfish, and using microsatellites and RAPDs 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})) varied with each marker in *G. curmuca*. The overall F_{ST} was high for RAPD (here G_{ST} = 0.2286) than allozymes (0.0510) and microsatellites (F_{ST}=0.0689 and R_{ST}=0.0729). 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 and Muneer (2005) in yellow catfish from the Western Ghats, India. Similar levels of F_{ST} from both AMOVA and F-statistics using allozyme and microsatellite markers in this study suggest that both sets of allele frequency distributions represent neutral markers in red-tailed barb. 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.*, 1996). Genetic distance values between populations using this battery of markers showed similar pattern in *G. curmuca*. Irrespective of the markers used, the topologies of the UPGMA dendrogram also exhibited similar pattern of genetic divergence in the present study, indicating population structure of this species is entirely consistent with all the three 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; Muneer, 2005).

In bottleneck analysis, allozyme (IAM model) and microsatellite (TPM model) markers in *G. curmuca* exhibited concordant results in both Wilcoxon test and mode shift test. This indicates that the causative factor(s) of the genetic bottleneck had the same effect on the coding (allozymes) and non-coding

(microsatellites) region of DNA. Similar trend has been reported in other cyprinid (*Cirrhinus mrigala*) with these two classes of markers by Chauhan *et al.* (2007).

The three methods in the present study probably might have generated markers pertaining to different parts of red-tailed barb genome. Similarity in genetic divergence values with all the three markers indicated the robustness of the techniques applied; this reinforces reliability of interpretations and confirms existence of three genetically discrete stocks of red-tailed barb. Although the three techniques could clearly discriminate the populations, microsatellites 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 red-tailed barb 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 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; Ali *et al.*, 2004; Muneer, 2005; 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 *Gonoproktopterus curmuca*. The non-denaturing PAGE coupled with silver staining could resolve the alleles of even 2bp difference in the present study.

Finally, the present findings of genetic divergence levels with three marker types in *G. curmuca* suggest that the populations of Periyar, Chalakkudy and Chaliyar Rivers 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 red-tailed barb resource in the Western Ghats region, India. 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 bottleneck analysis). The study emphasizes the need for stock-wise management of natural populations of *G. curmuca*. The stock-wise propagation-assisted rehabilitation should involve brood stock of three rivers (Periyar, Chalakkudy and Chaliyar Rivers) maintained separately. The hatchery-bred progeny will have to be released in three rivers without any chance of mixing of the stocks. Continued screening of microsatellite variation within different populations of *G. curmuca* will further help in monitoring the rehabilitation programme of the species.

Chapter 6

SUMMARY

- A private allele was recorded in allozyme analysis (*LDH-2**; R_f value 78) in Chaliyar River population with an allele frequency of 0.4714.

- 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 *EST-1**, *G_6PDH**, *LDH-2** and *MDH-2** in Periyar population and *EST-4** in Chaliyar 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 genetic differentiation among the populations. F_{ST} for overall populations was 0.0510, indicating that 5.1% genetic variation exists among populations. The pair wise F_{ST} between populations also differed significantly ($P<0.001$) from zero for all the pairs of riverine locations indicating significant heterogeneity between populations. The analysis of molecular variance (AMOVA) using allozyme data also indicated strongly significant genetic differentiation (5.18%) among populations of *G. curmuca*.

- Pair-wise genetic distance values between the populations of Periyar and Chalakkudy Rivers was 0.0.329; between Chalakkudy and Chaliyar Rivers 0.0501; and between Periyar River and Chaliyar River 0.0702. This indicates the Periyar and Chalakkudy populations are closely related compared to Chaliyar 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 *G. curmuca* were established by constructing a UPGMA dendrogram.

- The BOTTLENECK analysis using allozymes (IAM model) indicated clear mode shift of allele diversity in all the populations in contrast to the expected L-shaped distribution. The probability values also indicated significant genetic bottleneck in all populations of *G. curmuca*.

- For **Microsatellite** analysis, forty primers, from six resource species belonging to Order Cypriniformes were used for cross-species amplification in *G. curmuca*. Eight primers out of 40 gave scorable banding pattern after PCR amplifications. These 8 primers produced 9 presumptive microsatellite loci. Among these, 8 loci *viz.*, *CcatG1-1*, *MFW01*, *MFW11*, *MFW19*, *MFW26*, *MFW72*, *Ppro48*, and *Ppro126* were confirmed to contain repeats after sequencing. All the 8 microsatellite loci were polymorphic (100%).

- A total of 46 alleles were produced in microsatellite analysis across all the populations. In Periyar population, the number of observed alleles was 46 whereas in Chalakkudy and Chaliyar populations, 40 and 43 alleles were recorded respectively.

- There were nine private alleles in microsatellites analysis. Out these, four private alleles were observed in Periyar River, two in Chalakkudy River and three in Chaliyar River.

- In Periyar population, the mean observed heterozygosity (H_{obs}) and expected heterozygosity (H_{exp}) values were 0.5148 and 0.6067 respectively. In Chalakkudy River, these values were 0.5360 and 0.5996 respectively and in Chaliyar population, 0.5239 and 0.5619 respectively. 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 *MFW11*, *MFW19*, *MFW72* and *Ppro48* in Periyar River; *MFW01*, *MFW19*, *MFW72*, and *Ppro126* in Chalakkudy population; and *CcatG1-1*, *MFW11*, *MFW19*, *MFW72*, and *Ppro126* in Chaliyar River as a result of heterozygote deficiency. The positive value of F_{IS} at almost all the loci indicated inbreeding in populations of *G. curmuca* in almost all the loci.

- 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 F_{ST} and R_{ST} estimates for overall and pair-wise populations were highly significant ($P<0.0001$) indicating a significant level of genetic differentiation among the populations of *G. curmuca*. The R_{ST} and F_{ST} estimates were very similar indicating that the step-wise mutation model (SMM) has not contributed significantly in population differentiation. The analysis of molecular variance (AMOVA) using microsatellite data also indicated strongly significant genetic differentiation (6.73%) among populations of *G. curmuca*.

- Nei's (1978) unbiased genetic identity and distance were estimated between pairs of three populations of *G. curmuca* 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 set of samples.

- The BOTTLENECK results using microsatellites under two-phased mutation model (TPM) also indicated clear mode shift of allele diversity in all the populations in contrast to the expected L-shaped distribution. The probability values also indicated significant genetic bottleneck in all the three populations.

- In **RAPD** analysis, 31 oligonucleotide decamers were selected from 80 primers (4 kits-20 primers each from kit OPA, OPAA, OPAC and OPAH) in primary screening; however, only 9 primers were selected based on repeatability, sharpness and intensity of bands *viz.*, OPA-15, OPA-16, OPAA-07, OPAA-08, OPAC-05, OPAC-06, OPAH-03, OPAH-17, and OPAH-19 for population genetic analysis. A total of 117 bands were detected consistently with all 9 decamer primers in three populations. The size of the fragments ranged from 0800 to 6500bp. The number of

fragments generated per primer ranged from 04 to 20. Of the 117 RAPD fragments, 52 (44.44%) were found to be shared by individuals of all three populations. The remaining 65 fragments were found to be polymorphic (55.56%). In Periyar River, a total of 88 different fragments were detected; in Chalakkudy River population 82 fragments and in Chaliyar population a total of 72 fragments were detected.

- Forty one RAPD fragments were identified as stock specific markers with 9 primers. Of these, eighteen fragments were exclusive to Periyar population; fifteen fragments specific to Chalakkudy River, and eight fragments were recorded only in Chaliyar population.

- The average gene diversity or heterozygosity (H) was 0.1848 for overall primers. The mean value (H) in Periyar River was 0.0558, in Chalakkudy population, 0.1640; and in Chaliyar River 0.1008. No RAPD locus pairs showed linkage disequilibrium.

- The average value of coefficient of genetic differentiation (G_{ST}) 0.2286 for overall primers among populations was significant ($P<0.05$).

- Nei's (1978) unbiased genetic identity and distance were estimated between pairs of three populations of *G. curmuca*. Based on the genetic distance value, a dendrogram depicting the phylogenetic relationships among three populations of *G. curmuca* were constructed following UPGMA method. The results and pattern of dendrogram were concordant with that obtained from allozyme and microsatellite analyses in this species.

- In **conclusion**, the genetic markers (allozymes, microsatellites and RAPDs) were found to be powerful tools to analyze the population genetic structure of the red-tailed barb, *Gonoproktopterus curmuca*. These three classes of markers demonstrated clear cut genetic differentiation between pairs of 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 and genetic bottleneck revealed by the two co-dominant markers - allozymes and microsatellites. The study emphasizes the need for stock-wise, propagation assisted-rehabilitation of the natural populations of red-tailed barb, *Gonoproktopterus curmuca.*

Chapter 7

CONCLUSION

Contents

7.1 Conservation and management of natural populations of *Gonoproktopterus curmuca* based on the present findings

The goal of this study was to determine whether the natural populations of endangered red-tailed barb, *Gonoproktopterus curmuca* from three riverine systems are genetically distinct. Such information is essential in stock restoration programmes of the species for the safe use of broodstock from various geographic sources for captive breeding. The present study has generated important information on the genetic variation and stock structure of the red-tailed barb, endemic to the Western Ghats, India. Three genetically discrete stocks of the species have been identified for the first time using allozymes, microsatellites, and RAPDs and it is a significant step towards realizing the goal of management of fishery and conservation of populations of this cyprinid 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 a particular habitat. Hence, conservation and fishery management strategy need to be stock-specific. The results of the present study pointed out the need to identify the most suitable conservation and management strategic plans for the genetically distinct populations of endangered *G. curmuca.*

Low genetic variability (heterozygote deficiency and deviation from HWE) coupled with inbreeding (positive value of F_{IS}) was observed in this study in all the three populations of red-tailed barb with different types of genetic markers,

which could be the consequence of genetic bottleneck, resulting from over-exploitation and habitat destruction (Anon., 1998). As these factors would lead to a reduction in reproductive fitness (Padhi and Mandal, 2000) and efforts to increase the genetic diversity of red-tailed barb should be given high priority for conservation of the species. As the populations of *G. curmuca* have already shown signs of genetic bottleneck, hence a rapid action plan for its conservation and rehabilitation may be formulated and implemented at the earliest, 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 or supportive breeding programmes.

G. curmuca is a much sought after ornamental species and the over-fishing is done mainly to meet the increasing demand of the aquarium trade. To support the ornamental trade, emphasis must be given to popularize the breeding and larval rearing techniques that have already been developed by the Regional Agricultural Research Station (RARS), Kerala Agricultural University (KAU), Kumarakam, Kerala. This would not only reduce the

fishing pressure in natural habitats, but also assure continuous supply of same-sized and quality specimens for the aquarium industry.

7.2 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
    a. Imposing ban on fishing practices targeted for red-tailed barb, particularly during breeding seasons.
    b. Stock assessment of *G. curmuca* in different rivers and imposing quota systems for maintaining the population size.
    c. Banning the sale of under-sized / juveniles of red-tailed barb specimens.
    d. Restrict the fishing gear for not catching small and immature barbs and prevent the use of explosives, chemicals for fishing and poisoning.
    e. Maintaining minimum water level in the rivers (in case there are dams and weirs) and declaring certain stretches of rivers as sanctuaries.

7.3 Action plan suggested for 'propagation-assisted, stock-specific restocking' of red-tailed barb with the help of 'supportive breeding programme'

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

- Brood stock of red-tailed barb collected from different rivers must be tagged and maintained in separate ponds in the holding facility.

- The existing farm and hatchery facilities at the state or central government institutes/universities or NGOs can be utilized, for the supportive breeding of stocks of red-tailed barb.
- 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.
- Use of cryopreserved milt, collected from different males and pooled (from the same stock) would be useful for increasing the effective population size and recovery of endangered populations of this barb. 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.
- Different genetic stocks should be bred separately and ranched into respective rivers from where they are collected.
- Stretches of rivers harbouring resident population or that can serve as a potential sanctuary, may be selected for ranching of red-tailed barb populations.
- Assessing the impact of ranching through monitoring the parameters like catch per unit effort / area through experimental fishing.
- Changes in genetic variation *i.e.* allele frequencies especially the occurrence of rare alleles over a course of time may be monitored. 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.

7.4. Current status of *ex-situ* conservation of *Gonoproktopterus curmuca*

- *G. curmuca* collected from wild was successfully bred in captivity for the first time by the RARS, Kumarakom, Kerala in a project funded by the NBFGR. 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 red-tailed barb was developed by the NBFGR - RARS team that gave high hatching rates (86% of control) and larval survival (81% of control). Milt collected from more than 120 healthy males were pooled (population wise), cryopreserved and maintained in the gene bank.
- Other related issues remain to be addressed to fully understand the dynamics of *G. curmuca* include a better understanding of the basic biology, population dynamics and ecology in order to make the link between spawning and feeding grounds and to evaluate spawning waves and associated larval peaks in natural habitats. This should facilitate an analysis of adaptive traits in order to ensure the confident placement of populations of *G. curmuca* into specific genetically structured hierarchies.

In **conclusion,** the markers and stock structure data generated in the present study can provide an essential component for formulating meaningful conservation strategies for red-tailed barb as mentioned above. This, along with the existing protocols on captive breeding and milt cryopreservation can be integrated into a package for conserving genetic diversity and rehabilitation of the natural populations of *Gonoproktopterus curmuca.*

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APPENDICES

1. **Publications**

2. **NCBI accessions submitted**

动物学报 50(4):686 - 690, 2004
Acta Zoologica Sinica

Microsatellite DNA markers to assess population structure of red tailed barb Gonoproktopterus curmuca *

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用微卫星标记评估红尾鲃的种群结构 *

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摘 要 本文检测了三种鲤科鱼的16对微卫星引物在红尾鲃中的适用性，其中6对引物可以成功扩增，且5个位点具有多态性。对采自两条不同河流的标本，通过检测这些多态微卫星位点的遗传变异情况，评估了它们在红尾鲃种群结构分析的适合性。结果显示这5个多态位点在上述两个样本中的平均表观杂合度分别是0.293和0.471。这两个样本显著的基因异质性表明我们所确定的微卫星标记可用于红尾鲃的种内遗传分化研究 [动物学报 50 (4): 686 - 690, 2004]。

关键词 红尾鲃 微卫星 遗传变异
Key words Red tailed barb, *Gonoproktopterus curmuca*, Microsatellite, Genetic variation

Red tailed barb *Gonoproktopterus curmuca* (Hamilton-Buchanan, 1807) is endemic to the rivers originating exclusively from southern part of the Western Ghats in Peninsular India (Gopalakrishnan and Ponniah, 2000). The Western Ghats are recognized as one of the twenty five biodiversity "hotspots" of the world (Myers et al., 2000). *G. curmuca* has commercial value as a food fish as well as for ornamental trade and also considered as a potential species for aquaculture. The sharp decline in abundance of *G. curmuca* and its endangered status is of serious concern (Gopalakrishnan and Ponniah, 2000). For the significance attached to the species, effective conservation and propagation-assisted rehabilitation strategies need to be planned. However, such an approach needs data on the genetic variation and population structure of *G. curmuca* across its natural distribution. To generate population genetics data, identification of polymorphic markers with consistent scorable alleles is a crucial step (Fergusan et al., 1995). Until now, no information is available on any class of genetic markers in *G. curmuca*.

Microsatellites are short tandem repeat motifs with high level of allelic polymorphism and co-dominant inheritance, useful for direct assessment of pattern and distribution of genetic variability at intra specific level (O'Connell and Wright, 1997). The flanking sequences of microsatellites within related taxa are highly conserved. The potential of these markers is enhanced when primers designed for one species amplify homologous loci in other species (Scribner and Pearce, 2000). Successful amplification of homologous microsatellite loci has been demonstrated in some cyprinid fishes (Zheng et al., 1995; Mohindra et al., 2001; Lal et al., 2004). The present study examines cross-species amplification of

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primers, developed for three cyprinids in *G. curmuca*. The objective was to identify polymorphic microsatellite loci and evaluate suitability of the identified loci in population structure analysis of *G. curmuca*.

1 Materials and methods

1.1 Sampling sites and sample collection

The *G. curmuca* specimens were obtained through commercial catches from two rivers, Chalakkudi (Vazhachal, n = 15), Periyar (Bhuthathanketuu, n = 14). The riverine locations were chosen to cover geographically distant populations of *G. curmuca*. The samples were obtained during July 1999 to August 2001 and total length ranged from 200 mm to 600 mm ;collection was done at actual fishing sites. Blood samples, collected through caudal puncture, were fixed in 95 % ethanol (1 :5) and stored at 4 C till use.

1.2 PCR amplification and electrophoresis

Total genomic DNA was extracted from blood samples following the procedure of Ruzzante et al. (1996). PCR amplifications were performed (MJ Research thermal cycler PTC-200) in a final volume of 25 μl, containing 25 - 50 ng of genomic DNA, 1 × PCR buffer (10 mmol Tris-HCl, pH 9.0; 50 mmol KCl; 0.01 % gelatin), 2.0 mmol $MgCl_2$, 0.2 mmol of each dNTP, 5 pmol of each primer and 1.5 units of *Taq* DNA polymerase.

Amplification conditions were 94 C for 5 min followed by 25 cycles at 94 C for 30 s, T_a for 30 s and 72 C for 1 min, with a final extension of 72 C for 4 min. After amplification, 8 μl of PCR products were electrophoresed on non-denaturing polyacrylamide (19 :1 acrylamide bisacrylamide) gels (size 10 cm ×10.5 cm, Amersham Biosciences Ltd.). The gel concentration was optimised according to allele size for better resolution. Electrophoresis was done at 4 C with 1 × TBE buffer for 5 hours at 150 V. The gels were silver stained (Silver Staining Kit, Amersham Biosciences, USA) to visualize microsatellite loci and allelic patterns ;and known DNA size marker (*MspI* cut *pBR*322 DNA) was run in every gel. The size of the amplified products was determined with ID Elite (Amersham Biosciences) software. The alleles with dinucleotide repeats could be resolved and were designated according to PCR product sizes. Genotype of each individual at each locus was assigned manually.

1.3 Screening of primers and genetic diversity analysis

Microsatellite primers from *Cyprinus carpio*, *Barbodes gonionotus* and *Catla catla* were tested for amplification of homologous loci (Table 1). The three species are termed as resource species in the study. This cross-species amplification experiment was done with eight specimens of *G. curmuca*. The optimum annealing temperature to get scorable band pattern was determined through experimental standardization for each primer pair. The primers yielding scorable amplified product were again evaluated with larger sample size (29 individuals, from 2 rivers) to evaluate their suitability in quantification of genetic divergence in *G. curmuca*. The data was analyzed using software Genetix 4.02 (Belkhir et al., 1997) to obtain allele frequencies, mean number of alleles per locus, heterozygosity values, expected (He) and observed (Ho). Tests for conformity to Hardy-Weinberg expectations (probability and score test) were performed by the Markov chain method with parameters dememorization = 1 000, batches = 100 and iteration = 100 (Genepop ver. 3.3, probability test). Genetic homogeneity of four sample sets was determined through an exact test (G based test) that assumes random samples of genotypes (Genepop ver. 3.3, Genotype differentiation test) (Raymond and Rousset, 1995b). This test is performed on genotype tables and possible non-independence of alleles within genotypes will not affect test validity (Raymond and Rousset, 1995c; Goudet et al., 1996).

2 Results

Of the 16 heterologous primer pairs tested, six (23.00 %) provided successful amplification of homologous loci in *G. curmuca* (Table 1). It is evident (Table 2) that the optimum annealing temperature (T_a C) observed in *G. curmuca* differed from that reported in the resource species for respective primer pair. Primer *Bgon*22 amplified but produced monomorphic band in all the individuals tested.

In the present study, five polymorphic microsatellite loci (*MFW*1, 11, 19, 26, *CcatG*1-1), exhibiting 2 to 5 alleles, could be successfully identified for *G. curmuca*. The parameters of genetic variation at each locus and over all loci differed between the two sample sets (Table 3). The observed heterozygosity values over all loci were 0.293 (Chalakkudi) and 0.471 (Periyar). Mean number of alleles per locus ranged from 4.20 (Chalakkudi) to 4.40 (Periyar). The probability test provided the evidence that the observed allele frequencies significantly (P < 0.05) deviated from that expected under Hardy-Weinberg equilibrium. Deviation was observed in both the sample sets, at three to four loci (Table 3) with significant deficiency of heterozogotes. Except at locus *MFW*1 (Chalakkudy samples), the score test also confirmed significant heterozygote deficiency at other three loci.

Significant heterogeneity (P < 0.05) in genotype proportions was observed at three out of five loci

Table 1 Primers of Microsatellite loci tested for cross species amplification in G. curmuca

Species	No. of primer pairs tested	Locus	Genbank accession No.	Reference	Successful primer pair amplified in *G. curmuca* No. (%)
Catla catla	1	*Ccat* G1	AF045380	Naish and Skibinski, 1998	1 (100)
Cyprinus carpio	10	MFW1, 2, 9, 11, 15, 17, 19, 20, 24, 26		Crooijmans et al., 1997	4(20)
Barbodes gonionotus	5	Bgon 22, 69, 75, 79, 17	-	Chenuil et al., 1999	1(20)
Total tested	16				6(23.00)

Table 2 Characteristics of amplified microsatellite loci in G. curmuca

		Resource species			*G. curmuca*	
Species	Locus	Primer sequence(5′→3′)	Repeat motif	T_a(°C)	T_a(°C)	No. of alleles
Cyprinus	*MFW1*	GTCCAGACTGTTCATCAGGAG	CA	55	59	5
carpio		GAGGTGTACACTGAGTCACGC				
	MFW11	GCATTTGCCTTGATGGTTGTG	CA	55	58	5
		TCGTCTGGTTTAGAGTGCTGC				
	MFW19	GAATCCTCCATCATGCAAAC	CA	55	51	6
		CAAACTCCACATTGTGCC				
	MFW26	CCCTGAGATAGAAACCACTG	CA	55	57	4
		CACCATGCTTGGATGCAAAAG				
Catla catla	*CcatG1-1*	AGCAGGTTGATCATTTCTTCC	$[GATA]_n\cdots$	61	51	5
		TGCTGTGTTTCAAATGTTCC	$\cdots[CCA]_n$			
Barbodes	*Bgon 22*	TCTTGTTGATCACACGGACG	CCT		55	1
gonionotus		ACAGATGGGGAAAGAGAGCA				

Table 3 Parameters of genetic variability for each microsatellite locus in G. curmuca samples from two locations

Locus homogeneity	River	Size range(bp)	No. of alleles at each locus	Ho	He	HW(p)	Genotype(p)
MFW1	Ch	175 - 190	4	0.333	0.571	0.0029*	0.1185
	Pr	175 - 195	5	0.714	0.745	0.1391	
MFW11	Ch	180 - 201	5	0.267	0.718	0.0003*	0.5254
	Pr	180 - 201	5	0.143	0.704	<0.0001*	
MFW19	Ch	215 - 220	3	0.200	0.487	0.0101**	0.0005*
	Pr	201 - 225	6	0.786	0.791	0.0199**	
MFW26	Ch	147 - 160	4	0.267	0.649	0.0005*	0.0008*
	Pr	147 - 157	3	0.286	0.582	0.0020*	
CcatG1-1	Ch	185 - 201	5	0.400	0.660	0.0319**	0.0370**
	Pr	190 - 201	4	0.429	0.599	0.0204**	
Mean over Prall loci	Ch		4.20	0.293	0.617		<0.0001*
	Pr	-	4.40	0.471	0.684	-	

(Ch = Chalakudy, Pr = Periyar). The observed (Ho) and Hardy-Weinberg expected (He) heterozygosity values with associated probability (p); probability (p) of genotype homogeneity between samples is given. Significant probability values are marked, ** $P<0.05$, * Critical probability level adjusted for sequential bonferroni correction.

(Table 3). After the sequential bonferroni correction ($P<0.0071$) was made to the probability levels, still two loci (*MFW*19 and 26) exhibited significant heterogeneity. Combined probability over all the loci was less than 0.0001 (Table 3). G_{st} for small sample size (Nei and Chesser, 1983) of 0.049 provided further evidence of population substructuring *in G. curmuca*.

3 Discussion

The study demonstrates successful cross-priming of microsatellite loci in red tailed barb, *G. curmuca* and identified five polymorphic loci. The result is consistent with the earlier reports, suggesting the possibility of using primers interspecifically among cyprinids (Zheng et al., 1995). Mohindra et al. (2001) demonstrated amplification of homologous microsatellite locus in *Labeo rohita* using primer developed for other cyprinid *Catla catla*. Successful identification of polymorphic microsatellite markers for *Cirrhinus mrigala* was achieved through use of primers of other cyprinid fishes (Lal et al., 2004).

The presence of null alleles could be one of the possible factors responsible for the observed heterozygote deficiency. Null alleles are not represented in PCR amplification due to mutation at primer binding site and contribute towards homozygote excess (Paetkau and Strobeck, 1995). Raymond and Rousset (1995a) suggested the score test was more powerful than the probability test when the alternate hypothesis of interest is heterozygote deficiency. Interestingly, except at locus *MFW*1 (Chalakkudy samples), the results of the score test were consistent with the probability test. This may not be a conclusive interpretation for the absence of null alleles, however it suggests the likelihood of deficiency of heterozygotes at least at three loci in both populations. If true, a serious concern is that the assumptions underlying the Hardy-Weinberg equilibrium relevant to natural population of *G. curmuca* are violated (Ferguson et al., 1995). One of the reasons could be reduction in effective breeding population size in *G. curmuca* possibly due to overexploitation, restricted migrations and habitat alterations, etc.

Genetic heterogeneity was tested on the tables based on the genotype rather than allele frequencies in view of the observed nonconformity to Hardy-Weinberg expectations (Raymond and Rousset, 1995c; Goudet et al., 1996). The various estimates provided strong evidence that the two sample sets were not drawn from the same random mating gene pool. Analysis of larger sample sizes from more geographical locations will provide fine scale assessment of population structure of *G. curmuca* and also more insight into the observed homozygote excess. In the given situation, cautious use of the identified loci is suggested. The method of estimating null allele frequencies (Brookfield, 1996) may help in deriving appropriate conclusions.

In conclusion, the present study identified five polymorphic microsatellite loci that exhibit promise to determine genetic divergence in natural populations of *G. curmuca*. This will also provide monitoring mechanism against the possible genetic bottlenecks; the populations may be facing and help to plan strategy for rehabilitation of declining natural resources.

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Short Communication

Primers from the orders Osteoglossiform and Siluriform detect polymorphic microsatellite loci in sun-catfish, *Horabagrus brachysoma* (Teleostei: Bagridae)

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Summary

Horabagrus brachysoma (sun-catfish, Bagridae, Siluriformes) is a valuable ornamental and food fish. The stock structure of *H. brachysoma*, necessary to conserve its declining natural populations, is not known. Twenty-five primers developed for four fish species belonging to the orders Siluriform (3) and Osteoglossiform (1) were tested and eight primers amplified microsatellite loci in *H. brachysoma*. The results demonstrate that cross-priming between fish species belonging to different families and even to different orders can yield microsatellite loci. Five of eight primers each amplified two loci. However, the loci that had repeat motifs after sequencing were considered only for genotyping. Finally, eight loci were polymorphic with three to seven alleles. Individual fish genotype data (n = 42; 21 each in two rivers) at each locus was analysed. Significant genetic heterogeneity was detected at six loci. The identified loci exhibited potential for use in population genetics application in *H. brachysoma*.

Horabagrus brachysoma is endemic to the rivers of Western Ghats in Peninsular India. The fish is endangered through exploitation as an ornamental and food fish, which has led to a decline in natural abundance (Gopalakrishnan and Ponniah, 2000). Microsatellites are among the most useful markers for detecting genetic variation and provide options of using the primers developed for one species to amplify loci in other related species (review by Scribner and Pearce, 2000). A survey of 161 species (http://www.absc.usgs.gov/research/genetics/heterologous_primers.htm) across taxonomic groups indicates limited reports on use of primers between families (31, polymorphism = 17) and orders (4, polymorphism = 1). Microsatellite loci conserved between families and orders can be useful in evolutionary studies and in generating population genetics data for a wide range of species.

Primers developed for four fish species (resource species) from the orders Siluriform (3) and Osteoglossiform (1) were examined to amplify microsatellite loci in sun-catfish (Table 1). The study aim was to identify polymorphic microsatellite loci and assess their suitability for population structure analysis of *H. brachysoma*.

Specimens of yellow catfish *H. brachysoma* were obtained from commercial catches of the Chalakkudy and Nethravati rivers. The blood samples, extracted through caudal puncture, were stored in 95% ethanol. Total DNA was extracted following the procedure of Ruzzante et al., 1996. A cross-priming experiment was performed on eight specimens (n = 4/river). Procedures for PCR reaction, electrophoresis and genotyping were followed as described in Lal et al. (2004). The optimum annealing temperature to obtain a scorable band pattern was determined through experimental standardization for each primer pair.

Eight primer pairs exhibited amplification in *H. brachysoma* (Table 1). The optimum annealing temperature to obtain scorable band patterns in *H. brachysoma* differed from that reported for the respective primer pair in resource species (Table 2). The study demonstrated successful cross-priming of microsatellite loci between fish species that are distantly or not related. Certain sequences flanking the tandem repeats could be conserved between the various families of the order Siluriform. Interestingly, some microsatellite sequences from the primitive order osteoglossiform have remained conserved in the order Siluriform, of relatively later evolutionary origin.

The eight primers amplified 13 scorable loci (Table 2). The additional five loci came from the primers Phy07, Cma4, Cga06, D33, and D38 that amplified two loci each. Before using for genotyping, it was essential to confirm whether both loci for these primers had repeat motifs. Following the sequence observations, the eight loci (Phy01, Phy05, Phy07-1, Cma3, Cma4, Cga06-1, D33-2 and D38-1) that confirmed the presence of repeat motifs were used for genotyping (Table 2); these loci were polymorphic, with three to seven alleles. To assess genetic variation, 42 samples (n = 21/river) were individually genotyped at each locus. Genetix 4.05 (Belkhir et al., 1997) was used to obtain observed (Ho) and expected (He) heterozygosities at each locus, mean overall loci, and mean number of alleles per locus for the two sample sets (Table 3). Deviations from the Hardy–Weinberg Equilibrium and linkage disequilibrium were tested using Markov chain approximation in GENEPOP 3.4 (Raymond and Rousset, 1995). The probability of conformity to the Hardy Weinberg expectations indicated significant deviation ($P < 0.05$) in both samples after the Bonferroni correction was applied to the probability levels (Table 2). Positive Fis values at these loci revealed the heterozygote deficiency. There was no evidence of linkage disequilibrium.

Genetic homogeneity was tested based on genotype (GENEPOP 3.4; Raymond and Rousset, 1995) and the combined probability over all loci indicated significant divergence between the two sample sets. With sequential Bonferroni correction made to the probability levels, six loci exhibited significant ($P < 0.0063$) heterogeneity (Table 3). G_{st} for small sample size (Genetix 4.05; Belkhir et al., 1997) was 0.0752.

Table 1
Primers of microsatellite loci tested for cross-priming in *Horabagrus brachysoma*

Species	Tested (no.)	Loci	GenBank accession no.	Reference	Amplified in *H. brachysoma* no.(%)
Order Siluriform					
Pangasius hypophthalmus (Pangasidae)	4	Phy 01, 03, 05, 07	AJ131380 AJ131381 AJ131382 AJ131383	Volckaert et al., 1999	3 (75.00)
Clarias macrocephalus (Claridae)	4	Cma 01,02,03,04		Na-Nakorn et al., 1999	2 (50.00)
Clarias gariepinus (Claridae)	7	Cga 01, 02,03,05,06,09,10		Galbusera et al., 1996	1 (14.20)
Order Osteoglossiform					
Scleropages formosus (Osteoglossidae)	10	D11,13,14,16,33,35,37,38,42,72	AF219953 AF219954 AF219955 AF219957 AF219961 AF219962 AF219963 AF219964 AF219965 AF219966	Yue et al., 2000	2 (20.00)
Total	25				8 (32.00)

Table 2
Annealing Temp. (Ta°C) in resource species (Res. sp.) and optimized for *H. brachysoma*: Parameters of genetic variability at individual and mean over all loci in Chalakkudi (Ch) and Nethravati (Ne) samples: agreement to HW expectations (P) and genic homogeneity (P). Significant *(P < 0.05) **(After Sequential Bonferroni correction. Lessios, 1992)

Locus	Res. sp. *H. brachysoma*	Ta°C	River	Size range (bp)	No. of alleles/locus	Ho	He	HW (P)	Genic homogeneity (P)
Phy01	65	56	Ch	162–196	7	0.909	0.793	0.0836	< 0.0001**
			Ne	162–190	6	0.619	0.632	< 0.0830*	
Phy05	60	55	Ch	146–170	6	0.667	0.774	0.0743	0.0015**
			Ne	146–166	5	0.714	0.703	0.1381	
Phy07-1	68	55	Ch	270–285	4	0.191	0.588	0.0003**	0.0360
			Ne	270–285	4	0.143	0.761	0.0001**	
Cma3	50	45	Ch	147–170	7	0.571	0.744	0.0070*	< 0.0001**
			Ne	147–166	6	0.524	0.747	0.0030**	
Cma4-2	48	56	Ch	172–182	4	0.286	0.516	0.0250*	0.0380*
			Ne	172–182	4	0.238	0.398	0.0465*	
Cga06		57	Ch	218–244	5	0.524	0.641	0.0380*	< 0.0001**
			Ne	218–244	5	0.567	0.634	0.0080*	
D33-2	55	53	Ch	192–212	3	0.238	0.289	0.4483	0.0011**
			Ne	200–212	3	0.667	0.559	0.0146*	
D38-1	50	55	Ch	252–310	4	0.905	0.653	0.0070*	0.0000**
			Ne	252–295	3	0.810	0.602	0.4733	
Mean overall loci			Ch		4.875	0.536	0.614		< 0.0001**
			Ne		4.375	0.589	0.623		

The various estimates provided evidence that genetic variation detected at the identified microsatellite loci can be significant in determining the population structure of *H. brachysoma*.

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Allozyme Variation in a Threatened Freshwater Fish, Spotted Murrel (*Channa punctatus*) in a South Indian River System

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Samples of the spotted murrel (Channa punctatus) *were collected from three rivers of Tamil Nadu and Kerala. The allozyme variation of* C. punctatus *was investigated by polyacrylamide gel electrophoresis. Eighteen enzymes were detected, but only 10 (EST, PGM, G3PDH, G6PDH, SOD, GPI, ODH, GDH, XDH, and CK) showed consistent phenotypic variations. Allele frequencies were estimated at the 18 polymorphic loci representing 10 enzymes. Two rare alleles, EST-4*C and G6PDH-2*C, were noted in the Tamirabarani and Kallada populations but were absent in the Siruvani population. The allele frequencies of the Tamirabarani and Kallada populations were similar, except for a few loci. Among the three populations, the maximum genetic distance (0.026) and* F_{ST} *(0.203) were found between the geographically distant Siruvani and Kallada populations. Overall the study showed that among the three populations, the Tamirabarani and Kallada have similar genetic structures.*

KEY WORDS: *Channa punctatus*; allozyme; population; heterozygosity.

INTRODUCTION

The spotted murrel, *Channa punctatus* (Bloch, 1793), commonly called the snakehead, is an important freshwater, food fish of Southeast Asia. It is found in rivers,

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ponds and lakes of India, Sri Lanka, Pakistan, Nepal, Bangladesh, Myanmar, Malaysia, China, Tahiti, and Polynesia (Jayaram, 1981). It has been identified as having potential for aquaculture in derelict and swampy water, since it is an air-breathing fish. It commands good consumer preference due to taste, high protein content and very few intramuscular spines (Haniffa *et al.*, 2002). It breeds naturally during southwest and northeast monsoons in India. Parameswaran and Murugesan (1976) reported that induced-bred murrels never exhibited parental care, but we found breeding behavior and parental care under induced breeding conditions using different ovulating agents (Haniffa *et al.*, 2004). The fecundity of this species is very low (3000 to 4000) compared with the major Indian carps, but it varies with the size of the fish.

Over the last 10 years, its wild population has declined steadily, mainly because of overexploitation, loss of habitat, introduction of alien species, disease, pollution, siltation, poisoning, dynamite, and other destructive fishing (CAMP, 1998). As a result, *C. punctatus* is listed among 66 low-risk, near-threatened fish species of India, according to IUCN status (CAMP, 1998). Information on the genetic structure of cultivable fish is necessary for optimizing identification of potential broodstock, stock enhancement, selective breeding programs, management for sustainable yield, and conservation of biodiversity. Previous studies have provided detailed knowledge of the length-to-weight relationship (Haniffa *et al.*, 2006), embryology and development (Haniffa *et al.*, 2002), biochemical composition (Singh and Singh, 2002; Sehgal and Goswami, 2001), hematology (Pandey *et al.*, 1981), courtship behavior (Haniffa *et al.*, 2004), and breeding (Haniffa and Sridhar, 2002) for this species. Basic knowledge of the levels of genetic variation within and among the populations is poorly represented, although a very few studies have been made on *C. punctatus*. Rishi *et al.* (2001) studied the LDH polymorphism of *C. punctatus* populations sampled from three natural bodies of water at Haryana (India), and Nabi *et al.* (2003) studied the genetic structure of *C. punctatus* populations collected from locations on the Rohilkhand plains of India using transferrin as a marker. In our previous study we analyzed the genetic variability of three *C. punctatus* populations using RAPD markers (Nagarajan *et al.*, 2006). In this study, we used allozymes to investigate the genetic variability of three *C. punctatus* populations collected from three south Indian rivers.

MATERIALS AND METHODS

Fish Sampling

The majority of the rivers in the states of Tamil Nadu and Kerala originate from the Western Ghats, a mountain range in India that starts south of the Tapti River near the border of Gujarat and Maharashtra and goes approximately 1600 km through the states of Maharashtra, Goa, Karnataka, Kerala, and Tamil Nadu to

Kanyakumari at the southern end of the Indian peninsula. Recognized as one of the 21 biodiversity hotspots of the world, it harbors a rich and diversified fish fauna characterized by many rare and endemic species. In the present study we collected *C. punctatus* samples from three rivers of the Western Ghats, the Siruvani, Tamirabarani, and Kallada. The Tamirabarani River originates from the peak of the Periya Pothigai hills of the Western Ghats at Thirunelveli District (8.4°N, 77.44°E, Thirunelveli, Tamil Nadu). The Kallada River enters Kerala and runs into the Quilon and nearby districts (8.54°N, 76.38°E, Quilon, Kerala; Fig. 1). The Siruvani River originates about 500 km away from the Tamirabarani and Kallada rivers in the Western Ghats (11.00°N, 77.00°E, Coimbatore, Tamil Nadu). Of the three rivers, the Tamirabarani and Kallada have a rich fish biodiversity (Martin *et al.*, 2000; Kurup *et al.*, 2004). From each population, 60 fish samples were used for the present study. Liver tissues were dissected from the fish and were immediately stored at −80°C prior to analysis.

Extract Preparation

Adequate portions (250 mg) of liver tissue were first minced and homogenized using a glass homogenizer under cold condition. A buffer solution containing sucrose (50%), 0.2 M Tris HCl (pH 7.2), EDTA (64 mg/100 mL) and double-distilled water was used as homogenizing medium in selected proportions to the sample weight (250 mg/mL). The homogenates were then centrifuged at 12,000 rpm for 1 h at 4°C. The supernatant was again spun at 12,000 rpm for 40 min. After the second centrifugation, the supernatant was collected and used for further analyses.

Electrophoresis and Staining

Vertical polyacrylamide gel electrophoresis was used for the separation of allozymes at different enzyme loci. Gels consisted of 3.9% acrylamide and 3.36% bis-acrylamide, and electrophoresis was run at 30 mA and 150 V at 4°C with Tris-Boric acid-EDTA buffer (pH 8.0). The bands of each enzyme were revealed by incubating the gels in the dark at 37°C in the presence of specific histochemical staining solution until sharp bands were visualized. The locus and allele designations were followed according to the standardized genetic nomenclature for protein-coding loci (Shaklee *et al.*, 1990).

Statistical Analysis

Genetic variation between the populations was assessed by the following standard measures; number of polymorphic loci (P), allele frequency, observed heterozygosity per locus (H_o), expected heterozygosity per locus (H_e), fixation index (F_{IS}),

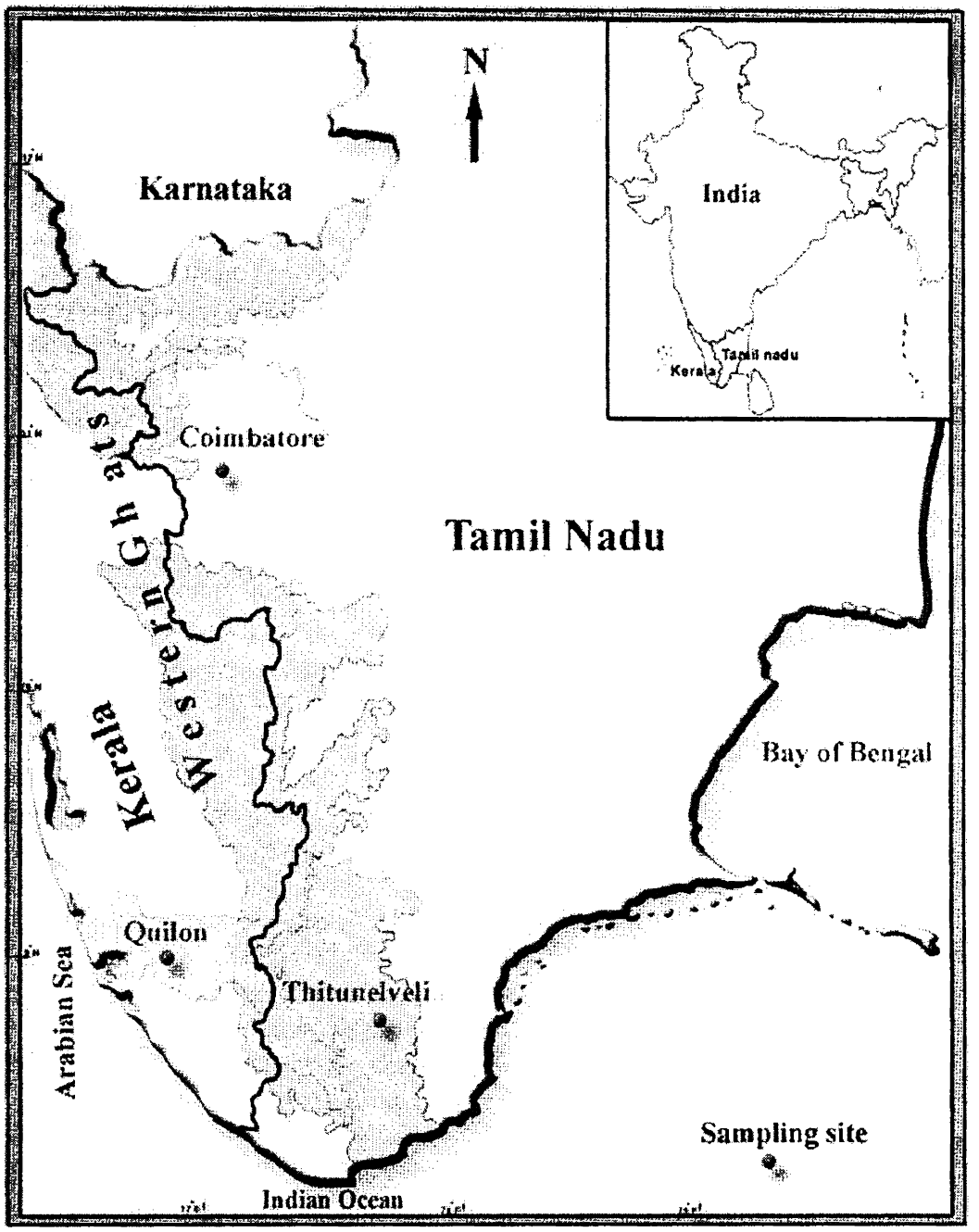

Fig. 1. Sampling sites of *C. punctatus* populations used in this study.

genetic diversity (F_{ST}) for the overall populations, and Nei's genetic distance (D_N) using the program PopGene 1.31 (Yeh *et al.*, 1999). Deviations from Hardy–Weinberg equilibrium of each locus for each population were tested by the Markov chain method of exact probability test using the program GenePop 3.3 (Raymond and Rousset, 1995). The *P* values were corrected using the Bonferroni correction

(Rice, 1989). AMOVA (analysis of molecular variance) and population pairwise F_{ST} were computed using the program Arlequin (Schneider *et al.*, 2002). Isolation by distance (IBD) between populations was confirmed by Mantel's tests (Mantel, 1967) using the program IBD 2.1 (Jensen *et al.*, 2005).

RESULTS

In this study, we checked for 18 enzymes in *C. punctatus*, but only 16 showed their presence in liver samples. The enzymes that could not be detected were aspartate amino transferase (AAT) and hexokinase (HK). Of the 16 enzymes detected, 12 showed phenotypic variations and 4 were monomorphic: glutamate dehydrogenase (GLUDH), adenylate kinase (AK), malic enzyme (MEP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Of the 12 that showed variation, lactate dehydrogenase (LDH) and isocitrate dehydrogenase (ICDH) exhibited inconsistent phenotypic patterns. The remaining 10 showed consistent phenotypic variation and were therefore useful for genetic analysis: EST, PGM, G3PDH, G6PDH, SOD, GPI, ODH, GDH, XDH, and CK. They are coded by 27 putative loci (Table I). A total of 38 alleles were detected from the 18 polymorphic loci of 10 enzyme systems, and their frequencies are presented in Table II. Of the 18 polymorphic loci, *EST-4** and *G6PDH-2** contained three alleles; the others had two alleles. The *EST-4** and *G6PDH-2** loci showed the allele C in very low frequencies, *EST-4** in the Tamirabarani population and *G6PDH-2** in Kallada, but they were absent in the Siruvani population. The allele frequencies were very similar in the Tamirabarani and Kallada populations. The frequency of the *ODH-1**, *GDH-2**, *EST-4**, and *G6PDH-2** loci alleles in the Siruvani population was significantly different from that of the Tamirabarani and Kallada populations.

The observed heterozygosity ranged from 0.194 in the Siruvani population to 0.236 in the Tamirabarani population. The expected heterozygosity was 0.262 in Siruvani and 0.327 in Kallada (Table III). Of the 38 polymorphic loci from the three populations, 31 showed Hardy-Weinberg equilibrium, and 7 deviated significantly after the Bonferroni corrections (Rice, 1989). These statistically significant values were produced at *PGM-3** and *GDH-3** in the Siruvani population, *ODH-2**, *GDH-2**, and *XDH-2** in the Tamirabarani population, and *GDH-1** and *GDH-3** in the Kallada population. In each of the statistically significant cases, the fixation index (F_{IS}) was very high, indicating significant heterozygote deficiency at the population level. The mean F_{IS} per population was 0.262, ranging from 0.226 in the Tamirabarani population to 0.301 in the Kallada population (Table IV). The F_{ST} for the overall population ranged from 0.007 in *PGM-2** to 0.068 in *ODH-1**, with a mean of 0.028, indicating that about 2.8% of the total genetic variation exists between populations due to population differentiation (Table IV). AMOVA analysis revealed 12.46% variation among the populations (Table V). We obtained 13.55% variation when we combined the Tamirabarani and Kallada populations

Table I. Allozymes Screened in *Channa punctatus*

S. no.	Enzyme	Abbreviation and enzyme code	Subunit structure	Locus	Monomorphic or Polymorphic
1	Esterase	EST 3.1.1	Monomeric	*EST-1**	Monomorphic
				*EST-2**	Monomorphic
				*EST-3**	Monomorphic
				*EST-4**	Polymorphic
				*EST-5**	Polymorphic
2	Phosphoglucomutase	PGM 5.4.2.2	Monomeric	*PGM-1**	Polymorphic
				*PGM-2**	Polymorphic
				*PGM-3**	Polymorphic
3	Glycerol-3-phosphate dehydrogenase	G3PDH 1.1.1.8	Dimeric	*G3PDH**	Polymorphic
4	Glucose-6-phosphate dehydrogenase	G6PDH 1.1.1.49	Dimeric	*G6PDH-1**	Monomorphic
				*G6PDH-2**	Polymorphic
5	Superoxide dismutase	SOD 1.15.1.1	Dimeric	SOD-1*	Polymorphic
				*SOD-2**	Polymorphic
6	Glucose-6-phosphate isomerase	GPI 5.3.1.9	Dimeric	*GPI**	Polymorphic
7	Octanol dehydrogenase	ODH 1.1.1.73	Dimeric	*ODH-1**	Polymorphic
				*ODH-2**	Polymorphic
				*ODH-3**	Monomorphic
8	Glucose dehydrogenase	GDH 1.1.1.47	Monomeric	*GDH-1**	Polymorphic
				*GDH-2**	Polymorphic
				*GDH-3**	Polymorphic
9	Xanthine dehydrogenase	XDH 1.1.1.204	Dimeric	*XDH-1**	Monomorphic
				*XDH-2**	Polymorphic
				*XDH-3**	Polymorphic
10	Creatine kinase	CK 2.7.3.2	Dimeric	*CK-1**	Polymorphic
				*CK-2**	Monomorphic
				*CK-3**	Monomorphic
				*CK-4**	Monomorphic

as one group to compare with the Siruvani population. There was only 2.87% variation between the Tamirabarani and Kallada populations (interpopulational variation). Among the three populations, the maximum genetic distance and F_{ST} were found between the Siruvani and Kallada populations (Table VI). We checked for a correlation between the geographic distance and corresponding F_{ST} value for the three populations using the IBD program. A significant positive correlation was obtained ($r = 0.59$, $P < 0.02$), confirming that these three populations are in isolation by distance. The overall analysis indicated that the Tamirabaran population is genetically closer to the Kallada population, whereas the Siruvan population is closer to the Tamirabarani population.

Table II. Estimated Allele Frequency at 18 Polymorphic Loci in Three Populations of *C. punctatus*

Locus	Allele	Siruvani	Tamirabarani	Kallada
EST-4	A	0.833	0.589	0.544
	B	0.167	0.394	0.428
	C	0.000[a]	0.017[b]	0.028[b]
EST-5	A	0.739	0.544	0.522
	B	0.261	0.456	0.478
PGM-1	A	0.650	0.556	0.511
	B	0.350	0.444	0.489
PGM-2	A	0.639	0.556	0.544
	B	0.361	0.444	0.456
PGM-3	A	0.650	0.600	0.578
	B	0.350	0.400	0.422
G3PDH-1	A	0.689	0.611	0.544
	B	0.311	0.389	0.456
G6PDH-2	A	0.672	0.533	0.567
	B	0.328	0.439	0.411
	C	0.000[a]	0.028[b]	0.022[b]
SOD-1	A	0.678	0.600	0.522
	B	0.322	0.400	0.478
SOD-2	A	0.7000	0.861	0.678
	B	0.3000	0.139	0.322
GPI-1	A	0.717	0.583	0.544
	B	0.283	0.417	0.456
ODH-1	A	0.833[a]	0.556[b]	0.589[b]
	B	0.167	0.444	0.411
ODH-2	A	0.833	0.806	0.611
	B	0.167	0.194	0.389
GDH-1	A	0.800	0.639	0.589
	B	0.200	0.361	0.411
GDH-2	A	0.822[a]	0.733[a,b]	0.567[b]
	B	0.178	0.267	0.433
GDH-3	A	0.783	0.639	0.567
	B	0.217	0.361	0.433
XDH-2	A	0.672	0.828	0.689
	B	0.328	0.172	0.311
XDH-3	A	0.750	0.589	0.511
	B	0.250	0.411	0.489
CK-1	A	0.667	0.533	0.533
	B	0.333	0.467	0.467

Note. Values with different superscripts (a,b) in the same row are significantly different ($P < 0.05$).

DISCUSSION

The genetic variability in the three natural populations of *C. punctatus* was evident in this study using allozyme markers. The allozyme allele frequency of the Siruvani population was significantly different from that of the Tamirabarani and Kallada populations at *ODH-1**, *GDH-2**, *EST-4**, and *G6PDH-2** loci, but the Tamirabarani and Kallada populations were closely similar. The variation in allele frequency in the populations can be due to environmental factors such as

Table III. Genetic Variability Estimates of Three *C. punctatus* Populations

S. No	Parameter	Siruvani	Tamirabarani	Kallada
1	Sample size	60	60	60
2	Number of loci found	27	27	27
3	Number of polymorphic loci	18	18	18
4	Observed heterozygosity	0.194 ± 0.152	0.236 ± 0.195	0.227 ± 0.176
5	Expected heterozygosity	0.262 ± 0.196	0.300 ± 0.226	0.327 ± 0.237
6	Observed number of alleles	1.667 ± 0.480	1.741 ± 0.594	1.741 ± 0.594
7	Effective number of alleles	1.439 ± 0.342	1.561 ± 0.444	1.6388 ± 0.465

Table IV. Fixation Index for Polymorphic Loci in Three *C. punctatus* Populations

	Fixation Index (F_{IS})			
Locus	Siruvani	Tamirabarani	Kallada	Overall F_{ST}
EST-4	0.300	0.196	0.316	0.0086
EST-5	0.107	0.194	0.286	0.0395
PGM-1	0.292	0.280	0.378	0.0137
PGM-2	0.254	0.280	0.373	0.0073
PGM-3	0.603*	0.398	0.180	0.0204
G3PDH-1	0.119	0.018	0.462	0.0147
G6PDH-2	0.269	0.234	0.258	0.0118
SOD-1	0.289	0.028	0.198	0.0168
SOD-2	0.101	0.303	0.135	0.0352
GPI-1	0.261	0.017	0.194	0.0230
ODH-1	0.120	0.190	0.449	0.0683
ODH-2	0.120	0.539*	0.252	0.0521
GDH-1	0.236	0.061	0.633*	0.0370
GDH-2	0.316	0.546*	0.186	0.0542
GDH-3	0.575*	0.013	0.548*	0.0363
XDH-2	0.118	0.493*	0.119	0.0247
XDH-3	0.378	0.082	0.244	0.0419
CK1	0.200	0.196	0.196	0.0162
Mean	0.259	0.226	0.301	0.0281

*Locus deviates significantly from Hardy-Weinberg equilibrium after Bonferroni correction.

Table V. AMOVA of Three *C. punctatus* Populations

	Percentage of variation*			
Source of variation	No grouping	Two groups[a]	Two groups[b]	Two groups[c]
Among groups	12.46	13.55	−1.76	−12.98
Among populations within groups	—	2.87	13.71	21.66
Within populations	87.54	83.58	88.05	91.32

[a]Tamirabarani + Kallada versus Siruvani.
[b]Tamirabarani + Siruvani versus Kallada.
[c]Tamirabarani versus Kallada + Siruvani.
*$P < 0.01$.

Table VI. Genetic Distance and Genetic Diversity Between *C. punctatus* Populations

Population	Siruvani	Tamirabarani	Kallada
Siruvani	—	0.152	0.203
Tamirabarani	0.017	—	0.027
Kallada	0.026	0.007	—

Note. Below diagonal, Nei's unbiased genetic distance. Above diagonal, F_{ST} genetic diversity value.

temperature, alkalinity, and pollution (Ponniah, 1989). The role of temperature in maintaining alleles at different frequencies has been proved in natural populations (Nyman and Shaw, 1971) and experimentally (Mitton and Koeh, 1975). Two rare alleles, *G6PDH-1*C* and *EST-4*C*, were found in the Tamirabarani and Kallada populations with low frequencies, though sample size was sufficient. This represents an inherent genetic stock difference between the Siruvani and the Tamirabarani/Kallada populations (Levy and Neal, 1999). The rare alleles can be utilized as genetic markers for selection of a candidate stock for controlled breeding programs (Lester and Pante, 1992). Significant deviations from Hardy-Weinberg expectations were found at *XDH-2**, *G3PDH-1**, and *GDH-3** in Siruvani; *XDH-1**, *ODH-1**, and *PGM-1** in Tamirabarani; and *SOD-1* in Kallada because of an excess of homozygotes. The excess of homozygotes is also confirmed by positive fixation index values (Table IV). Homozygote excess for allozyme has been reported quite commonly in many fish species (Engelbrecht and Mulder, 2000; Steenkamp *et al.*, 2001). Several hypotheses have been mentioned to explain homozygote excess in fish species, including inbreeding, population admixture (Wahlund effect), or the presence of a nonexpressed allele (Appleyard *et al.*, 2001; Ward *et al.*, 2003).

The population structure of freshwater organisms is primarily dependent on the distribution of the river systems, as has been reported by several authors (Ikeda *et al.*, 1993; Hara *et al.*, 1998). The present study also showed a significant correlation between genetic distance and geographic distance, confirmed by the Mantel test. The three populations used in the present study were collected from three different rivers, all of which originate in the Western Ghats. The Tamirabarani and Kallada rivers originate at the south end of the Western Ghats and are geographically closer to each other than to the Siruvani, which originates more than 500 km away from the other rivers. The results of this study consistently showed that the Tamirabarani and Kallada populations were genetically closer to each other, compared with the Siruvani population. It supports the concept that genetic differentiation is primarily dependent on geographic isolation. Theoretically, the result of the present study shows a certain level of gene flow between the Tamirabarani and Kallada populations when compared with the Siruvani population. In fact,

natural interbreeding between the Tamirabarani and Kallada populations was impossible, and there has been no record of transplantation of *C. punctatus* between the two rivers. It is likely that these two populations have come from a single stock introduced into these two rivers in the past, or migration or transportation between the two rivers could be a possibility.

In conclusion, allozyme analysis revealed, as did the RAPD markers used by Nagarajan *et al.* (2006), that of the three *C. punctatus* populations, the Tamirabarani and Kallada populations have similar genetic structures. The estimated values of average number of alleles, percentage of polymorphic loci, and heterozygosity for populations are considered indicators of the actual level of genetic variability of the species. Genetic variability data may also be used as a tool by an aquaculturist for stock selection for selective breeding programs. The present study provides basic information about the genetic structure of these three populations that can be used to improve the quality of the populations by selective breeding or out-breeding programs, and it would also help to conserve the population. Microsatellite markers, however, would be better suited than isozyme and RAPD analysis to detect population bottlenecks and losses of variation due to inbreeding, with allele richness being a more sensitive variability measure than mean heterozygosity (Bentzen *et al.*, 1996; Wright and Bentzen, 1994). Hence, further research on microsatellite markers for these populations is in progress.

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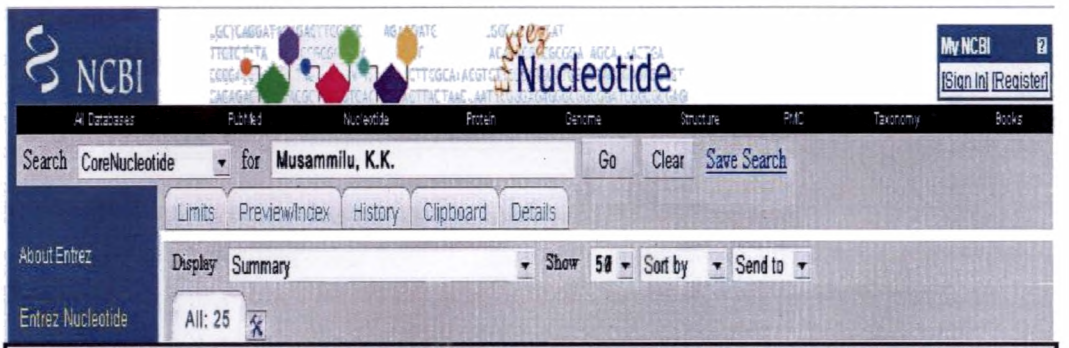

Microsatellite sequences (25 nos.)		
Sl. No.	**Accession Numbers**	**Details**
1	**DQ780014**	Gopalakrishnan, A., **Musammilu, K.K.**, Abdul Muneer, P.M., Mohindra, V., Lal, K.K., Basheer, V.S., Punia, P. and Lakra, W.S. *Gonoproktopterus curmuca* clone Gcur MFW01 microsatellite sequence. 182bp linear DNA; 01-AUG-2006
2	DQ780015	Mohindra, V., **Musammilu, K.K.,** Gopalakrishnan, A., Abdul Muneer, P.M., Lal, K.K., Basheer, V.S., Punia, P. and Lakra,W.S. *Gonoproktopterus curmuca* clone Gcur G1 microsatellite sequence. 240bp linear DNA; 01-AUG-2006.
3	DQ780016	Gopalakrishnan, A., Abdul Muneer, P.M., **Musammilu, K.K.,** Mohindra, V., Lal, K.K., Basheer, V.S., Punia, P. and Lakra,W.S. *Horabagrus brachysoma* clone Hbr Cga06 microsatellite sequence. 192bp linear DNA; 01-AUG-2006.
4	DQ780017	Mohindra, V., Abdul Muneer, P.M., **Musammilu, K.K.,** Gopalakrishnan, A., Lal, K.K., Basheer, V.S., Punia, P. and Lakra,W.S. *Horabagrus brachysoma* clone Hbr Cma03 microsatellite sequence. 166bp linear DNA; 01-AUG-2006.
5	DQ780018	Mohindra, V., Abdul Muneer, P.M., **Musammilu, K.K.,** Gopalakrishnan, A., Lal, K.K., Basheer, V.S., Punia, P. and Lakra, W.S. *Horabagrus brachysoma* clone Hbr Cma04 microsatellite sequence. 136bp linear DNA; 01-AUG-2006.
6	DQ780019	Mohindra, V., Abdul Muneer, P.M., **Musammilu, K.K.,** Gopalakrishnan, A., Lal, K.K., Basheer, V.S., Punia, P. and Lakra, W.S. *Horabagrus brachysoma* clone Hbr D33 microsatellite sequence. 208bp linear DNA; 01-AUG-2006.
7	DQ780020	Mohindra, V., Abdul Muneer, P.M., **Musammilu, K.K.,** Gopalakrishnan, A., Lal, K.K., Basheer, V.S., Punia, P. and Lakra, W.S. *Horabagrus brachysoma* clone Hbr D38 microsatellite sequence. 299bp linear DNA; 01-AUG-2006.
8	DQ780021	Gopalakrishnan, A., Abdul Muneer, P.M., **Musammilu, K.K.,** Mohindra, V., Lal, K.K., Basheer, V.S., Punia, P. and Lakra, W.S. *Horabagrus brachysoma* clone Hbr Phy01 microsatellite sequence. 181bp linear DNA; 01-AUG-2006.
9	DQ780022	Gopalakrishnan, A., Abdul Muneer, P.M., **Musammilu, K.K.,** Mohindra, V., Lal, K.K., Basheer, V.S., Punia, P. and Lakra, W.S. *Horabagrus brachysoma* clone Hbr Phy05 microsatellite sequence. 162bp linear DNA; 01-AUG-2006.
10	DQ780023	Gopalakrishnan, A., Abdul Muneer, P.M., **Musammilu, K.K.,** Mohindra, V., Lal, K.K., Basheer, V.S., Punia, P. and Lakra, W.S. *Horabagrus brachysoma* clone Hbr Phy07 microsatellite sequences. 275bp linear DNA; 01-AUG-2006.
11	EF582608	Mohindra, V., **Musammilu, K.K.**, Gopalakrishnan, A., Basheer, V.S., Lal, K.K., Punia, P., Abdul Muneer, P.M. and Lakra, W.S. *Gonoproktopterus curmuca* isolate Gcur MFW11 microsatellite sequence. 176 bp linear DNA; 30-May-2007.

Sl. No.	Accession Numbers	Details
□ 12	EF582609	Mohindra, V., **Musammilu, K.K.,** Gopalakrishnan, A., Basheer, V.S., Lal, K.K., Punia, P., Abdul Muneer, P.M. and Lakra, W.S. *Gonoproktopterus curmuca* isolate Gcur MFW19 microsatellite sequence. 201bp linear DNA; 30-MAY-2007.
□ 13	EF582610	Mohindra, V., **Musammilu, K.K.,** Gopalakrishnan, A., Basheer, V.S., Lal, K.K., Punia, P., Abdul Muneer, P.M. and Lakra, W.S. *Gonoproktopterus curmuca* isolate Gcur MFW26 microsatellite sequence. 165bp linear DNA; 30-MAY-2007.
□ 14	EF582611	Mohindra, V., **Musammilu, K.K.,** Gopalakrishnan, A., Basheer, V.S., Lal, K.K., Punia, P., Abdul Muneer, P.M. and Lakra, W.S. *Gonoproktopterus curmuca* isolate Gcur MFW72 microsatellite sequence. 171bp linear DNA; 30-MAY-2007.
□ 15	EF582612	Mohindra, V., **Musammilu, K.K.,** Gopalakrishnan, A., Basheer, V.S., Lal, K.K., Punia, P., Abdul Muneer, P.M. and Lakra, W.S. *Gonoproktopterus curmuca* isolate Gcur Ppro48 microsatellite sequence. 251bp linear DNA; 30-MAY-2007.
□ 16	EF582613	Mohindra, V., **Musammilu, K.K.,** Gopalakrishnan, A., Basheer, V.S., Lal, K.K., Punia, P., Abdul Muneer, P.M. and Lakra, W.S. *Gonoproktopterus curmuca* isolate Gcur Ppro126 microsatellite sequence. 246bp linear DNA; 30-MAY-2007.
□ 17	EU272893	Gopalakrishnan, A., Lijo, J., **Musammilu, K.K.,** Basheer, V.S., Mohindra, V., Lal, K.K., Punia, P. and Lakra, W.S. *Labeo dussumieri* clone Lduss_Bgon22 microsatellite sequence. 118 bp linear DNA, 04-DEC-2007.
□ 18	EU272894	Gopalakrishnan, A., Lijo, J., **Musammilu, K.K.,** Basheer, V.S., Mohindra, V., Lal, K.K., Punia, P. and Lakra, W.S. *Labeo dussumieri* clone Lduss_MFW26 microsatellite sequence. 145bp linear DNA ; 04-DEC-2007.
□ 19	EU272895	Gopalakrishnan, A., Lijo, J., **Musammilu, K.K.,** Basheer, V.S., Mohindra, V., Lal, K.K., Punia, P. and Lakra,W.S. *Puntius denisonii* clone Pdeni_MFW02 microsatellite sequence. 164 bp linear DNA; 04-DEC-2007.
□ 20	EU272896	Gopalakrishnan, A., Lijo, J., **Musammilu, K.K.,** Basheer, V.S., Mohindra, V., Lal, K.K., Punia, P. and Lakra, W.S. *Puntius denisonii* clone Pdeni_MFW11 microsatellite sequence. 164 bp linear DNA; 04-DEC-2007.
□ 21	EU272897	Gopalakrishnan, A., Lijo, J., **Musammilu, K.K.,** Basheer, V.S., Mohindra, V., Lal, K.K., Punia, P. and Lakra, W.S. *Puntius denisonii* clone Pdeni_MFW17 microsatellite sequence. 203bp linear DNA; 04-DEC-2007
□ 22	EU272898	Gopalakrishnan, A., Lijo, J., **Musammilu, K.K.,** Basheer, V.S., Mohindra, V., Lal, K.K., Punia, P. and Lakra, W.S. *Puntius denisonii* clone Pdeni_MFW19 microsatellite sequence. 257bp linear DNA; 04-DEC-2007
□ 23	EU272899	Lijo, J., Gopalakrishnan, A., **Musammilu, K.K.,** Basheer, V.S., Mohindra, V., Lal, K.K., Punia, P. and Lakra, W.S. *Puntius denisonii* clone Pdeni_MFW20 microsatellite sequence. 148bp linear DNA; 04-DEC-2007
□ 24	EU272900	Lijo, J., Gopalakrishnan, A., **Musammilu, K.K.,** Basheer, V.S., Mohindra, V., Lal, K.K., Punia, P. and Lakra, W.S. *Puntius denisonii* clone Pdeni_MFW26 microsatellite sequence. 115bp linear DNA; 04-DEC-2007.
□ 25	EU272901	Lijo, J., Gopalakrishnan, A., **Musammilu, K.K.,** Basheer, V.S., Mohindra, V., Lal, K.K., Punia, P. and Lakra, W.S. *Puntius denisonii* clone Pdeni_R6 microsatellite sequence. 166bp linear DNA; 04-DEC-2007.