REVIEW OF LITERATURE

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

Organisms are incessantly undergoing micro and macro evolutionary processes both at molecular and organismal levels. Actually, the process of evolution starts at the molecular level, more precisely from a single base of the DNA molecule and ends up in variations at the organismal level. Genes are the factors, which determine the phenotypic characters of any organism. Thus, the variations that happen to the genes in turn produce individuals, which are different either at the molecular level or at the organismal level. These individuals may form separate groups within the species itself and such groups are the fundamental genetic units of evolution. These intraspecific groups were called as 'stocks' and fishery biologists started using these stocks as a basis to manage commercially important marine organisms. Shaklee et al. (1990) defined a stock as "a panmictic population of related individuals within a
single species that is genetically distinct from other such populations". Therefore, in any management regime, identification of discrete stocks becomes a critical element (Ihssen et al., 1981a; Fetterolf, 1981).

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

Of these, morphometric investigations are based on a set of measurements of the body form (Hubbs and Lagler, 1947). Study on the life history, morphology and electrophoretic characteristics of five allopatric stocks of lake white fish showed that morphometry can be used to distinguish individuals of different stocks (Ihssen et al., 1981b), although the branching patterns for morphometrics versus biochemical variation were different. For selection of brood stock in genetic improvement programmes of certain penaeids, one or two morphometric variables could be identified, giving accurate estimate of tail weight (Lester, 1983; Goswami et al., 1986). A study of Pacific white shrimp, Penaeus vannamei from different commercial hatcheries found significant differences in all morphometric traits between sites, indicating that environmental differences affected growth as well as shape of the shrimps (Chow and Sandifer, 1992). But, in a study using canonical discriminant analysis of morphometric and meristic characters to identify cultured tilapias, the results did not support the use of morphometric characters for differentiating tilapia strains and introgressed hybrids (Pante et al., 1988). These conventional data sets can be biased and they have got several weaknesses too. (i) They tend to be in one direction only (longitudinal) lacking information of depth and breadth, (ii) they often produce uneven and biased areal coverage of the body form, (iii) repetition of
landmarks often occur, (iv) many measurements extends over much of the body and (v) the amount of distortion due to preservation cannot be easily estimated in case of soft bodied organisms (Sathianandan, 1999). To overcome these problems, a new method called the “truss network” was developed in which an even areal coverage over the entire fish form was possible (Humphries et al., 1981). This method can discriminate stocks of fishes and prawns on the basis of size free shape derived from distance measures. Here, the forms may be standardized to one or more common reference sizes by representing measured distances on some composite measure of body size and reconstructing the form using the distance values predicted at some standard body size. The composite mapped forms are suitable for biorthogonal analysis of shape differences between forms (Sathianandan, 1999). Truss network analysis on chinook salmon demonstrated shape differences among the three naturally occurring populations (Winans, 1984). This method was introduced among prawns to study the shape differences among them (Lester and Pante, 1992) and a machine vision system was developed for the selection of brood stock using the truss network (Perkins and Lester, 1990). A comparison of the conventional morphometrics and truss network analysis done on the blunt snout bream, finally described the truss network analysis as a better tool for probing evolutionary processes or elucidating relationships among populations (Li et al., 1993).

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

2.1. Genetic Markers

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

The various marker types available for fisheries and conservation applications (Park and Moran, 1994) represent a bewildering array of choices for the uninitiated. The development of new markers has been necessary for species with little detectable variation among individuals using the old markers. However, relative novelty and not the attributes of the markers themselves have often dictated marker choice (Utter et al., 1991). There is no single marker type that is appropriate for all applications and a genetic marker system should be based on the characteristics of a particular species (interacting with the attributes of the marker type) rather than how recently they have been developed (Ferguson and Danzmann, 1998). In fact, a combination of mitochondrial and nuclear markers is the most powerful approach (Ward and Grewe, 1994). Attributes of the species (genetic effective population size-contemporary and historical (Ne)), amount of gene flow (migration) in combination with those of the marker loci themselves could be used to choose an appropriate marker system. Other important factors influencing marker choice are cost and sampling requirements (Ferguson et al., 1995).
Genetic markers are basically of 2 types - protein and DNA. In 1960's initial studies involved proteins such as haemoglobin and transferrin. However, very soon the attention was turned to enzymatic protein (allozyme) variation on which most subsequent studies have been based (Ferguson et al., 1995). A new technique based on molecular characters to identify the stocks was also developed in the early nineties (Williams et al., 1990, Welsh and McClelland, 1990; Penner et al., 1993; Jeffreys et al., 1985; Tautz, 1989).

2.1.1. Allozyme markers

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

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

Studies have been successfully carried out to assess levels of genetic differentiation and gene flow at the intra specific level in several important fish species using allozyme/isozyme electrophoresis (Richardson, 1982; Menezes et al., 1992; Begg et al., 1998; Appleyard and Mather, 2000; McGlashan and Hughes, 2000; Cook et al., 2002; Salini et al., 2004) and the taxonomic uses of enzyme electrophoresis are also well known (Avise, 1974; Ferguson, 1980). Many workers have already demonstrated the use of allozymes and other proteins as genetic markers for the identification of fish stocks or species (Simonarsen and Watts, 1969; Fujio and Kato, 1979; Mulley and Latter, 1980; Grand and Utter, 1984) and in fish breeding (Moav et al., 1978). Significant differences in the allelic frequencies among populations of a species clearly indicate that these were not interbreeding but isolated populations (Ayala and Keiger, 1980; 1984). The significance of similar worldwide reports of genetic diversity in fishes and shellfishes was well evaluated in the international symposia held in 1971 (Ligny, 1971). Later, the special significance of the genetic stock concept at various levels of fisheries management and various
techniques for detection of genetic stocks were re-evaluated in the international symposia held in 1981, the proceedings of which were published as a special issue [Can. J. Fish. Aquat. Sci. Vol., 38 (12), 1981]. Using allozyme genetic tags, six genetically heterogeneous stocks were detected in the flounder populations of Newfoundland region (Fairbairn, 1981). Ridgway et al. (1970) reported the esterase polymorphism in the Atlantic herring and Shaklee and Salini (1985) in barramundi, _Lates calcarifer_. These studies are relevant not only to evolutionary biology but also to the management of these stocks, providing information to adjust regulations according to observed stock structure.

Many authors have studied the extent of genetic differentiation and population structure using allozyme markers in fish species. A homogeneous stock structure was reported in European hake, _Merluccius merluccius_ (Mangaly and Jamieson, 1978). Coelho et al. (1995) studied the genetic structure and differentiation among populations of two cyprinids _Leuciscus pyrenaicus_ and _L. caroliterti_. Some investigators made a comparative study of different populations of chum salmon: Wilmot et al. (1994) compared Western Alaskan and Russian Far East stocks; Winans et al. (1994) studied Asian stocks; Phelps et al. (1994) the Pacific North West populations; Kondzela et al. (1994) compared stocks of South East Alaska and Northern British Columbia. Allozyme markers have been employed in other salmon by different groups: In sockeye salmon (Wood et al., 1994, in Canada; Varnavskaya et al., 1994a, in Asia and North America; 1994b in Alaska, British Colombian and Kamchatka lake in Russia; in Atlantic salmon (Cross and Challanin, 1991; Skaala et al., 1998); in odd year pink salmon, _Oncorhynchus gorbuscha_ (Shaklee and Varnavskaya, 1994); and in Chinook salmon, _Oncorhynchus tshawytscha_ (Verspoor et al., 1991; Youngson et al., 1991; Adams, 1994). Using allozyme markers, distinct genetic stocks of cultured tilapia in Fiji were identified by Appleyard and Mather (2000). Similarly, significant genetic differentiation was detected in North Australian mackerel (Begg et al., 1998); in _Barbus callensis_ (Berrebi et al., 1995); in African and Iberian populations of _Cobitis_ (Perdices, 1995) and in North Atlantic tusk, _Brosme brosme_ (Johansen and Naevdal, 1995). Recently, Peres et al. (2002) reported genetic variability patterns in _Hoplias malabaricus_ in fluvial and lacustrine environments in
the upper Paraná floodplain. Musyl and Keenan (1996) found small genetic differences in the Australian catfish, *Tandanus tandanus* between a Brisbane River (east flowing) and a Condamine River site (west flowing). They also found lower than expected levels of genetic divergence among some eastern and western Australian populations of the perch, *Macquaria ambigua*. McGlashan and Hughes (2000) reported significant levels of genetic subdivision among 16 populations of the Australian freshwater fish, *Craterocephalus stercusmuscarum* using 7 polymorphic allozyme loci and sequence information on the ATPase gene of mitochondrial DNA. McGlashan and Hughes (2002) also showed that populations of subspecies *Craterocephalus stercusmuscarum fulvus* separated by a mountain range in Australia were genetically more similar than populations of *C.s.fulvus* and *C.s. stercusmuscarum* which inhabit a contiguous coastal margin. The same authors in 2002 reported extensive genetic subdivisions across the range of the Australian freshwater fish, *Pseudomugil signifer* using 6 polymorphic allozyme loci. Cook et al. (2002) reported large and significant genetic variation in *Macrobrachium australiensis* among the 4 major catchments in Western Queensland, Australia, using 6 polymorphic allozymes. Genetic variation throughout the geographic range of the tropical shad, hilsa *Tenualosa ilisha* was analysed using allozyme marker by Salini et al. (2004).

Review of the relevant literature reveals that work on biochemical genetics of Indian fishes is scanty in comparison to the work done in many parts of the rest of the world. Chandrasekhar (1959) has studied the profile of blood proteins of five Indian carps. Krishnaja and Rege (1977, 1979) undertook electrophoretic studies on the genetics of two species of Indian carp and their fertile hybrids. Sarangi and Mandal (1996) reported isozyme polymorphism in diploid and tetraploid Indian major carps, *Labeo rohita*. Goapalakrishnan et al. (1997) identified species-specific esterase markers in rohu and mrigal, while Singh et al. (2004) identified allozyme markers helpful in population genetic analysis of *Cirrhinus mrigala*. Examples of other important biochemical genetic studies at the intraspecific level in fish from Indian waters are that of mullet, *Mugil cephalus* (Vijayakumar, 1992; Menezes et al., 1990); oil sardine, *Sardinella longiceps* (Venkitakrishnan, 1992; Menezes, 1994a; 1994b);
mackerel (Menezes et al., 1990); Pomfret (Menezes, 1993) hilsa from the Ganges River (above and below Farakka barrage) and Brahmaputhra River (Lal et al., 2004a) and Lactarius lactarius (Gopalakrishnan et al., 2004c). The above-mentioned investigations identified distinct genetic stocks of M. cephalus and L. lactarius from Indian waters while low genetic divergence was reported in sardines, mackerel, hilsa and pomfrets. The above examples reveal that biochemical genetic techniques are efficient in differentiating genetic variation in natural stock of fish/shellfish species. The phenomenon of the very low-level genetic variation and close genetic homogeneity was reported even in distant geographic populations in species of penaeidae from Indian waters using allozymes as reported in other parts of the world (Bindhu Paul, 2000; Rebello, 2002).

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

2.1.2. Molecular markers

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

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

2.1.2.1. Random amplified polymorphic DNA (RAPD)

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

The principle behind RAPD analysis is that at low annealing temperatures or high magnesium concentrations, a primer is likely to find many sequences within the template DNA to which it can anneal. Depending on the length and complexity of the genome of an organism, there can be numerous pairs of these sequences and they will be arranged inversely to and within about two kilobases of each other. Considering this, PCR will amplify many random fragments that can vary in size when different species, subspecies, populations or individuals are analyzed and this will constitute the basis of identification. A single primer is used to amplify the intervening region between two complementary, but inversely oriented, sequences. Suitable primers include random GC-rich 10 mers and polymers complementary to random repeats. Priming sites are randomly distributed throughout the genome and polymorphism in such sites results in differing amplification products, detected by the presence or
absence of fragments. Hence, RAPDs are treated as dominant markers. Polymorphisms result from either size changes in the amplified region or base changes that alter primer binding. The RAPD technique apart from single copy fraction, also amplifies highly repetitive regions that may accumulate more nucleotide mutations compared with those encoding allozyme variants, offering a wider potential for assessing inter-population genetic differentiation. Thus, several authors reported specific RAPD markers, useful for distinguishing intra-species population or between closely related species, in organisms where allozymes have proven to show low-resolution power to assess genetic differences (Black et al., 1992 and Cognato et al., 1995).

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

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

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

RAPD analysis has several advantages over other procedures. These include relatively shorter time (1-2 days) required to complete analysis after standardization, ability to detect extensive polymorphisms, low cost, simplicity, rapidity, need for minute amounts of genomic DNA (~30ng), random primers required for analysis, simpler protocols and involvement of non - invasive sampling for tissue analysis. There is no need for molecular hybridization and the technique allows examination of
genomic variation without prior knowledge of DNA sequences (Welsh and McClelland, 1990; Williams et al., 1993; Liu et al., 1999a). RAPD-PCR technique has been shown to give a high resolution especially in separating species complexes and sibling species, in detecting cryptic pairs of species and in confirming close relationships between species.

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

2.1.2.2. Microsatellites

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

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

Generally, microsatellite loci are abundant and distributed throughout the eukaryotic genome (Tautz and Renz, 1984) and each locus is characterized by known DNA sequence. These sequences consist of both unique DNA (which defines the locus) and of repetitive DNA motifs (which may be shared among loci). The repetitive
elements consist of tandem reiterations of simple sequence repeats (SSRs) and are typically composed of two to four nucleotides such as (AC)n or (GATA)n where n lies between 5 and 50 (DeWoody and Avise, 2000). Within vertebrates, the dinucleotide repeats -GT and CA- are believed to be the most common microsatellites (Zardoya et al., 1996). Study of single locus microsatellites requires specific primers flanking the repeat units, whose sequences can be derived from (i) genomic DNA libraries or (ii) from available sequences in the gene banks.

The high variability, ease and accuracy of assaying microsatellites make them the marker of choice for high-resolution population analysis (Estoup et al., 1993). Microsatellites with only a few alleles are well suited for population genetic studies, while the more variable loci are ideal for genome mapping and pedigree analysis and the fixed or less polymorphic microsatellite loci are used to resolve taxonomic ambiguity in different taxa (Carvalho and Hauser, 1994). Highly polymorphic microsatellite markers have great potential utility as genetic tags for use in aquaculture and fisheries biology. They are powerful DNA markers for quantifying genetic variations within and between populations of species (O’Connell et al., 1998). They may prove particularly valuable for stock discrimination and population genetics due to the high level of polymorphism compared with conventional allozyme markers (Bentzen et al., 1991; Wright and Bentzen, 1994). Microsatellite DNA markers are among the most likely to conform to the assumption of neutrality and have proven to be powerful in differentiating geographically isolated populations, sibling species and sub-species (Zardoya et al., 1996). The qualities of microsatellites make them very useful as genetic markers for studies of population differentiation and stock identification (reviewed in Park and Moran 1994; Wright and Bentzen, 1994; O’Reilly and Wright, 1995), in kinship and parentage exclusion (Queller et al., 1993; Kellog et al., 1995; Hansen et al., 2001) and in genome mapping (Lee and Kocher, 1996). Microsatellites are also being used as genetic markers for identification of population structure, genome mapping, pedigree analysis; and to resolve taxonomic ambiguities in many other animals besides fishes (Garcia et al., 1996; Nelson et al., 2002; Naciri et al., 1995; Wallick et al., 1999; Brooker et al., 2000; Sugaya et al., 2002; Ciofi et al., 2002; Shaw et al., 1999; Supungul et al., 2000; Norris et al., 2001).
Various authors have reported microsatellite polymorphisms and sequences in some marine and freshwater fish species for population genetic analysis (Estoup et al., 1993; Rico et al., 1993; Brooker et al., 1994; Garcia de Leon et al., 1995; Presa and Guyomand, 1996; Appleyard et al., 2002; Han et al., 2000; Ball et al., 2000; Kirankumar et al., 2002). The development of polymorphic microsatellite markers to determine the population structure of the Patagonian tooth fish, *Dissostichus eleginoides*, has been reported by Reilly and Ward (1998). Microsatellite polymorphisms have been used to provide evidence that the cod in the northwestern Atlantic belong to genetically distinguishable populations and that genetic differences exist between the northwestern and southeastern cod populations (Bentzen et al., 1996). O'Connell et al. (1997) reported that microsatellites, comprising (GT)$_n$ tandemly repeated arrays, were useful in determining the patterns of differentiation in freshwater migratory populations of rainbow trout *Oncorhynchus mykiss* in Lake Ontario. Takagi et al. (1999) identified four microsatellite loci in tuna species of genus *Thunnus* and investigated genetic polymorphism at these loci in Northern Pacific populations. In a cichlid, *Eretmodus cyanostictus*, Taylor et al. (2001) determined four polymorphic microsatellite loci for studying nine populations in Lake Tanganyika. Appleyard et al. (2002) examined seven microsatellite loci in Patagonian Tooth fish from three locations in the Southern Ocean. Gold et al. (2002) analysed the population structure of king mackerel (*Scomberomorus cavalla*) along the east (Atlantic) and west (Gulf) coasts of Florida using seven microsatellite loci. O'Connell et al. (1998) reported the investigation of five highly variable microsatellite loci for population structure in Pacific herring, *Clupea pallasi* collected from 6 sites in Kodiak Island. Similarly, many others have reported studies of polymorphic microsatellite loci to evaluate population structure of different fish species (Beacham and Dempson, 1998; McConnell et al., 1995; Reilly et al., 1999; Perez-Enriquez et al., 1999; Ball et al., 2000; Appleyard et al., 2001; Brooker et al., 2000; Colihuque, 2003; Ruzzante et al., 1996).

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

Although microsatellite DNA analysis via PCR is an ideal technique for answering many population genetic questions, the development of species-specific primers for PCR amplification of alleles can be expensive and time-consuming, as it involves construction of genomic libraries, screening of clones with microsatellite sequences and designing microsatellite primers. However, there are reports which point to the fact that flanking sequences of some microsatellite loci are conserved among related taxa so that primers developed for one species can be used to amplify homologous loci in related species. The conservation of flanking regions of microsatellite sequences among closely related species has been reported by a number of groups (Moore et al., 1991; Schlotterer et al., 1991; Estoup et al., 1995; Zheng et
al., 1995; Presa and Guyomard, 1996; Scribner et al., 1996; May et al., 1997; Coltman et al., 1996; Pepin et al., 1995). Such an approach can circumvent extensive preliminary work necessary to develop PCR-primers for individual loci that continues to stand in the way of quick and widespread application of single locus microsatellite markers. Thus, by using heterologous PCR primers the cost of developing similar markers in related species can be significantly reduced.

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

A number of attempts have been made to study the cross species amplification of microsatellite loci in fishes. Scribner et al. (1996) isolated cloned microsatellites from Atlantic salmon genomic libraries and used them for cross-species amplification and population genetic applications in other salmon species. May et al. (1997) reported microsatellite genetic variation through cross species amplification in sturgeons Acipenser and Scaphirhynchus. Takagi et al. (1999) reported that microsatellite primers isolated from one tuna might be used to amplify microsatellite loci in other tuna species especially those of the genus Thunnus. Microsatellites from rainbow trout Oncorhynchus mykiss have been used for the genetic study of salmonids (Morris et al., 1996; Small et al., 1998; Beacham and Dempson, 1998). Heterologous primers have been used to characterize bull trout by using three sets of primers from sockeye salmon, rainbow trout and brook trout (Kanda and Allendorf, 2001), for several Salvelinus species using primers of Salvelinus fontinali; for Brook charr (Angers and Bernatchiz, 1996), for Poecilia reticulata by using primers of Poecilia occidentalis (Parker et al., 1998) and Oreochromis shiranus and O. shiranus chilwae by using primers of Nile tilapia (Ambali, 1997). There are some reports in which the
flanking sequences are conserved between families of the same order. Primers of stickleback and cod have been used in *Merlangius merlangius* (Gadidae) (Rico et al., 1997); that of rainbow trout (Family: Salmonidae) in whitefish, *Coregonus nasus* (Patton et al., 1997); and primers of goldfish, *Carassius auratus* in nine species of cyprinids (Zheng et al., 1995). Yue and Orban (2002) developed 15 polymorphic microsatellite loci in silver crucian carp *Carassius auratus gibelio* and reported, eleven out of 15 primer pairs cross-amplified in the genome of common carp (*Cyprinus carpio*). Zardoya et al. (1996) through a classical study demonstrated that microsatellite flanking regions (MFRs) contain reliable phylogenic information and they were able to recover with considerable confidence the phylogenetic relationship within Family Cichlidae and other families of the suborder Labroidei from different parts of the world including India. In India, Mohindra *et al.* (2001 a, b; 2002 a, b, c) have carried out cross-species amplification of *C. catla* G1 primer in *Catla catla* from Gobindsagar, *Laboe dero, L. dyocheilus L. rohita* and *Morulius calbasu*, and sequenced the loci in these species. Das and Barat (2002a, b, c) also carried out characterisation of dinucleotide microsatellite repeats in *Laboe rohita*. In an endemic cyprinid of the Western Ghats (*Laboe dussumieri*), Gopalakrishnan *et al.* (2002) sequenced microsatellite loci by cross-species amplification of *C. catla* G1 primer. Kirankumar *et al.* (2002) reported the complete sequence of a repeat like region in Indian rosy barb (*Puntius conchonius*). The cross-species amplification of microsatellite in *Puntius denisonii* using primers developed for other of other cyprinid fishes was reported by Lijo John (2004). Successful identification of polymorphic microsatellite markers for *Cirrhinus mrigala* and *Gonoprotoperus curmuca* was achieved through use of primers from other cyprinid fishes (Lal *et al.*, 2004; Gopalakrishnan *et al.*, 2004a).

Advantages of microsatellites such as short size range, uninterrupted stretches of identical repeat units, high proportion of polymorphisms, insights gained in understanding the mutational process which helps in developing statistical procedures for inter-population comparisons, their abundance in fish genomes, the availability of methodologies for cloning of microsatellites, have all resulted in their abundant use in fisheries research. Tetranucleotide microsatellites are also very useful for paternity
and forensic investigations in humans. The advantageous properties of microsatellites has led to modern developments such as digital storage, automated detection and scoring systems such as automated DNA sequences, fluorescent-imaging devices etc. (O’Connell and Wright, 1997). Disadvantages of microsatellites include the appearance of shadow or stutter bands, presence of null alleles (existing alleles that are not observed using standard assays); homoplasy; and too many alleles at certain loci that would demand very high sample size for analysis (Mohindra et al., 2001a). Also, microsatellite flanking regions (MFRs) sometimes contain length mutations which may produce identical length variants that could compromise microsatellite population level studies (and comparisons of levels of variation across species for homologous loci) and phylogenetic inferences as these length variants in the flanking regions can potentially minimize allele length variation in the repeat region (Zardoya et al., 1996).

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

2.2. Genetic markers in catfishes

Genetic markers have been used to distinguish species as well as for stock structure analysis in catfishes also. Allozyme markers have been used to obtain genetic evidence for the validity of two species of African catfishes, *Clarias gareipinus* and *Clarias anguillaris*, Teugels et al. (1992) examined electrophoretic variation at 13 protein loci in two West African populations of both species. Agnese et al. (1997) described genetic variation at 25 protein loci in two sympatric samples from the Senegal River for these two species. Rognon et al. (1998) reported allozyme variation in both species at 25 allozyme loci. Van der Bank et al. (1992) carried out a
comparative biochemical genetic study of three populations of domesticated and wild African catfish, *Clarias gariepinus*. Genetic relationship of glucose phosphate isomerase-B phenotypes were analysed in channel catfish, *Ictalurus punctatus* by Goudie et al. (1995). Thermal stability of soluble malate dehydrogenase (sMDH) was analyzed by Monteiro et al. (1998) in fish belonging to the Order Siluriformes. Population genetic structure of baung, *Mystus nemurus* were analyzed in Malaysia using allozyme markers (Siraj et al., 1998). Pouyaud et al. (2000) studied the phylogeny of the Family Pangasiidae and verified the presently used classification based on morphological data. In marine catfishes, Suzuki and Phan (1990a, b) studied intra-specific variation and inter-specific relationships of 6 ariid species from Brazil, using 10 allozymes from eye lens and skeletal muscle proteins. Gopalakrishnan et al. (1996) identified 7 allozyme markers to resolve a taxonomic ambiguity in *Tachysurus (Arius) maculatus*, *T. subrostratus* and in other species of marine catfishes.

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

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

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

*Horabagrus brachysoma*, the species selected for the present study was listed as 'endangered' according to the latest IUCN categorization in the NBFGR-CAMP workshop held in 1997 (Anon., 1998). The species has been short-listed for initiating a 'stock-specific propagation assisted rehabilitation programme' in rivers where it is naturally distributed. In connection with this, captive breeding and milt cryopreservation techniques have been developed for the species by National Bureau of Fish Genetic Resources (NBFGR) in collaboration with Regional Agricultural Research Station (RARS) of Kerala Agricultural University at Kumarakom (Gopalakrishnan et al., 2004b). However, no attempts have been made to study the stock structure and basic genetic profile of the species that are essential for good fishery management, conservation and rehabilitation of this species. Available information on this species relates only to length-weight relationships (Kumar et al., 35...
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1999). This prompted me to take up the present work with a view to identifying polymorphic allozyme, RAPD and microsatellite markers in order to obtain a detailed picture of the population structure of the species distributed in three rivers in the Western Ghats.