MOLECULAR PROFILING OF SOME ECONOMICALLY IMPORTANT FISHES OF KASHMIR

DISSERTATION

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Certificate

This is to certify that the dissertation entitled **"Molecular Profiling of Some Economically Important Fishes of Kashmir"** is the original and bonafide research work of **Hilal Ahmad Ganaie**, Research Scholar, Post-graduate Department of Zoology, University of Kashmir, Srinagar. This work has been carried out under my guidance and supervision and has not been previously submitted for the award of any degree, diploma or any other distinction.

It is further certified that Hilal Ahmad Ganaie has put in the required attendance in the Department and fulfils all the statutory requirement for the award of M.Phil degree in Zoology.

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DEDICATED TO MY

FAMILY AND TEACHERS

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All the words in the lexicon will be meaningless, if I fail to divulge my extreme sense of regards to my adorable parents for their sacrifice, prayers and blessings. Where would I be without them? I wish to acquiescence the gratefulness beyond accountability to my adorable parents whose affection and morale boosting during every phase of my life, indeed actuated me to make this chimeral expectancy true. Whatever I am today, I entirely owe it to them.

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HILAL AHMAD GANAIE

CONTENTS

LIST OF TABLES

.

LIST OF PLATES

Plate No.	Title of the Plates
1	*Cyprinus carpio communis.*
2	*Cyprinus carpio specularis.*
3	*Oncorhynchus mykiss.*
4	Fishermen selling live fishes in Hazratbal Market.
5	Trout Fish Farm at Harwan Srinagar.
6	PCR Machine.
7	Gel Doc.
8	RAPD profile of the fishes

Chapter 1

Introduction

1.1. Genes and Genomes

The term "Genome" was coined in 1920 by Winkler as a hybrid of Gene and Chromosome. It signifies the full set of chromosomes and all the genes contained therein. Genomic DNA is tightly and orderly packed in the process called DNA condensation to fit the small available volumes of the cell. In eukaryotes, DNA is located in the cell nucleus, as well as small amounts in mitochondria and chloroplasts. In prokaryotes, the DNA is held within an irregularly shaped body in the cytoplasm called the nucleoid. The genetic information in a genome is held within genes, and the complete set of this information in an organism is called its genotype. A gene is a unit of heredity and is a region of DNA that influences a particular characteristic in an organism.

In many species, only a small fraction of the total sequence of the genome encodes protein. For example, only about 1.5% of the human genome consists of protein-coding exons, with over 50% of human DNA consisting of non-coding repetitive sequences The reasons for the presence of so much noncoding DNA in eukaryotic genomes and the extraordinary differences in genome size, or *C-value*, among species represent a long-standing puzzle known as the "C-value paradox". However, DNA sequences that do not code protein may still encode functional non-coding RNA molecules, which are involved in the regulation of gene expression.

Structural proteins that bind DNA are well-understood examples of non-specific DNA-protein interactions. Within chromosomes, DNA is held in complexes with structural proteins. These proteins organize the DNA into a compact structure called chromatin. In eukaryotes this structure involves DNA binding to a complex of small basic proteins called histones, while in prokaryotes multiple types of proteins are involved. The histones form a disk-shaped complex called a nucleosome, which contains two complete turns of double-stranded DNA wrapped around its surface. These non-specific

interactions are formed through basic residues in the histones making ionic bonds to the acidic sugar-phosphate backbone of the DNA, and are therefore largely independent of the base sequence. Chemical modification of these basic amino acid residues includes methylation, phosphorylation and acetylation. These chemical changes alter the strength of the interaction between the DNA and the histones, making the DNA more or less accessible to transcription factors and changing the rate of transcription. Other non-specific DNA-binding proteins in chromatin include the high-mobility group proteins, which bind to bent or distorted DNA. A distinct group of DNA-binding proteins are bind to single-stranded DNA. These binding proteins seem to stabilize single-stranded DNA and protect it from forming stem-loops or being degraded by nucleases.

1.2. Deoxyribonucleic Acid (DNA)

Deoxyribonucleic acid (DNA) is a nucleic acid containing the genetic instructions used in the development and functioning of all known living organisms (with the exception of RNA viruses). The DNA segments carrying this genetic information are called genes. Likewise, other DNA sequences have structural purposes, or are involved in regulating the use of this genetic information. Along with RNA and proteins, DNA is one of the three major macromolecules that are essential for all known forms of life. DNA consists of two long polymers of simple units called nucleotides, with back bones made of sugars and phosphate groups joined by ester bonds. These two strands run in opposite directions to each other and are therefore anti-parallel. Attached to each sugar is one of four types of molecules called nucleo bases (or simply bases). It is the sequence of these four nucleobases along the backbone that encodes information. This information is read using the genetic code, which specifies the sequence of the amino acids within proteins. The code is read by copying stretches of DNA into the related nucleic acid RNA in a process called transcription.

Within cell's DNA is organized into long structures called chromosomes. During cell division these chromosomes are duplicated in the process of DNA replication, providing each cell its own complete set of chromosomes. Eukaryotic organisms (animals, plants, fungi and protests) store most of their DNA inside the cell nucleus and some of the DNA in the organelles, such as mitochondria or chloroplasts.

In contrast, prokaryotes (bacteria and archaea) store their DNA only in the cytoplasm. Within the chromosomes, chromatin proteins such as histones compact and organize DNA. These compact structures guide the interactions between DNA and other proteins, helping to control which parts of the DNA are to be transcribed. DNA is a long polymer made from repeating units called nucleotides. As first discovered by James D. Watson and Francis Crick, the structure of DNA of all species comprises two helical chains each coiled round the same axis, and each with a pitch of 34 A^0 (3.4 nanometers) and a radius of 10 A^0 (1.0 nanometers). According to another study, when measured in a particular solution, the DNA chain measured 22 to 26 A^0 wide (2.2 to 2.6 nanometers), and one nucleotide unit measured 3.3 A^0 (0.33 nm) long. Although each individual repeating unit is very small, DNA polymers can be very large molecules containing millions of nucleotides. For instance, the largest human chromosome, chromosome number 1, is approximately 220 million base pairs long.

In living organisms DNA does not usually exist as a single molecule, but instead as a pair of molecules that are held tightly together. These two long strands entwine like vines, in the shape of a double helix. The nucleotide repeats contain both the segment of the backbone of the molecule, which holds the chain together, and a nucleobases, which interacts with the other DNA strand in the helix. A nucleo base linked to a sugar is called a nucleoside and a base linked to a sugar and one or more phosphate groups is called a nucleotide.

Polymers comprising multiple linked nucleotides (as in DNA) are called a polynucleotide.

The backbone of the DNA strand is made from alternating phosphate and sugar residues. The sugar in DNA is 2-deoxyribose, which is a pentose (five-carbon) sugar. The sugars are joined together by phosphate groups that form phosphodiester bonds between the third and fifth carbon atoms of adjacent sugar rings. These asymmetric bonds mean a strand of DNA has a direction. In a double helix the direction of the nucleotides in one strand is opposite to their direction in the other strand: the strands are antiparallel. The asymmetric ends of DNA strands are called the 5' (five prime) and 3' (three prime) ends, with the 5' end having a terminal phosphate group and the 3' end a terminal hydroxyl group. One major difference between DNA and RNA is the sugar, with the 2-deoxyribose in DNA being replaced by the alternative pentose sugar ribose in RNA. The DNA double helix is stabilized primarily by two forces: hydrogen bonds between nucleotides and base-stacking interactions among the aromatic nucleobases. The four bases found in DNA are adenine (A), cytosine (C), guanine (G) and thymine (T). These four bases are attached to the sugar/phosphate to form the complete nucleotide.

The nucleobases are classified into two types: the purine: A and G, being fused five- and six- membered heterocyclic compounds, and the pyrimidine: the six-membered rings C and T. A fifth pyrimidine nucleobases, Uracil (U), usually takes the place of thymine in RNA and differs from thymine by lacking a methyl group on its ring. Uracil is not usually found in DNA, occurring only as a breakdown product of cytosine. In addition to RNA and DNA a large number of artificial nucleic acid analogues have also been created to study the proprieties of nucleic acids, or for use in biotechnology.

1.3. DNA Profiling

DNA profiling also called DNA testing, DNA typing, or genetic fingerprinting, is a technique employed by forensic scientists to assist in the identification of individuals by their respective DNA profiles. DNA profiles are encrypted sets of numbers that reflect a person's DNA makeup, which can also be used as the person's identifier. DNA profiling should not be confused with full genome sequencing. It is used in, for example, parental testing and criminal investigation.

The DNA profiling technique was first reported by Jeffrey's (1984) at the University of Leicester in England, and is now the basis of several national DNA databases. Dr. Jeffrey's genetic fingerprinting was made commercially available in 1987, when a chemical company, Imperial Chemical Industries (ICI), started a blood-testing centre in England.

1.4. DNA Profiling Technique

The process begins with a sample of an individual's DNA (typically called a "reference sample"). The most desirable method of collecting a reference sample is the use of a buccal swab, as this reduces the possibility of contamination. When this is not available (e.g. because a court order may be needed and not obtainable) other methods may need to be used to collect a sample of blood, saliva, semen, or other appropriate fluid or tissue from personal items (e.g. toothbrush, razor, etc.) or from stored samples (e.g. banked sperm or biopsy tissue). Samples obtained from blood relatives (biological relative) can provide an indication of an individual's profile, as could human remains which had been previously profiled.

A reference sample is then analyzed to create the individual's DNA profile using one of a number of techniques, discussed below. The DNA profile is then compared against another sample to determine whether there is a genetic match.

1.4.1. PCR analysis

With the invention of the polymerase chain reaction (PCR) technique, DNA profiling took huge strides forward in both discriminating power and the ability to recover information from very small (or degraded) starting samples. PCR greatly amplifies the amounts of a specific region of DNA, using oligo nucleotide primers and a thermostable Taq DNA polymerase. Early assays such as the HLA-DQ alpha reverse dot blot strips grew to be very popular due to their ease of use, and the speed with which a result could be obtained. However they were not as discriminating as Restriction Length Fragment Polymorphism (RFLP). It was also difficult to determine a DNA profile for mixed samples, such as a vaginal swab from a sexual assault victim. Fortunately, the PCR method was readily adapted for analyzing VNTR (Variable Number Tandem Repeats), particularly Short Tendam Repeats (STR) loci.

1.4.2. Random Amplification of Polymorphic DNA (RAPD) Analysis

RAPD is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8–12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction.

No knowledge of the DNA sequence for the targeted genome is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared. Because it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such

as short tandem repeats. In recent years, RAPD has been used to characterize, and trace, the phylogeny of diverse plant and animal species.

RAPD markers are decamer (10 nucleotide length) DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence and which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way. It is used to analyze the genetic diversity of an individual by using random primers.

1.4.3. Short Tendam Repeats (STR) Analysis

The method of DNA profiling used today is based on PCR and uses **S**hort **T**andem **R**epeats (STR), a type of VNTR. This method uses highly polymorphic regions that have short repeated sequences of DNA (the most common is 4 bases repeated, but there are other lengths in use, including 3 and 5 bases). Because unrelated people almost certainly have different numbers of repeat units, STRs can be used to discriminate between unrelated individuals. These STR loci (locations on a chromosome) are targeted with sequence-specific primers and amplified using PCR. The DNA fragments that result are then separated and detected using electrophoresis. There are two common methods of separation and detection, capillary electrophoresis (CE) and gel electrophoresis. Each STR is polymorphic, but the number of alleles is very small. Typically each STR allele will be shared by around 5 - 20% of individuals. The power of STR analysis comes from looking at multiple STR loci simultaneously. The pattern of alleles can identify an individual quite accurately. Thus STR analysis provides an excellent identification tool. The more STR regions that are tested in an individual the more discriminating the test becomes.

1.4.4. Amplified Fragment Length Polymorphism (AmpFLP) Analysis:

The technique, AmpFLP, was also put into practice during the early 1990s. This technique was also faster than RFLP analysis and used PCR to amplify DNA samples. It relied on variable number tandem repeat (VNTR) polymorphisms to distinguish various alleles, which were separated on a polyacrylamide gel using an allelic ladder (as opposed to a molecular weight ladder). Bands could be visualized by silver staining the gel. One popular locus for fingerprinting was the D1S80 locus. As with all PCR based methods, highly degraded DNA or very small amounts of DNA may cause allelic dropout (causing a mistake in thinking a heterozygote is a homozygote) or other stochastic effects. In addition, because the analysis is done on a gel, very high number repeats may bunch together at the top of the gel, making it difficult to resolve. AmpFLP analysis can be highly automated, and allows for easy creation of phylogenetic trees based on comparing individual samples of DNA. Due to its relatively low cost and ease of set-up and operation, AmpFLP remains popular in lower income countries.

1.4.5. Variable Number Tandem Repeat (VNTR) Analysis

A Variable Number Tandem Repeat (VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat. These can be found on many chromosomes, and often show variations in length between individuals. Each variant acts as an inherited allele, allowing them to be used for personal or parental identification. Their analysis is useful in genetics and biology research, forensics and DNA fingerprinting.

There are two principal families of VNTRs: microsatellites and minisatellites. The former are repeats of sequences less than about 5 base pairs in length (an arbitrary cut off), while the latter involve longer blocks. It has five applications in fisheries and aquaculture:

1. Species Identification
2. Genetic variation and population structure study in natural populations

3. Comparison between wild and hatchery populations

4. Assessment of demographic bottleneck in natural population

5. Propagation assisted rehabilitation programmes.

1.4.6. Restriction Fragment Length Polymorphism (RFLP) Analysis:

RFLP (Restriction Fragment Length Polymorphism) is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated. In RFLP analysis, the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis. Although now largely obsolete due to the rise of inexpensive DNA sequencing technologies, RFLP analysis was the first DNA profiling technique inexpensive enough to see widespread application. In addition to genetic fingerprinting, RFLP was an important tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing.

Most RFLP markers are co-dominant (both alleles in heterozygous sample will be detected) and highly locus-specific. An RFLP probe is a labelled DNA sequence that hybridizes with one or more fragments of the digested DNA sample after they were separated by gel electrophoresis, thus revealing a unique blotting pattern characteristic to a specific genotype at a specific locus. Short, single- or low-copy genomic DNA or cDNA clones are typically used as RFLP probes. The RFLP probes are frequently used in genome mapping and in variation analysis (genotyping, forensics, paternity tests, hereditary disease diagnostics, etc.).

1.5. Fish Blood and Scales as an Ideal Source of DNA

Fish blood and scales are an excellent and reliable source of high quality DNA with a number of advantages. In most cases DNA is obtained from the collection of blood from the fish. In the case of rare or endangered species, this method is not advisable. Therefore, fin tissue is often used for the DNA extraction because sampling is relatively fast, logistically simple, and is non-lethal. A small piece of tissue provides plenty of DNA for PCR and restriction digests. Most importantly, collection of fish fin does not harm the fish. This is very important consideration especially relevant regarding rare and the ornamental species. Fish fin samples are used for DNA- based studies on genetic diversity, mating systems and parentage determination of fish populations with minimal disturbances.

There are a number of different procedures for the preparation of genomic DNA. They all start with some form of cell lysis, followed by deproteination and recovery of DNA. The main difference between various approaches lies in the extent of deproteination and in molecular weight of the DNA produced. Methods used to isolate DNA are dependent on the source, age, and size of the sample. Despite the wide variety of methods used, all of them generally aim to separate DNA present in the nucleus of the cell from other cellular components.

The presence of proteins, lipids, polysaccharides and other organic or inorganic compounds in the DNA preparation can interfere with DNA analysis methods. They can also reduce the quality of DNA. The extraction methods to efficiently purify DNA from various sources have to be adapted depending on factors such as sample size, the freshness of the sample, and the biochemical content of the cell from which DNA is being extracted. If the sample is small (for example sperm or a single hair) the method must be different from that used to isolate DNA from a couple of milligrams of tissues or milliliters of blood. The freshness of the sample also affects he extraction technique.

Extraction methods are also variable according to the biochemical content of the source cells. For example, in the case of bacteria, the main biochemical present in a cell extract is protein, DNA and RNA. Therefore, phenol extraction or protease treatment, followed by removal of RNA with ribonuclease, leaves a pure DNA sample. These treatments may not be sufficient if the cell also contain significant quantities of other biochemicals.

1.6. Genome Expression

DNA usually occurs as linear chromosomes in eukaryotes and circular chromosomes in prokaryotes. The set of chromosomes in a cell makes up its genome; the human genome has approximately 3 billion base pairs of DNA arranged into 46 chromosomes. The information carried by DNA is held in the sequence of pieces of DNA called genes. Transmission of genetic information in genes is achieved via complementary base pairing. For example, in transcription, when a cell uses the information in a gene, the DNA sequence is copied into a complementary RNA sequence through the attraction between the DNA and the correct RNA nucleotides. Usually, this RNA copy is then used to make a matching protein sequence in a process called translation, which depends on the same interaction between RNA nucleotides. In alternative fashion, a cell may simply copy its genetic information in a process called DNA replication.

Chapter 2

Review of Literature

The present work dealing with the Molecular Profiling of fishes has been studied across the globe right from early nineteenth century. In order to have an idea about the previous work carried on the aspect it is necessary to have a clear idea about the previous work so an attempt was made to review the available printed literature on the subject. As it is not possible to review all the work done so far, therefore, the important ones   are reviewed in the following pages. In order to have a clear understanding the literature has been revised under two headings.

2.1.   Molecular Profiling of fishes.

2.2.   RAPD analysis in fishes.

2.1. Molecular Profiling of fishes

This review will deal with some general features of fish genomes, briefly covering investigations carried out in the laboratory more than half a century ago. This line of investigation started just after we had shown that the CsCl (Cesium Chloride) "main band" of the bovine genome was not characterized by a continuous variation of GC levels (GC is the molar ratio of guanine and cytosine in DNA), as generally believed, but could be fractionated into three "major DNA components" by using preparative ultracentrifugation in $Cs_2SO_4/Ag+$ (Filipski *et al.*, 1973). Calf DNA preparations having molecular weights of 5 to 7×10^6 have been fractionated by preparative Cs_2SO_4—Ag+

density gradient centrifugation into a number of components. These may be divided into three groups:

i. The main DNA component (1.697 g/cm3; all densities quoted are those determined in CsCl density gradients), the 1.704 and 1.709 g/cm3 components form about 50, 25 and 10% of the genome, respectively; they are characterized by having symmetrical CsCl bands and melting curves, both of which have standard deviations close to those of bacterial DNAs of comparable molecular weight, and by their G + C contents being equal to 39, 48 and 54%, respectively; after heat-denaturation and reannealing, their buoyant densities in CsCl are greater than native DNA by 12, 10 and 3 mg/cm^3, respectively.

ii. The 1.705, 1.710, 1.714 and 1.723 g/cm^3 components represent 4, 1.5, 7 and 1.5% of the DNA, respectively, and exhibit the properties of "satellite" DNAs; their CsCl bands and melting curves have standard deviations lower than those of bacterial DNAs; after heat-denaturation and reannealing, their buoyant densities are identical to native DNA, except for the 1.705 g/cm^3 component, which remains heavier by 5 mg/cm^3; in alkaline CsCl, only the 1.714 g/cm^3 component shows a strand separation.

iii. A number of minor components, forming 1% of the DNA, have been recognized, but they have not been investigated in detail; two of them (1.719 and 1.699 g/cm^3) might correspond to ribosomal cistrons and mitochondrial DNA, respectively.

This approach had previously allowed us to achieve a complete separation of the major satellites of mouse and guinea pig from the corresponding main bands by using a sequence specific ligand, Ag+, which differentially binds to the short sequences forming the two satellites (Corneo *et al.*, 1968).

During the **first phase** of research on fish genomes (1976–1991), the main experimental approaches consisted in analytical and preparative ultracentrifugation in CsCl and in Cs_2SO_4/Ag+, respectively (Ag+ was later followed by another sequence-specific ligand, BAMD, bis (acetato-mercury-methyl) dioxane). After an **intermezzo** (1992– 2001) in which investigations increasingly took advantage of the available nucleotide sequences, a **second phase** (2002–present) developed on the basis of completely (or almost completely) sequenced fish genomes.

2.1.1. The first phase

It is a very rare event, yet it may happen, that the very first experiment in a given field of research is a revelation. This was the case for the first analytical ultracentrifugation ever done on a fish DNA, that of toadfish (*Opsanus tau*), which showed the most symmetrical CsCl profile exhibited by a vertebrate DNA. The sample came from Woods Hole, through the courtesy of Audrey Haschemeyer, who had been working on that fish for different reasons. The perfect symmetry of the CsCl profile was in sharp contrast with the strong asymmetry of DNAs from all the 13 warm-blooded vertebrates explored. Since very low asymmetries were also found in another fish, salmon (*Salmo irideus*), in two amphibians, and in two reptiles (although to a lesser extent in the case of *Testudo graeca*), it was concluded, on the basis of the striking differences between the genomes of cold- and warm-blooded vertebrates, that "large-scale changes in the genome organization have taken place during the evolution of vertebrates" (Thiery *et al.,* 1976). As far as fishes are concerned, the analytical ultracentrifugation approach was later extended to 33 species from 12 orders of Teleostei and 1 order of Chondricthyes (Hudson *et al.,* 1980) and later on, to 122 species from 21 orders of Osteichthyes and 3 orders of Chondricthyes (Bernardi and Bernardi, 1990a,b, 1991). The Cs_2SO_4 preparative ultracentrifugation in the presence of sequence specific ligands was also used in several cases. While detailed results from this first phase of fish genomics

can be found in the papers, the major conclusions that could be drawn were the following:

(i)   The compositional heterogeneity and band symmetry of fish DNAs explored by Hudson *et al*., (1980) was comparable to those of bacterial genomes and of the isolated "major components" of DNAs from warm-blooded vertebrates, namely the DNAs from individual isochore families; isochores, first so named by Hudson *et al*., (1980), are the long, fairly homogeneous genome regions, originally estimated as longer than 300 kb, from which DNA fragments are derived during preparation; (Macaya *et al*., 1976);

(ii)   One fish, *Arothron diadematus*, having the typical small genome of Tetraodontids (0.4–0.5 pg; Hinegardner and Rosen, 1972), was shown to comprise "single copy" sequences that represented as much as 87% of its DNA (Pizon *et al*., 1984);

(iii)   The genomes of fishes living at high temperatures and those which live in temperate waters showed differences in their genomes, the former exhibiting GC-rich components that were absent in the latter (Bernardi and Bernardi, 1986);

(iv)   A positive correlation was found between increasing DNA heterogeneity of fish genomes and increasing staining contrast of chromosomal bands (Medrano *et al*., 1988);

(v)   The modal buoyant density of all species investigated covered a very broad range, 1.695–1.708 g/cm3, corresponding to a GC range of 36% to 49% ;

(vi)   Different ranges of average GC were found in different fish orders, some orders (Cyprinodontiformes) exhibiting wide, others (Cypriniformes) narrow ranges;

(vii)   A negative correlation was found between GC and genome size (the last three conclusions were drawn by Bernardi and Bernardi, 1990 a, b).

The investigations just mentioned established some basic points, which definitely differentiated fish genomes from the genomes of warm-blooded vertebrates. Indeed, while the genomes of warm blooded vertebrates were compositionally very heterogeneous (five major components could be fractionated from a typical mammalian genome, the human genome; Bernardi *et al*., 1985; Costantini *et al*., 2006), the genomes of fishes were very homogeneous, often more homogeneous than bacterial genomes (in spite of the fact that the latter are about 100 times smaller in size than the genome of Tetraodontids) or single major components from mammalian genomes (Hudson *et al*., 1980). On the other hand, the broad compositional range of the modal buoyant densities (and average GC levels) of fish genomes was in sharp contrast with the very narrow range covered by mammalian and avian genomes. The results obtained on the Tetraodontid fish showed that at the low end of the broad spectrum of genome sizes of fish, interspersed repeated sequences had almost disappeared and intergenic sequences had shrunk very considerably (Pizon *et al.,* 1984). Indeed, the negative correlation holding between GC and genome size is probably due to the progressive decrease of intergenic and intronic sequences that are GC-poorer than coding sequences. An evidence for the appearance of GC rich components in the genomes of fishes living at high temperatures was obtained, mimicking somehow the genomes of warm-blooded vertebrates (Bernardi and Bernardi, 1986). While for a long time the chromosomes of fishes and amphibians were considered to show no banding (Cuny *et al*., 1981), it was demonstrate that Giemsa banding became increasingly apparent as DNA heterogeneity increased (Medrano *et al*., 1988). All these properties were discovered by using classical approaches of molecular biology, such as ultracentrifugation, reassociation kinetics and

Giemsa staining. The points mentioned so far were established between 1976 (Thiery *et al*., 1976) and 1991 (Bernardi and Bernardi, 1990 a, b, 1991) in the Laboratory of Molecular Genetics of the Jacques Monod Institute in Paris (1998). The work started in Laboratory of Molecular Evolution in the Stazione Zoologica of Naples, the findings raised important questions as to what happened in evolution at the transition between cold- and warm-blooded vertebrates (Bernardi, 2004, 2007).

2.1.2. Intermezzo

The time between the first and the second phase of fish genomics saw some additional progress in terms of general conclusions. The main findings were:

(i)   That in the case of Tetraodontiformes it was not only the intergenic sequences that decreased in amount, as already shown by Pizon *et al.,* (1984), but also the introns (Brenner *et al*., 1993);

(ii)   That gene density, already shown to be bimodal in the human genome (since a high gene concentration characterized the GC-rich "genome core", a low gene concentration the GC-poor "genome desert"; Mouchiroud *et al*., 1991; Zoubak *et al*., 1996), also was bimodal in the much less heterogeneous genomes of cold-blooded vertebrate (Caccio *et al.*, 1994);

(iii)   That data from the literature indicated a higher CpG level in fishes and amphibians compared to mammals and birds (Jabbari *et al*., 1997);

(iv)   That two different methylation levels existed in vertebrates, a lower one in mammals and birds and a higher one in amphibians and fishes (Jabbari *et al*., 1997); in fact, this correlation between body temperature and methylation level, not only separated warm- from cold-blooded vertebrates, but, as shown later by Varriale and Bernardi (2006) also fishes living at different temperatures;

(v)   That increasing the number of fish genomes explored from 122 to 201 (Bucciarelli *et al*., 2002) did not show significant differences in the properties defined by Bernardi and Bernardi (1990 a), suggesting that the features established by these investigations were unlikely to change by further increasing the fish sample.

2.1.3. The second phase

The availability in the last few years of largely or fully sequenced fish genomes, those of zebrafish (*Brachidanio rerio*), medaka (*Oryzas latipes*), stickleback (*Gasterosteus aculeatus*) and pufferfish (*Tetraodon nigroviridis*) allowed to investigate in detail these genomes in both isochore patterns and gene distribution. Fortunately, these fishes belong to four distant orders and their genomes are spread in composition over almost the entire GC range of fish genomes so making the results generally valid.

These investigations produced isochore maps, using the approach previously applied to the human genome (Costantini *et al*., 2006) that were drastically different not only from those of mammals (in that only two major isochore families were essentially present in each genome vs. five in the human genome), but also from each other, in that different isochore families were represented in different genomes (Costantini *et al.,* 2007). Obviously, this explained the previous observations of a narrow spread of DNA heterogeneity in fish genomes, as well as the wide range of average GC levels. Moreover, it also confirmed the negative correlation of such GC levels with genome size.

Gene density distributions for these fish genomes were also obtained and shown to follow the expected increase in gene density with increasing isochore GC although with a shallower gradient compared to the human genome. The only exception was zebrafish, in which the L1 isochore family had a higher gene concentration than the L2 family; this may be however due to the relatively small gene sample used and the small amount of DNA in L2 isochores. Finally, a remarkable conservation of the average size of the

isochores (which match replicon clusters in case of human chromosomes (Costantini and Bernardi, 2008); and the average GC levels of isochore families in both fish and human genomes (Costantini *et al*., 2007). These recent results are of interest for at least two different reasons. The first one is that they confirmed and extended previous findings (Bernardi and Bernardi 1990 a, b). The second reason is that they led to the discovery of some novel features of isochores that have important implications as far as genome evolution is concerned.

Along the first line, one should mention the results concerning the compositional patterns. Indeed, the two major compositional features of fish genomes, the wide intergenomic spread of base composition and the narrow intragenomic distribution were confirmed on the basis of genome sequences. This provided a more detailed picture as compared to the results obtained by the ultracentrifugation approach previously used. In each fish genome essentially only two isochore families were present, one of them being predominant (although less in case of pufferfish), whereas in the human genome five families are present. On the other hand, the compositional spread of the fish genomes analyzed was so wide that there was essentially no overlap between the isochore families of zebrafish and pufferfish. Both the narrow intra genomic distribution and the wide compositional spread of fish genomes can be understood in terms of adaptation to environmental factors. While the narrow intra genomic distribution may be visualized as an adaptation to a particular ecological niche, the wide compositional spread implies not only a response to changes in environmental conditions and new adaptations, but also the existence of compositional transitions involving whole genomes. As already pointed out (Bernardi and Bernardi, 1990b), such transitions may occur among the genomes of fishes belonging to different orders, or even to different families and genera, independent of geological time. In some cases, selective advantages linked to compositional transitions could be identified. For

instance, the differences found between two Tetraodontids, *Fugu* and *Tetraodon*, may be correlated with the different body temperatures of these closely related fishes. The former "cold" marine fish showed distributions of DNA (Jabbari and Bernardi, 2004) and coding sequences that do not reach the high GC level as in the case of the latter, a tropical freshwater fish. Another example, also associated with body temperature differences, is the higher GC level attained by *Gillichthys seta*, a gobid living in tide pools with temperatures up to 44 °C, vs. *Gillichthys mirabilis*, a congeneric species living at about 10–20 °C (Bucciarelli *et al*., 2002).

As far as, novel features of isochores are concerned, one should mention:

(i)   The conservation of isochore size in vertebrates may be explained by the fact that human isochores are replicon clusters (Costantini and Bernardi, 2008), with GC-rich isochores correspond to early replicating units, GC-poor isochores to late replicating units and

(ii)   That while the "whole-genome shifts" lead to different patterns of isochore families, the latter are centered on the same GC values from fish to human. The reasons for such conserved properties are being investigated. In any case, the newly discovered features of isochores which are evolutionarily conserved in vertebrates reinforce the idea that isochores represent "a fundamental level of genome organization" (Eyre-Walker and Hurst, 2001).

The common carp (*Cyprinus carpio* L.) is the most extensively cultured fish species in China and throughout the world (Sun and Liang, 2004). Although the production value of this species has dramatically increased in recent decades, there has been no corresponding advance in genetic research or improvement of common carp breeding. The field of aquaculture has also developed rapidly in recent decades.

However, few studies have focused on the genetics of common carp with high production capabilities or the possibility of adverse effects on elevated production. To provide a framework for further in-depth genetic studies, it is necessary to construct genetic linkage maps. Such in-depth genetic studies may include the mapping of interested commercial traits to a particular region of a genome, comparative genomics, physical cloning of mutants, and whole genome sequencing (Cnaani *et al*., 2004; Lander and Botstein, 1989; Naruse *et al*., 2004). Fortunately, significant advances have been made in the construction of genetic linkage maps and the identification of QTL locations of common carp in the last few decades. Developments include the construction of three genetic linkage maps using molecular markers (Cheng *et al*., 2009; Sun and Liang, 2004; Zhang *et al*., 2010) and comparative maps (Zheng *et al*., 2011 a). In addition, important commercial traits of the common carp have also been identified on these maps in recent years. Genetic analysis of German mirror carp (Hou *et al*., 2007) has been carried out and the genes involved in the control of quantitative traits by QTL.

However, there are some problems with these previous studies. SNPs (single-nucleotide polymorphisms) are the most common type of variation in the genome. SNPs provide the best genome coverage for analyzing performance and production of traits. Genome with high-density SNP coverage is a powerful tool for whole genome association studies because it allows the detection of linkage disequilibrium (Liu *et al*., 2011).

2.2. Random Amplification of Polymorphic DNA (RAPD) Analysis in Fishes

In less than half a century, molecular markers have totally changed the view of nature and in this process they have evolved themselves. Within the last decade, technological advancement has increasingly supported the use of genetics in determining population diversity. Many molecular techniques are now available, which allow ecologists and evolutionary biologists to determine

the genetic architecture of a wide variety of closely related individuals. Recently, the development of molecular techniques has created new possibilities for the selection and genetic improvement of livestock. The discovery of the PCR had a major impact on the research of eukaryotic genomes and contributed to the development and application of various DNA markers (Mulcahy *et al.*, 1993; Marle-Koster and Nel, 2003; Schlotterer, 2004). However, progress is limited by availability of useful genes or identified markers that are linked to important traits. Thus, identification of sufficient numbers of molecular markers is critically important for gene mapping, for marker assisted selection (MAS) and for eventual cloning of beneficial genes from different fish species (Liu *et al.*, 1999).

Molecular markers derived from polymerase chain reaction (PCR) amplification of genomic DNA are an important part of the toolkit of evolutionary geneticists. RAPD (randomly amplified polymorphic DNA) markers that were shown to genetically link to a trait of interest could be used for individual and pedigree identification, pathogenic diagnostics and trait improvement in genetics and breeding programmes (Yoon and Kim, 2001; Holsinger *et al.*, 2002). Random oligonucleotide primers produce RAPDs that have been used extensively as molecular markers (Koh *et al.*, 1999; Shikano and Taniguchi, 2002). RAPDs also have the advantage that no prior knowledge of the genome is necessary for successful application (Welsh and McClelland, 1990; Williams *et al.*, 1990; Fischer *et al.*, 2000; Klinbunga *et al.*, 2000). Information on the genetic structure of fish species is useful for optimizing identification of stocks, stock enhancement, breeding programs, management for sustainable yield and preservation of genetic diversity (Dinesh *et al.*, 1993; Garcia and Benzie, 1995; Tassanakajon *et al.,* 1997, 1998). RAPD markers have been used for phylogenetic studies for species and subspecies identification of fish (Bardakci and Skibinski, 1994; Borowsky *et al.*, 1995; Sultmann *et al.*, 1995; Partis and Wells, 1996), for gynogenetic fish

identification (Chen and Leibenguth, 1995; Corley-Smith *et al*., 1996) and for gene mapping studies in fish (Postlethwait *et al*., 1994; Kazianis *et al*., 1996). DNA polymorphisms have been extensively employed as a means of assessing genetic diversity in aquatic organisms. RAPD fingerprinting offers a rapid and efficient method for generating a new series of DNA markers in fishes (Foo *et al*., 1995).

2.2.1. Random Amplification of Polymorphic DNA Analysis in Carp:

Common carp (*Cyprinus carpio*) varieties (e.g. races, strains, breeds and stocks) ''developed through a combination of forces including geographic isolation, adaptation accumulation of mutations and natural as well as human selection pressures'' (Hulata, 1995). Classical taxonomic analysis divides the currently existing common carp forms into three categories:

  i.   European (*Cyprinus carpio carpio*),

 ii.   Far Eastern (*C. carpio haematopterus*) and

iii.   South East Asian (*C. carpio viridiviolaceus*) (Kirpitchnikov, 1999).

The Middle and Eastern European region is home for many common carp varieties. However, their origin and relatedness are not well described. Despite the commercial importance of the species, genetic data on common carp stocks are relatively scarce. Analysis of protein polymorphisms was performed on some populations (Csizmadia *et al*., 1995; Kohlmann and Kersten, 1999). However, reports have only been published recently on common carp genotypes using RAPDs (Dong and Zhou, 1998) and microsatellite markers (Crooijmans *et al*., 1997; Aliah *et al*., 1999; Tanck *et al*., 2000; 2001; Desvignes *et al*., 2001; David *et al*., 2001; Lehoczky *et al*., 2002). The genetic analysis of two famous common carp varieties from Hungary (the entire Attala and Dinnyes broodstocks) and their comparison with over 100 individuals collected from various other sources has been reported by Bartfai *et al*., (2002). Results obtained with both types of DNA markers showed lack of major differences between the genetic structures of the two stocks:

heterozygosity values and allele frequencies were very similar. Concerns about the four commercially important Indian carp species (*rohu, catla, mrigal and calbasu*) belong to the Family Cyprinidae and Order Cypriniformes (Talwar and Jhingran, 1991). These species may have a common ancestral origin (Khanna, 1988). Barman *et al.,* (2002) evaluated the use of the RAPD assay as a source of genetic markers to generate species-specific RAPD profiles for four species of Indian carp and to estimate genetic variation among these four species. They demonstrated that *calbasu* is the closest to rohu and the farthest from *mrigal.* Common carp (*Cyprinus carpio L.*) belongs to Cyprinidae, the largest family among freshwater teleosts (Nelson, 1994), for which the world's annual total catch in 1999 was estimated above 15.6 million metric tons, compared to the 2.3 million tons of salmonids (FAO, 2001).

The silver crucian carp (*Carassius auratus gibelio*), is a triploid gynogenetic species, which provides a unique model system for understanding evolutionary genetics and for elucidating the regulatory mechanisms underlying diverse reproduction modes in vertebrates (Yang *et al*., 1999; Zhou *et al*., 2000a). Several features, such as the existence of males (Fan and Shen, 1990) and two reproduction modes (Zhou *et al*., 2000b), place this particular silver crucian carp on an intermediate evolutionary step between species with uni and bisexual reproduction systems. A total of 88 polymorphic fragments were scored from 24 primers after excluding bands that were monomorphic in all five clones. Zhou *et al*., (2001) selected three RAPD markers (RA1-PA, RA2-EF and RA4-D) produced by Opj-1, and two RAPD DNA fragments (RA3-PAD and RA5-D) produced by Opj-7 for molecular cloning and sequencing. Sequence data indicated that there were identical 801-bp nucleotide sequences in the shared marker RA1-PA cloned respectively from clones P and A. Further studies on carp could help ecologists and biologists to determine not only their taxonomic status but the genetic diversity of their populations and their relatedness to domesticated stocks (Bartfai *et al*., 2002).

2.2.2. Random Amplification of Polymorphic DNA Analysis in Catfish

Catfish (*Ictaluridae*) is a commercially important warm water fish species, which is distributed all over the world. Consequent to the rapid increase in hatchery-reared catfish production, there is a need to understand the genetic composition of natural catfish populations in order to evaluate the range and exact of latent genetic effects induced by hatchery operations. Little information is available on the phylogenetic relationships among the few catfish populations in Korea. RAPD technique is one of the most frequently used molecular methods for taxonomic and systematic analyses of various organisms (Bartish *et al.*, 2000), and has provided important applications in catfish.

The channel catfish (*Ictalurus punctatus*) is the most important cultured fish in the United States, accounting for over 50% of all aquaculture production. It is also one of the important sporting fish in many southern states. Liu *et al.*, (1998) studied the segregation of RAPD markers in F1 hybrids and backcross hybrids in this species to evaluate the feasibility of using RAPD markers for both intra specific mating plans and inter specific hybrid mating plans. An additional 100 RAPD primers were evaluated for their usefulness in catfish. Of the 100 primers, 42 were good; 33 were of medium quality; and 25 were poor (Liu *et al.*, 1999). A total of 462 new polymorphic RAPD markers were identified in this study. Additionally, no differences were detected in RAPD profiles between the two reciprocal F hybrids despite dramatic phenotypic differences between the two reciprocal hybrids due to paternal predominance (Dunham *et al.*, 1982). One hundred randomly amplified polymorphic DNA RAPD primers have been used to identify DNA-based genetic polymorphism for constructing a genetic linkage map of catfish. Overall polymorphism was low among strains within a species for both channel catfish and blue catfish (*Ictalurus furcatus*). However, considerably higher

levels of polymorphism were detected between channel catfish and blue catfish.

Chong *et al*., (2000) represented the first application of the amplified fragment length polymorphism (AFLP) technique and the RAPD technique in the study of genetic variation within and among five geographical populations of the Malaysian river catfish (*Mystus nemurus*). Nine RAPD primers detected a total of 42 polymorphic markers, respectively. Genetic similarity and diversity of cultured catfish (*Silurus asotus*) populations collected from two areas in western Korea were examined using RAPD-PCR (Yoon and Kim, 2001). Polymorphic bands in these populations ranged from 56.4 to 59.6%. Genetic similarity in cultured catfish populations may have been caused because individuals from two populations were reared in the same environment or by inbreeding over several generations. Kovacs *et al*., (2000), searched for sex-specific DNA sequences in the male and female genomes of African catfish by comparative RAPD assays performed on pooled DNA samples. Two sex-linked RAPD markers were identified from the male DNA pool and confirmed on individual samples, showing good agreement with phenotypic sex.

Chapter 3

Materials and Methods

In the present study, the molecular profiling of *Cyprinus carpio communis*, *Cyprinus carpio specularis* and *Onchorhynchus mykiss* was evaluated by doing the Random Amplification of Polymorphic DNA (RAPD**)** of the isolated genomic DNA. Two DNA extraction Procedures were followed for two different sources of DNA. The **Gen Elute ᵀᴹ Blood Genomic DNA Kit (Sigma Aldrich: Cat. No.NA2000**) was used to extract DNA from the blood of the fish. The method of Wasko *et al*., (2003) was also used to isolate the DNA from the scales of the *Oncorhynchus mykiss.* The fishes were acclimatized to the standard laboratory conditions. After acclimation, the DNA was isolated, checked for the purity, quantitation was done and then RAPD was carried out for the three fishes namely *Cyprinus carpio communis*, *Cyprinus carpio specularis* and *Oncorhynchus mykiss*. The entire experiment was designed in three parts as follows:

3.1. Part-1

This part includes collection, identification and acclimation of the fishes by standard methods. This part includes the following steps:

3.1.1. Experimental Animal

Two species of *Cyprinus carpio* L. (family: *Cyprinidae*), i.e. *Cyprinus carpio communis* and *Cyprinus carpio specularis* and Rainbow trout (*Oncorhynchus mykiss*) were chosen for the study because of their common availability throughout the year and edibility in Kashmir. Young specimens of

Cyprinus carpio communis, Cyprinus carpio specularis and *Oncorhynchus mykiss* (age: 1 year, weight: 300-400g and length: 20-25cm) were used in all experiments.

3.1.2. Identification of the Experimental Animal

The common carp can be distinguished from other Cyprinids by the heavy and strongly serrate spines in the anterior portion of its dorsal and anal fins, and by the presence of two rather long, fleshy barbles on each side of its upper jaw (Douglas, 1974). The mouth is terminal in the adult and sub terminal in the young (Page and Burr, 1991), colour and proportions are extremely variable, but scales are always large and thick. The different species i.e. *Cyprinus carpio communis* (Scale Carp; Plate 1) has regular concentric scales and *Cyprinus carpio specularis* (Mirror Carp; Plate 2) has large scales running along the side of the body in several rows with the rest of the body naked (Mcrimmon, 1968).

The following characters helped in the identification of the two *Cyprinus* fishes:

- The mirror carp, Cyprinus carpio specularis has its body covered, unevenly with large and bright scales. A large area of body is however naked.
- The scale carp, Cyprinus carpio communis has its body fully covered by regular arranged rows of scales.
- Cyprinus carpio specularis is an illophage, i.e. feeds at the bottom on decayed organic matter.
- The caudal fin of Cyprinus carpio communis is deeply emarginated.
- The caudal fin of Cyprinus carpio communis has 3 spines and 17-19 rays.

The *Oncorhynchus mykiss* (Plate 3) is a member of the salmon family of fishes and has the following characteristics:

- An elongate, laterally compressed body;

- A rounded snout, which becomes extended and the lower jaw turns up in breeding males;

- The back, upper sides and the top of the head are steel blue, blue-green, yellow-green to almost brown;

- The sides are silvery, white or pale yellow-green to grey, and marked with a pink blush to red band and many small black spots;

- The underside is silvery, white or grey to yellowish;

- The dorsal and caudal fins have radiating rows of black spots, while the remaining fins are buff with few spots; and stream dwelling and spawning brown trout display darker, more intense colours, whereas lake residents are lighter, brighter and more silvery.

3.1.3. Collection Site and Fish Collection

Adult specimens of the freshwater fishes *Cyprinus carpio communis* and *Cyprinus carpio specularis* were collected from the Dal Lake (north east Srinagar, 34^0 5'- 34^0 6'N latitude and 74^0 81'- 74^0 9'E longitude) by the local fishermen and then they sold them in the local market of Hazratbal, Srinagar.

The fishes were collected alive from the local fishermen during early morning hours (Plate 4). Fishes after collection were transported in specially designed containers with water.

The Rainbow trout was collected from the Trout farm at Harwan Srinagar and was brought to the lab in containers filled with water (Plate 5).

3.1.4. Acclimatization of the Fishes

After collection, the fishes were acclimatized to the laboratory conditions for 15 days. Specimens were kept in polypropylene troughs each with 2-3 individuals/30L of water. Water was kept oxygen saturated by aeration. The troughs were cleaned and the water was renewed regularly. Qualities of water (pH= 7.3± 0.6; Dissolved Oxygen=7.3 ± 0.4 ppm; Free CO_2 = 5.8± 0.4 ppm; Alkalinity= 106± 6.8 ppm) were measured according to the methods of APHA/ AWWA/ WPCF, (1998). Fishes were fed with commercial feed daily at least one hour prior to the replacement of the water.

3.2. Part-II

This part includes the isolation and quantitation of the DNA from the experimental animal by following standard protocols. This part includes the following steps:

3.2.1. Collection of Blood Sample from the Fish:

Prior to the collection of blood, all the glassware's and the tubes were kept in an autoclave to avoid the contamination of the bacterial and viral DNA. Blood samples were freshly collected from the fishes by puncturing the caudal vein with a micro syringe or by decapitating the caudal portion and exposing the blood vessel. This fresh blood was collected in the collection tubes in which 1% EDTA was poured as an anticoagulant in order to avoid the clotting of the blood.

3.2.2. Reagents and Solutions

The Gen Elute ^{TM} Blood Genomic DNA Kit (Sigma Aldrich; Cat. No. NA2000) provides the following reagents and solutions which were used for the isolation and purification of the DNA from the blood samples. The kit provides the following reagents and solutions for ten samples (Table 3.1):

- Resuspension Solution

- Lysis Solution C

- Column Preparation Solution

- Prewash Solution

- Wash Solution

- Elution Solution (10mM Tris-HCl, 0.5mM EDTA, pH 9.0)

- Proteinase K

- RNase A Solution

- Ethanol (95-100%)

3.2.3. Isolation of the DNA from the Blood of the Fish:

The isolation of the DNA was carried out by using Gen Elute ^{TM} Blood Genomic DNA Kit (**Sigma Aldrich: Cat. No. NA2000**). It provides high yields of remarkably pure DNA from blood samples of fishes which is suitable for most downstream processes. The protocol employs a single purification step to remove contaminating compounds, using a column preparation solution, wash solution and elution solution.

Concisely, the extraction procedures for fresh samples were done in the following manner:

1.   The blood was collected by puncturing a micro syringe into the caudal vein and transferred to EDTA anticoagulant tubes.

2.   The blood was then equilibrated to room temperature before beginning preparation.

3.   200µl of blood was transferred to 1.5ml microcentrifuge tube and 20µl of Proteinase K solution was added to the tube.

4.   For RNA-free genomic DNA, 20µl of RNase A solution was added.

5.   200µl of Lysis Solution C was added to the sample and vortex thoroughly for 15 seconds.

6.   The sample was then incubated for at 55° C for 10 minutes in a water bath.

7.   500 µl of Column Preparation solution was added to each pre-assembled GenElute Miniprep Binding Column and centrifuge at 12,000g for 1 minute. The flow was discarded through liquid.

8.   200µl of Ethanol was added to lysate (step 6). The solution was mixed thoroughly by vortexing for 5-10 seconds.

9.   Transfer entire content (of Step 8) into the treated column (Step 5).

10.   The Column was Centrifuge at 6500g for 1 minute and discard the tube containing flow-through liquid and place the column in a new 2 ml collection tube.

11.   First Wash of solution was done by adding 500 µl of Prewash Solution to the column and centrifuge at 6500g for 1 minute.

12.   For second Wash, 500µl of Wash Solution was added to the column.

13.   The Column was centrifuge for 3 minutes at 12000-16000g.

14.   DNA was eluted by adding 200µl of the Elution Solution directly into the centre of the Column.

15.   The Column was then centrifuged for 1 minute at 6500g.

To increase elution efficiency, the Column was incubated for 5 minutes at room temperature after adding the Elution Solution, then centrifuge.

The elute contains pure genomic DNA. The DNA can be stored for short term at 2 to 8^0 C and for long term storage, -20^0 C temperatures is recommended.

3.2.4. Isolation of the DNA from the Scales of the Fish:

Scale Collection

For the present study, the scales have also been used from Rainbow trout (*Oncorhynchus mykiss*). The scales were collected non-invasively by gentle scrapping on the caudal portion of the body with the forceps and the detached scales were collected in 2 ml tubes.

DNA was extracted from the fresh scales of the fish by using the following method. The protocol was followed according to Wasko *et al.,* (2003) with modifications.

1. Approximately 50 mg of scales were taken from the species and dried on a filter paper.

2. The scales were then cut into small pieces and placed in a 2 ml-Eppendroff tube containing 940 µl lysis buffer (200mM Tris HCl, pH-8.0; 100mM EDTA, pH- 8.0; 250mM NaCI), 30 µl Proteinase K (10mg/ml) and 30 µl 20% SDS.

3. The contents in the tubes were incubated at 48^0 C for 45-50 minutes in a water bath. The appropriateness of the incubation temperature was studied.

4. After incubation, an equal volume of phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the tube containing lysed scale cells.

5. The contents were then mixed properly by gently inverting the tube for 10 minutes to precipitate the proteins and other part of the nucleic acids.

6. The tubes were then rotated for 10 minutes at 9,200g. The top aqueous layer was transferred to a new 1.5 ml-Eppendroff tube, leaving interface and lower phase.

7. The DNA was then precipitated by adding equal volume of Isopropanol and 0.2 volume of 10M ammonium acetate and inverting the tubes gently several times.

8. The precipitated DNA was then pelleted by centrifugation at 13,200g for 10 minutes.

9. The supernatant was removed by pouring out gently, taking care to avoid loss of DNA pellet.

10.   The pellet was then washed briefly in 500 µl chilled 70% ethanol, air dried and resuspended in 20 µl sterile water/TE buffer.

3.3.   Quality Assessment of the Extracted Genomic DNA (gDNA):

After isolation of DNA, quantification and analysis of quality are necessary to ascertain the approximate quality of DNA obtained and the suitability of DNA sample for further analysis. This is important for many applications including gel electrophoresis, digestion of DNA by restriction enzymes or PCR amplification of target DNA.

Using the Beer Lambert Law it is possible to relate the amount of light absorbed to the concentration of the absorbing molecule. At a wavelength of 260 nm, the average extinction coefficient for double-stranded DNA is 0.020 $(\mu g/ml)^{-1} cm^{-1}$, for single-stranded DNA it is 0.027 $(\mu g/ml)^{-1} cm^{-1}$, for single-stranded RNA it is 0.025 $(\mu g/ml)^{-1} cm^{-1}$ and for short single-stranded oligo nucleotides it is dependent on the length and base composition. Thus, an optical density (OD) of 1 corresponds to a concentration of 50 µg/ml for double-stranded DNA. This method of calculation is valid for up to an OD of at least 2.

Spectrophotometric Determination:

The concentration of DNA sample can be checked by the use of UV spectrophotometry. Both RNA and DNA absorb UV light very efficiently making it possible to detect and quantify either at concentrations as low as 2.5ng/µl. The nitrogenous bases in nucleotides have an absorption maximum at about 260nm. Using a 1-cm light path, the extinction co-efficient for nucleotides at this wavelength is 20. The ratio of OD_{260} / OD_{280} should be determined to assess the purity of the sample. This method is however limited by the quantity of DNA and the purity of the preparation. Accurate analysis of the DNA preparation may be impeded by the presence of impurities in the sample or if the amount of DNA is too little. Based on this extinction coefficient, the absorbance at 260 nm in a 1-cm quartz cuvette of a 50µg/ml

solution of a double stranded DNA or a 40μg/ml solution of a single stranded RNA is equal to 1.

Procedure

The Spectrophotometric determination of DNA was done in the following steps:

1. 3 ml TE buffer was taken in a quartz cuvette for calibrating the spectrophotometer at 260 nm and 280 nm.
2. TE buffer was used as a blank in other cuvette of the spectrophotometer.
3. The DNA sample was diluted to 10-100 times with TE buffer and was mixed well.
4. The OD values at 260nm and 280 nm was noted on spectrophotometer.
5. The quantity of the DNA was calculated by the following formula

   Concentration of dsDNA = 50 x OD at 260 μg/ml.
6. The OD_{260} / OD_{280} ratio was also calculated.

A ratio in between 1.8-2.0 denotes that the absorption in the UV range is due to nucleic acids. A ratio lower than 1.8 indicates the presence of proteins and other UV absorbers like aromatic amino acids. A ratio higher than 2.0 indicates that the sample may be contaminated with phenol or chloroform.

3.4. Part-III

3.4.1. Basic Principle of PCR technique for RAPD analysis

RAPD analysis has been used effectively for initial assessment of genetic variation among fish species. Most parameters (concentration of reaction components, additives, different polymerases and thermal profiles) affecting RAPD-PCR should be examined, in an effort to increase pattern complexity (Diakou and Dovas, 2001). Fraga *et al*., (2002) analyzed the effect of changing concentrations of the primer, template DNA and Taq DNA polymerase with the goal of determining their optimum concentration for the standardization of the RAPD technique for genetic studies.

In RAPD analysis, the target sequences (to be amplified) are unknown. A primer is designed with an arbitrary sequence. In order to have sufficient PCR product, the ideal preconditions are as follows:

1. The primers must anneal in a particular orientation (such that they point towards each other)

2. They must anneal within a reasonable distance of one another.

3. For a RAPD reaction, a large fragment of DNA is used as the template in a PCR reaction containing many copies of a single arbitrary primer.

Polymerase Chain Reaction (PCR) can best be understood by following the principle of DNA replication. DNA polymerase uses single stranded DNA as template for the synthesis of a complementary new strand and it also requires a small section of double stranded DNA to initiate the synthesis. Thus, the starting point of DNA synthesis can be specified by supplying an oligonucleotide primer that anneals to the template at that point. Both strands can serve as templates for synthesis provided an oligonucleotide primer is applied for each strand. For PCR, the primers are chosen to flank the regions of DNA that is to be amplified so that newly synthesized strands of DNA, starting at each primer on the opposite strand. Thus, new primer sites are generated on each newly synthesized DNA strand.

Primer P-12 (Sigma Aldrich; Table 3.2), having the sequence 5'CCGAGCACCG3' was used to amplify the template DNA. Primer provides the free OH group necessary for binding of the DNA Polymerase.

3.4.2. PCR Running Procedure

A PCR programme was performed consisting of the following steps:

i. Initialization Step:

This step consists of heating the reaction to a temperature of $94\text{-}96^0$ C, which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

ii. Denaturation Step

This step is the first regular cycling event and consists of heating the reaction to 94-98^0 C for 20-30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded molecules.

iii. Annealing Step

The reaction temperature is lowered to 50-65^0 C for 20-40 seconds allowing annealing of the primers to the single stranded DNA template. Typically the annealing temperature is about 3-5^0 C below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer- template hybrid and begins DNA synthesis.

iv. Extension or Elongation Step

The temperature at this step depends on the DNA polymerase used; Taq DNA polymerase has its optimum activity temperature at 75-80^0 C, and commonly a temperature of 72^0 C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the extending DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule of thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, the amount of DNA target is doubled, leading to exponential amplification of the specific DNA fragment.

v.   Final Elongation

This single step is occasionally performed at a temperature of 70-74^0 C for 5-15 minutes after the last PCR cycle that any remaining single stranded DNA is fully extended.

vi. Final hold

This step at 4-15^0 C for an indefinite time may be employed for short term storage of the reaction.

3.4.3. Preparation of Master Mix

It involves making of a master mix for total number of PCR reactions to be set up so that the required amount of reaction mix can be distributed for each reaction.

The following components were added in the same order as given below:

Components	Per sample
Double distilled water	17.9 µl
PCR Buffer	1.5 µl
dNTPs mix	1.2 µl
Primer (**P-12**)	1 µl
MgCl$_2$	1 µl
Taq Polymerase	0.4 µl
Total Volume	**23 µl**

Setting up of Single PCR Reaction:

For each PCR reaction, the following components were added in the same order as given:

Components	Per sample
Master Mix	23 µl
DNA	2 µl
Total Volume	**25 µl**

PCR Programme

PCR reactions were carried out in a thermal cycler (Eppendorf: Plate 6) programmed for 94°C for 2 min., 40 cycles of 94°C for 1 min., 36°C for 30 sec., 72°C for 2 min. and finally 72°C for 7 min. The programme was run for 40 cycles.

3.4.4. Gel Electrophoresis

Amplified DNA fragments were separated by electrophoresis at 100 V on 1.2% agarose gel with Tris-borate-EDTA buffer (Sambrook *et al.*, 1989). Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA. The purpose of the gel might be to look at the DNA, to quantify it or to isolate a particular band. The DNA is visualized in the gel by addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and is fluorescent meaning that it absorbs invisible UV light and transmits the energy as visible orange light. Most agarose gels are made between 0.7% and 2%. A 0.7% gel will show good separation (resolution) of large DNA fragments (5–10 kb) and a 2% gel will show good resolution for small fragments (0.2–1 kb). Low percentage gels are very weak and may break when you try to lift them. High percentage gels are often brittle and do not set evenly.

Preparation of 1.2% Agarose gel

Weigh out 1.2 g of agarose into a 250 mL conical flask. Add 100 mL of 0.5xTBE, swirl to mix. It is good to use a large container, as long as it fits in the microwave, because the agarose boils over easily. Microwave for about 1 minute to dissolve the agarose. The agarose solution can boil over very easily. It is good to stop it after 45 seconds and give it a swirl. Leave it to cool on the bench for 5 minutes down to about 60°.

If it is boiled for a long time to dissolve the agarose then some water may be lost as water vapour. While the agarose is cooling, prepare the gel tank ready, on a level surface. Add 5 µl of ethidium bromide and swirl to mix.

The reason for allowing the agarose to cool a little before this step is to minimize production of ethidium bromide vapour. Ethidium Bromide is mutagenic and should be handled with extreme caution. Pour the gel slowly into the tank. Push any bubbles away to the side using a disposable tip. Insert the comb and check that it is correctly positioned. The benefit of pouring slowly is that most bubbles stay up in the flask. Leave to set for at least 30 minutes, preferably 1 hour, with the lid on.

Pour 0.5x TBE buffer into the gel tank to submerge the gel to 2–5 mm depth. This is the running buffer. Add an appropriate amount of loading buffer into each tube and leave the tip in the tube. The loading buffer gives colour and density to the sample to make it easy to load into the wells. Also, the dyes are negatively charged in neutral buffers and thus move in the same direction as the DNA during electrophoresis. This allows us to monitor the progress of the gel. The most common dyes are bromophenol blue. The first well was loaded with 2 µl of the marker.

The Step up ^{TM} 50 bp DNA Ladder (Table 3.3) was used to detect the size of the bands. This ladder consists of 20 bands of double stranded DNA fragments ranging from 50-1000 bp with size increment of 50 bp. The size of the ladder ranges from 0.15kb to 1kb. The other wells were loaded with 2 µl of DNA. The gel tank was closed, switched on the power-source and ran the gel at 100 V.

Visualisation

After the electrophoresis is complete, the DNA may be visualized with ethidium bromide which has intercalated into DNA, fluoresce under ultraviolet light. Photographs were taken of the gels, using Gel Doc (Bio Rad; Plate 7).

The Gel Doc™ XR$^+$ system is based on CCD high-resolution, high-sensitivity detection technology and modular options to accommodate a wide range of samples and support multiple detection methods including fluorescence and densitometry. The system is controlled by Image Lab™ software to optimize imager performance for fast, integrated and automated image capture of samples.

The system accommodates a wide array of samples. The system is an ideal accompaniment to PCR, purification, and electrophoresis systems, enabling image analysis and documentation of restriction digests, amplified nucleic acids, genetic fingerprinting, RAPDs, RFLPs, and protein purification and characterization.

The system supports fluorescence and colorimetric detection methods. The Gel Doc XR+ system consists of a darkroom hood, CCD camera and software-controlled motorized lens, UV and white light illuminators, filter slider with standard filter, and UV-protection shield.

Table 3.1: Various chemicals used for DNA analysis.

S.No	Name of Chemical used
1	Agarose
2.	Ammonium acetate
3.	Bromophenol blue
4.	Chloroform
5.	dNTPs (dATP, dTTP, dGTP, dCTP)
6.	Double distilled water
7.	EDTA
8.	Ethanol
9.	Ethidium bromide
10.	Gen Elute Genomic DNA Extraction kit
11.	Isoamyl alcohol
12.	Isopropanol
13.	$MgCl_2$
14.	NaCI
15.	Phenol
16.	PCR buffer
17.	Primer P-12 (5'CCGAGCACCG3')
18.	Proteinase K
19.	SDS
20.	Step up ^{TM} 50 bp DNA Ladder
21.	Taq DNA Polymerase
22.	TBE Buffer
23.	TE Buffer

Table 3.2: Description of Primer P-12

Name	P-12
Company	Sigma Aldrich
Sequence	(5'CCGAGCACCG3')
Storage	-20^0 C

Table 3.3: Description of Step Up ^{TM} 50 bp DNA Ladder

The Step Up^{TM} 50 bp DNA Ladder consists of 20 bands of double stranded DNA fragments ranging from 50-1000 bp with size increment of 50 bp.

Storage: -20^0 C

Marker Sizes (base pair):

1000	800	600	400	200
950	750	550	350	150
900	700	500	300	100
850	650	450	250	50

Chapter 4

Results

4.1. Quality Assessment of the Extracted DNA

The conventional genomic DNA extraction protocols need expensive and hazardous reagents for decontamination of phenolic compounds from the extracts and are only suited for certain types of tissues. This is a simple, rapid, less hazardous, cost-effective and high throughput protocol for extracting high quality DNA from blood of fishes by using **Gen Elute TM Blood Genomic Kit** for genomic DNA. Unlike most DNA preparations methods that require multiple steps and special handling, which increases the risk of error or contamination, this protocol employs a single purification step to remove contaminating compounds, using a column preparation solution, wash solution and elution solution.

In molecular biology, quantitation of nucleic acids is commonly performed to determine the average concentration of DNA or RNA present in a mixture, as well as its purity. Reactions that use nucleic acids often require particular amounts and purity for optimum performance. There are several methods to establish the concentration of a solution of nucleic acids, including Spectrophotometric quantification and UV fluorescence in presence of a DNA dye.

4.1.1. . Spectrophotometric Analysis

Nucleic acids absorb ultraviolet light in   a   specific   pattern. In a spectrophotometer, a sample is exposed to ultraviolet light at 260 nm, and a photo-detector measures the light that passes through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample.

4.1.2. Sample Purity

It is common for nucleic acid samples to be contaminated with other molecules e.g. proteins, organic compounds etc. The ratio of the absorbance at

260 and 280nm ($A_{260/280}$) is used to assess the purity of nucleic acids. For pure DNA, $A_{260/280}$ is about 1.8.

The isolated DNA had no signs of degradation and the Spectrophotometric comparison of absorbance at A_{260} / A_{280} nm provided a purity factor of 1.85-1.9, indicating its good quality.

The following table 4.1 gives the $A_{260/280}$ ratio, which signifies that the isolated DNA is pure.

Table 4.1: Absorbance ratio and Purity of the Isolated DNA.

S.No	Fish Species	OD at 260nm	OD at 280nm	$A_{260/280}$
1.	*Cyprinus carpio communis*	0.028	0.015	1.86
2.	*Cyprinus carpio specularis*	0.026	0.014	1.85
3.	*Oncorhynchus mykiss*	0.023	0.012	1.9

4.2. Quantitation of Genomic DNA from Fish Blood and Scales

The quantities of DNA isolated from the three fishes were measured spectrophotometrically by measuring the absorbance at 260nm. The following table 4.2 shows the absorbance of the particular fish at 260 and its proportional DNA concentration. The DNA concentration ranged from 1.15 to 1.40μg/ml, which is generally sufficient for PCR amplification and molecular genetic approaches. The variation in DNA concentration may be due to the fact that different amounts of the tissue present on the scales and blood of the fishes or due to differences in the quantity of genomic DNA in the sample. Moreover, the variation in DNA concentration may also be due to considerable variation in the size of the scale and the amount of dermis and epidermis on the outside and not in the collagen of the matrix of the scale. The following table 4.2 gives the yield of DNA obtained from the blood or scales of the fishes:

Table 4.2: Yield of DNA isolated from blood or scales of the fish

S.No	Fish Species	OD at 260nm	DNA Yield
1.	*Cyprinus carpio communis*	0.028	1.4 μg/ml of blood
2.	*Cyprinus carpio specularis*	0.026	1.3 μg/ml of blood
3.	*Oncorhynchus mykiss*	0.023	1.15 μg/mg of scale

4.3. Random Amplification of Polymorphic DNA (RAPD) Analysis

The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites. Therefore, amplification products from the same alleles may differ in length and will be detected as presence and absence of bands in the RAPD profile. The profile of RAPD bands is similar to that of low stringency minisatellite DNA fingerprinting patterns and is therefore also termed RAPD fingerprinting. On average, each primer directs amplification of several discrete loci in the genome so that allelism is not distinguishable in RAPD patterns.

Random amplification with short Primers is a useful technique in phylogenetics. The important point is that the banding pattern seen when the products of PCR with random primers are electrophoresed is a reflection of the overall structure of DNA molecule used as the template. If the starting material is total cell DNA, then the banding pattern represents the organization of the cell's genome. Difference between the genomes of the different organisms can be measured by PCR with RAPD technique. Two or more closely related

organisms would yield more similar banding patterns than those organisms that are more distant in evolutionary terms.

The banding patterns of the three fishes were different. The banding patterns of *Cyprinus carpio specularis* and *Cyprinus carpio communis* were somewhat more similar than the banding patterns of the *Oncorhynchus mykiss* (Plate 8).

The number of bands per individuals ranged from 04 to 06 (Table 4.3) and bands amplified ranged in size from 840 to 1000 bp (Table 4.4). The molecular size of the bands was estimated with a molecular DNA ladder (Step Up^{TM} 50 bp DNA Ladder), which is of 50- 1000 bp in length. The genetic variability of the fishes as analyzed from the Plate 8 can be interpreted in the following points:

1. There was a wide difference between the band patterns of the Cyprinus fishes to that of the Rainbow trout. This may be due to the fact that the Cyprinus fishes are the warm water fishes and the trout being the cold water fish.

2. There was also a close pattern of the bands in the *Cyprinus carpio specularis* and *Cyprinus carpio communis* as they have much similarity as both of them occur in the same family Cyprinidae.

3. Some of the bands in all the fishes were thick: in *Cyprinus carpio specularis,* a prominent band of 980 bp, in *Cyprinus carpio communis,* a prominent band of 930 bp and in *Oncorhynchus mykiss,* three successive bands (980, 940 & 930 bp), which indicates that these bands are highly amplified in comparison to the others.

Table 4.3: Primer sequence and number of DNA fragments obtained for the analyzed species.

Primer	Sequence	Number of DNA fragments		
		Cyprinus carpio specularis	*Cyprinus carpio communis*	*Oncorhync-hus mykiss*
P-12	5'CCGAGCACCG3'	**06**	**05**	**04**

Table 4.4: Size of the bands determined by comparing with the Step up^{TM} 50 bp DNA Ladder

Marker Size in bp.	*Cyprinus carpio specularis*	*Cyprinus carpio communis*	*Oncorhynchus mykiss*
1000	1000	1000	-
950	980	940	980
900	930	930	940
850	920	910	930
800	860	850	840
750	840	-	-
700	-	-	-

Cyprinus carpio specularis

Six RAPD fragments were obtained after the amplification with **P-12 primer** and all of them were polymorphic for *Cyprinus carpio specularis*. Size of DNA fragments were 840, 860, 930, 920, 980 and 1000 bp.

Cyprinus carpio communis

Five RAPD fragments were obtained after the amplification with **P-12 primer** and all of them were polymorphic for *Cyprinus carpio communis*. Size of DNA fragments were 850, 910, 930, 940 and 1000 bp.

Oncorhynchus mykiss

Four RAPD fragments were obtained after the amplification with **P-12 primer** and all of them were polymorphic for *Oncorhynchus mykiss*. Size of DNA fragments were 840, 930, 940 and 980 bp.
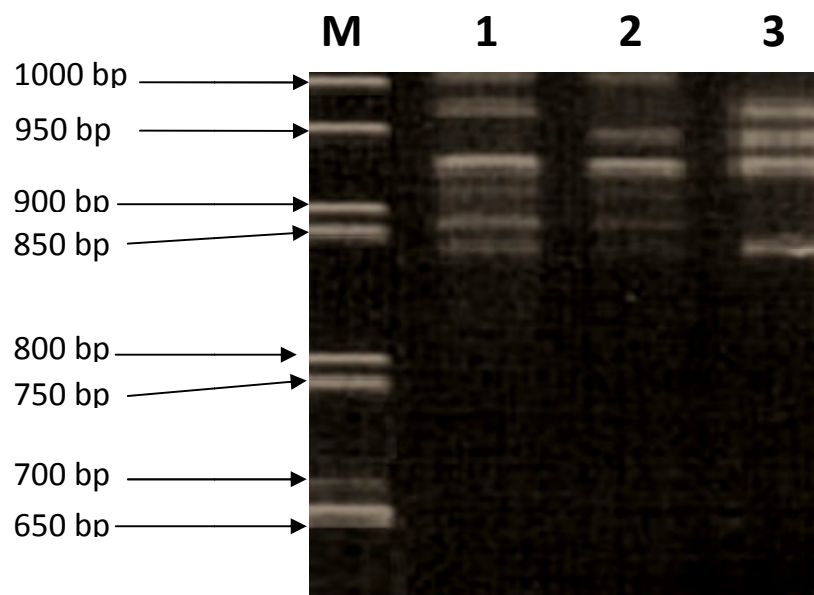
Plate 8.

A typical RAPD banding pattern amplified with primer **P-12** that resolved in 1.2% agarose gel and stained with Ethidium bromide. Template DNA was from Step up^{TM} 50bp DNA ladder (Lane M), *Cyprinus carpio specularis* (Lane 1), *Cyprinus carpio communis* (Lane 2) and *Oncorhynchus mykiss* (Lane 3).

Plate 1

Cyprinus carpio communis

Plate 2

Cyprinus carpio specularis

Plate 3

Oncorhynchus mykiss

Plate 4

Live fishes in the tubs for selling in Hazratbal Market.

Plate 5

Trout Fish Farm at Harwan Srinagar.

Plate 6

PCR Machine

PCR used for the Amplification of DNA

Plate 7

Gel Doc (Bio Rad)

The Gel Doc system enables quick, easy visualization and documentation of nucleic acid.

Chapter 5

Discussion

Genomic DNA of high quality and quantity is required to analyse genetic diversity by using molecular markers. A number of simplified protocols for DNA extraction have been reported such as Salting out Procedure by Miller *et al*., (1988); Chelex-based extraction by Walsh *et al*., (1991); Silica-guanidinium thiocyanate method by Carter and Milton (1993); Microwave Based extraction by Banerjee *et al.,* (1995); Boiling method by Valsecchi (1998) and Cetyltrimethylammonium bromide (CTAB) procedure by Tel-Zur *et al*., (1999) but majority of these methods are not suitable for the extraction of DNA from fish blood and scales.

Various genomic DNA extraction protocols have been optimized for blood samples (Lahiri *et al.,* 1992; Planelles *et al.,* 1996, Angelini *et al.,* 2000; Elgort *et al.,* 2004; Nasiri *et al.,* 2005; Pachot *et al.,* 2007; Budowle and Van Daal 2009;). Most of these methods were verified to be reproducible and yielded sufficiently high quality DNA for genetic analysis. In most of the above mentioned methods, enzymes or toxic organic solvents have been exploded (Ding 1992; Pachot *et al.,* 2007). Only a few methods have been noticed which are non-enzymatic and do not employ hazardous organic solvents (Lahiri and Nurnberger Jr 1991). To overcome the above mentioned remedies and to attain these aims, in the present study a very simple, inexpensive, rapid and less-hazardous protocol for extracting high quality DNA from fish blood by using **Gen Elute ᵀᴹ Blood Genomic DNA Kit** (**Sigma Aldrich: Cat. No. NA2000**), were followed.

The DNA extracted from the blood and scales of the fishes was of high quality and also the quantity of the extracted DNA was sufficient to carry out the RAPD analysis. It is noteworthy that the blood and scales both could be used as a source of DNA for molecular analysis.

The present study was used to estimate genetic variability in fish species from the Kashmir Valley by using RAPD. RAPD markers are efficient in species discrimination, particularly in cases where morphologic characteristics have low resolving power (Callejas and Ochando, 1998). Besides the classical application on estimates of genetic similarity among populations, RAPD markers have been used successfully to determine species-specific traits in fishes (Dinesh *et al*., 1993; Takagi and Taniguchi, 1995) and for reconstructing phylogenetic relations among species and subspecies (Bardakci and Skibinski, 1994).

The use of RAPD protocol to examine genetic variation and to generate DNA fingerprints of some fresh water fishes of Kashmir paved the way for more future studies to differentiate between fish species existing in fresh water bodies of Kashmir fresh water and their relation with others in India and abroad . The RAPD protocol was reliable, simple to set up, fast and large areas of genomic DNA screened as the study proved that. Consequently this study agrees with earlier studies using the same protocol (Welsh and McClelland, 1990; William *et al*., 1990). As well as it needs only minute amount of DNA, no prior information about DNA sequence required as that required in the study of mitochondrial and nuclear genes amplification and no hazardous radioactive chemicals used. These advantages make it more preferable than other techniques. The study agrees with Gomes *et al*., (1998). The RAPD-PCR amplification with a single decamer primer to produce a DNA fingerprint of DNA fragments was affecting essentially with Primer, DNA template and reaction conditions. So that these advantages in comparison to other DNA fingerprinting method, such as restriction fragment length polymorphism

(RFLP) (Fayazi, *et al.*, 2006) and Single Strand Conformation Polymorphism (SSCP) (Albadran, 2003) that need larger quantity of pure DNA, target gene or few loci of the gene and some information of specific primers or restriction enzymes. In spite of the morphological similarity among cyprinids species, molecular profiles showed that there was apparent variation in bands amplified by using the same RAPD primers. The number of bands on the agarose gel depends on the number of appropriately oriented and target sites present in DNA in that species or individual. The result is compatible with the results of Stacey *et al.*, (2007).The results of RAPD profiles showed strongly differentiated fingerprints of the two cyprinid species, so discrimination among the tested species was easy. This result coincided with the result of Welsh and McClelland (1990) and Hadreys *et al.*, (1992). This procedure established a method to differentiate between cyprinids and other fish families in Kashmir.

Although, Clark and Lanigan (1993) highlighted that the RAPD technique is less laborious compared with other fingerprinting techniques, producing results with low statistical error. In the other hand this method needs accurate work, and multiple decamer primers should be used to generate a number of molecular markers to establish fingerprints.

Therefore, DNA fingerprint similarity is being used to infer the level of genetic variation within and between natural populations (Lynch, 1990). This protocol was frequently used to this purpose as example Indian major Carps *Labeo rohita, calbasu, Catla catla* and *Cirrhinus mrigala* tested to investigate the genetic diversity using RAPD markers (Bardakci and Skibinski, 1994; Barman *et al.*, 2003). For many groups, even qualified taxonomist can't find characters for reliable identification for early life stages of fish species particularly in cyprinid species due to rapid growth and similar features among them, while using RAPD protocol would solve this problem. The fact that RAPD-PCR technique surveys numerous loci in the genome makes the method

particularly attractive for analysis of genetic distance and phylogeny reconstruction (Clark and Lanigan, 1993).

The polymorphism in genetic RAPD markers may stem out of deletion, insertion or substitution in priming sites that cause misleading priming (Williams *et al*., 1990). The additional advantage of RAPD fingerprinting lies in the fact that it is a sequence independent approach and each primer – DNA annealing will produce different spectrum of fragments from the PCR generating a species- specific fingerprint (Rastogi *et al*., 2007). Actually, the co-migrating dominant markers may not be homologous with one another. Secondly, the source of bands use presence or absence, although single change in the primer sequence or single substitution, insertion or deletion in gene sequence would alter the product of PCR while scoring that bands as presence or absence characters causes statistical error (Simmons *et al*., 2007).

The RAPD approach has also been used to create saturated genetic maps in fish species. Postlethwait *et al*., (1994) mapped 401 polymorphic DNA markers in zebrafish (*Danio rerio*). RAPD markers are also being used to construct genetic maps of *tilapia* species, *O. niloticus* and *O. aureus* (Naish *et al*., 1995) and rainbow trout, *Oncorhynchus mykiss* (Jackson *et al*., 1995).

The technique has also been used to study genetic variation in several fish species. Bardakci and Skibinski (1994) and Naish *et al*., (1995) used RAPD markers to discriminate between commercially important species, subspecies and strains of tilapia. RAPD markers were also generated for several tropical fish species representing 7 families (Dinesh *et al*., 1993). Furthermore, RAPD analysis revealed high levels of genetic variation among individuals from the same broodstock of sea bass (*Dicentrarchus labrax*) (Allegrucci *et al*., 1995). Finally, 721 strain-specific RAPD markers were identified in 2 laboratory strains of zebrafish (Johnson *et al*., 1994). RAPD markers are more suitable for clonal organisms than sexually reproducing

organisms. The ability of the RAPD technique to reveal intra-specific variation can be used in screening for the degree of inbreeding in commercial plant and animal species to prevent an increase in the frequency of deleterious recessive alleles in populations.

Although the RAPD method is relatively fast, cheap and easy to perform in comparison with other methods that have been used as DNA markers, the issue of reproducibility has been of much concern since the publication of the technique. In fact, ordinary PCR is also sensitive to changes in reaction conditions, but the RAPD reaction is far more sensitive than conventional PCR because of the length of a single and arbitrary primer used to amplify anonymous regions of a given genome. This reproducibility problem is usually the case for bands with lower intensity. The reason for bands with high or lower intensity is still unknown. Perhaps some primers do not perfectly match the priming sequence, amplification in some cycles might not occur and therefore bands remain fainter. The chance of these kinds of bands being sensitive to reaction conditions of course would be higher than those with higher intensity amplified with primers perfectly matching the priming sites. The most important factor for reproducibility of the RAPD profile has been found to be the result of inadequately prepared template DNA (Welsh and McClelland 1994). Differences between the template DNA concentration of 2 individuals DNA samples result in the loss or gain of some bands (Bardakci, 1996).

Since RAPD amplification is directed with a single, arbitrary and short oligonucleotide primer, DNA virtually from all sources is amenable to amplification. Therefore, DNA from the genome in question may include contaminant DNA from infections and parasites in the material from which the DNA has been isolated. Special care is needed for keeping out the DNA to be amplified from other sources of DNA.

Chapter 6

Conclusion

Random Amplification of Polymorphic DNA markers have found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with other methods. Therefore, RAPD technique can be performed in a moderate laboratory for most of its applications. Despite the reproducibility problem, the RAPD method is probably important as long as other DNA-based techniques remain unavailable in terms of cost, time and labour.

The effectiveness of RAPD in detecting polymorphism between and among different fish populations, their applicability in population studies and the establishment of genetic relationships among fish populations has been demonstrated. It is important to mention the fact that data results from RAPD assays can be extended to further dissect traits in a more refined way to exactly have the knowledge on specific genes and genetic pathways using other molecular methodologies. There is also the opportunity and need to study sequences of specific polymorphic bands to determine the genes detected by RAPD experiments. Further studies with other molecular methodologies are essential to clarify and confirm genetic relationships among fish species depicted using RAPDs.

The RAPD technique has an advantage over other systems of genetic documentation because it uses universal sets of primers and no preliminary work such as probe isolation, filter preparation, or nucleotide sequencing is necessary. RAPD has been used widely because of the following advantages:

- It requires no DNA probes and sequence information for the design of specific primers.

- It involves no blotting or hybridisation steps; hence, it is quick, simple and efficient.

- It requires only small amounts of DNA (about 10 ng per reaction) and the procedure can be automated.

- High number of fragments.

- Arbitrary primers are easily purchased.

Some limitations restrict practical application of RAPD analysis (e.g. dominance, reproducibility, homology inferences and artifact fragments). Some of its limitations are described below:

- Nearly all RAPD markers are dominant, i.e. it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). Codominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely.

- PCR is an enzymatic reaction, therefore, the quality and concentration of template DNA, concentrations of PCR components and the PCR cycling conditions may greatly influence the outcome. Thus, the RAPD technique is notoriously laboratory- dependent and needs carefully developed laboratory protocols to be reproducible.

- Mismatches between the primer and the template may result in the total absence of PCR product as well as in a merely decreased amount of the product. Thus, the RAPD results can be difficult to interpret.

- Lack of a prior knowledge on the identity of the amplification products.

- Problems with reproducibility (sensitive to changes in the quality of DNA, PCR components and PCR conditions).

- Problems of co-migration (do equal-sized bands correspond to the same homologous DNA fragment). Gel electrophoresis can separate DNA quantitatively, cannot separate equal-sized fragments qualitatively (i.e. according to base sequence).

Recommendations and Future Plans

By comparing the advantages and disadvantages of the RAPD markers, it is apparent that no one molecular marker is superior for all common applications and it is not possible to say that no variation exists on the basis of evidence from one or more markers. Unless the entire nuclear and mitochondrial genome is considered, this conclusion cannot be reached. Unfortunately increasing the number of alleles per locus does not always increase the probability of detecting significant differences. Thus it is recommended that at least two markers, mitochondrial and nuclear, should be utilized for each case. Finally, for routine applications in fisheries and aquaculture it may be better to focus on markers that are in widespread use by the scientific community.

Fishes exhibit a wide level of diversity in their morphology, behaviour, genomes and other aspects of biology. Hence this group is attractive for the study of many evolutionary questions related to various aspects. Fishes are also important to humans in economical, ecological and cultural point of view and the maintenance of fish diversity is essential and it is a challenge to man-kind for the sustenance. While considerable study has been done on elucidating this species diversity using novel and traditional morphological methods, and is continuing today, the DNA Barcoding sequence from the Cytochrome C

Oxidase I gene have proven very effective at aiding researchers of varied interests to identify species.

Therefore, during the Ph.D Course, a study of the Barcoding Gene Cytochrome C Oxidase I (COI) will be taken in order to observe the diversity of the fishes and thus helping in evolutionary lineages of the fishes.

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