GENETIC CHARACTERIZATION, CONTROLLED BREEDING AND DEVELOPMENT OF TRANSGENIC VARIETIES OF *PUNTIUS DENISONII* (DAY, 1865).

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DEDICATED TO

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&

My Family

DECLARATION

I hereby declare that this thesis entitled "**Genetic characterization, Controlled breeding and Development of transgenic varieties of *Puntius denisonii* (Day, 1865)**" is a bonafide record of research work done by me during the course of research and that the thesis has not formed the basis for the award to me of any degree, diploma, associateship, or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled "**Genetic characterization, Controlled breeding and Development of transgenic varieties of *Puntius denisonii* (Day, 1865)**" is a record of research work done independently by **Mr. MANOJ C.K.**, under my guidance and supervision that has not previously formed the basis for the award of any degree, fellowship, or associateship to him.

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LIST OF ABBREVIATIONS

IUCN	International Union for Conservation of Nature
RAPD	Random Amplified Polymorphic DNA
PCR	Polymerase Chain Reaction
PCA	Principal Component Analysis
CVA	Canonical Variance Analysis
GFP	Green Fluorescent Protein
CFP	Cyan Fluorescent Protein
BFP	Blue Fluorescent Protein
YFP	Yellow Fluorescent Protein
RFP	Red Fluorescent Protein
DNA	Deoxy ribo Nucleic Acid
PCs	Prinicipal Components
CFs	Canonical Functions
DF	Discriminant Function
rDNA	Ribosomal DNA
EDTA	Ethylene diamine tetra acetic acid
UPGMA	Unweighted pair group method of arithmetic mean
mtDNA	Mitochondrial DNA
LHRH	Luteinizing hormone releasing hormone
GnRHa	Gonadotropin releasing hormone analogue
CPE	Carp pituitary extract
HCG	Human Chorionic Gonadotropin
sGTH	Salmon Gonadotropin Hormone
GM	Genetically Modified
pCMV	Plasmid with cytomegalovirus promoter

1. INTRODUCTION

The demand for fish mainly for food, recreation, and ornamentals is steadily increasing. Natural fish populations have declined during the last several decades because of environmental degradation and over-fishing (Rottmann *et al*., 1991). India is one of the mega diversity hot spots contributing to the world's biological resources from the greater Himalayan range on the northern plain and long stretches of east as well as Western Ghats in the west. *Puntius denisonii* (Day, 1865) a highly sought after ornamental fish indigenous to the Western Ghats for domestic and international trade. The population structure of this fish which has been declared vulnerable by the IUCN recently has not been studied till date. Except a few studies conducted in its natural habitat, the captive breeding of *P. denisonii* in aquarium has not yielded promising results. The present study envisages to investigate the population structure of *P. denisonii* in rivers of Kerala and to develop controlled breeding techniques, inorder to preserve the genetic diversity and protect the wild ones from exploitation by supplying cultured ones to meet the increasing demand. Besides, an attempt has been undertaken to develop transgenic *P. denisonii* with green fluorescent protein.

Phenotypic variation between stocks is usually associated with the geographical region occupied by a species. Stock may be defined as a population or portion of a population, of which members are characterized by similarities which are not heritable, but are induced by the environment. It can be considered as a production or management unit. As the movement of individuals along the whole stretch of the river is difficult, the morphology of *P. denisonii* in the same river with different localities could also differ. Inter individual differences shown in habitat preference by *P. denisonii* were related to functional differences in body morphology and coloration. Phenotypic plasticity of fish allows them to respond adaptively to environmental change (Richard *et al.,* 1995). In long term, phenotypic variation in a species could lead to genotypic variation and this could even lead to the origin of a species. Otherwise, local populations of *P. denisonii*,

depending on distributional discontinuities and patterns of movement, can be associated into larger genetically related groupings or metapopulations (Verspoor, 1997).

A holistic or multiple approach, combining at least 1 phenotypic and 1 genotypic method can measure the similarities or dissimilarities of species under study and often allows apparent discrepancies implied by each method to be resolved (Begg and Waldman, 1999). Genetic markers are usually over sensitive to a low level of gene flow: Therefore, molecular markers alone are not sufficient to detect existing genetic variation among populations. Hence phenotypic and genotypic method i.e., Truss analysis and RAPD PCR are combined to determine stock relationships among *P. denisonii* individuals collected from the different rivers of Kerala. Combining the results obtained with these techniques provides considerable insight to the possible stock structure of the species. Genetic techniques of handling, conservation and breeding of *P. denisonii* should depend on knowing the amount of variation within each local reproductive unit, as fall in genetic diversity in a population could adversely affect its short-term viability (Leberg, 1990). Besides, this allows the channeling of conservation efforts to make better use of available resources. The conservation strategy for *P. denisonii* should endeavor to preserve its diversity in a particular location, since there might be local adaptations that would lose in competition with other populations, if introduced into the area.

However, body measurements that have been traditionally used to characterize stocks are recently being criticized because there are several biases and weaknesses inherent in traditional characters and measurement techniques. The predictive abilities of morphometric and meristic characters differ statistically and are likely to be lower for the meristic characters. A regionally unbiased truss network of morphometric measurements over the two-dimensional outline is used for analysis which gives more information about local body differences. Morphology is best described by multivariate techniques. Hence, after transforming absolute measurements to size-independent shape variables,

PCA, CVA and Cluster analysis was applied to reveal the population structure of this fish.

Molecular tools like RAPD analysis is valuable as a means to plan for long-term genetic diversity and for clarifying demographic and ecological issues early. The RAPD technique (Williams *et al.*, 1990) has been widely used in molecular biology laboratories and frequently applied to reveal population-genetic variation, divergence, and biogeography (Schaal and Leverich, 2001). Understanding relative levels of within- and among-population differentiation can help focus efforts on specific populations of *P. denisonii* in need of recovery as the occurrence of this fish in many of the river systems of Kerala has been greatly reduced.

Simple and reproducible fingerprints of complex genomes can be generated using universal random primers and polymerase chain reaction (PCR). It is not necessary to have prior genetic information of the fish for analysis. Each primer gives a different pattern of PCR products, each with the potential of detecting polymorphisms between strains of *P. denisonii*. Such polymorphisms are usually inherited in a Mendelian fashion and can be used as genetic markers. RAPD analysis is an efficient tool to differentiate geographically and genetically isolated populations of *P. denisonii*, and to verify the existence of locally adapted populations within the species that may have arisen either through genetic selection under different environmental conditions or genetic drift. Even with the limited survey of fish samples used for RAPD analysis, it is possible to identify the amount of genetic variability within the different stocks of *P. denisonii* and to detect percent of different polymorphic loci among the stocks.

The young ones of *Puntius denisonii* was collected from the river systems of Kerala where they breed naturally. But the ecology of breeding grounds are changing in such a fast pace that the fish are unable to cope. As a result the breeding grounds are losing their suitability to be used by the species, posing a threat of extinction. The widespread fragmentation of stream habitats resulting from land use activities, affected the distribution of *P. denisonii* in Kerala rivers. Being an ornamental fish indigenous to the Western Ghats which is

indiscriminately exploited, controlled spawning and artificial rearing of fry should be developed to supply hatchery bred *P. denisonii* to meet the increasing global demand.

Since the wild stocks of *P. denisonii* have been classified as vulnerable by the IUCN, there is a need for fast development of techniques of controlled propagation of this fish. Reproductive processes of fishes are by no means fully impaired in captivity. Usually, the progressive development of the gonads continues up to the final stages of gamete maturation, but the sequence is arrested only at the point of gamete release. Hormone-induced spawning is the only reliable method to induce reproduction in these fishes. To ease fishing pressure on wild stocks as part of recovery efforts and to meet the global demand by raising the cultured *P. denisonii*, the full developmental sequence, from egg to adult is described, as the information concerning its developmental biology is of much need to allow captive breeding of this species. The embryonic development and larval rearing of *Puntius denisonii* under laboratory conditions are explained.

Breeding of *P. denisonii* occurs in riverine habitats and the young ones are available in restricted centres like Irrity (Kannur) and Mukkam (Calicut) during a limited period of the year in Kerala. Much difficulty is experienced and high cost involved in transporting fish for domestic as well as international trade. Also, there is no certainty of a vigorous and healthy parentage of any collection of fish seed from natural environment. The need of artificial production of *P. denisonii* has grown tremendously in the recent years as there is scarcity of fish seed experienced in many of the previously identified natural habitats. Using the breeding and hatching techniques described in this preliminary study, it would be possible to meet to a large extent the requirements of captive bred quality seed of *P. denisonii* for domestic and foreign trade.

Through the use of transgenic technology, new varieties of ornamental fish with novel colors can be produced and supplied to meet the increasing world demand. The difficulty of commercialization of transgenic food fish has led this technology to be applied in ornamental fish industry. The availability of genes encoding fluorescent proteins such as GFP, RFP, BFP, YFP and CFP, has

enabled the production of green, red, blue, yellow or cyan fish in an almost endless variety of combinations. In the present study, an attempt has been made to develop transgenic varieties of *P. denisonii* incorporating the green fluorescent protein gene, which could possibly glow, if expressed, in the presence of blue light.

Transgenesis involves genomic alteration of an organism through insertion, modification or deletion of a gene of our interest. Electroporation has been shown to be the most effective means of gene transfer in fish since a large number of fertilized eggs can be treated in a short time (Inoue *et al*., 1990; Chen *et al*., 1995). Dot blot method was utilized to identify putative transgenic individuals in the present study. It is expected that further studies may produce high valued, stable transgenic lines of *P. denisonii* with uniform fluorescence ready for trade in the near future.

To conclude, the main objectives of the present work are (1) to find out the population structure and genetic diversity of wild *P. denisonii* collected from different river systems of Kerala using Truss analysis and RAPD-PCR, (2) to develop controlled breeding techniques for producing hatchery bred *P. denisonii* to meet the increasing market demand and to conserve the wild genetic diversity and (3) to develop transgenic varieties of *P. denisonii* with green fluorescent protein gene, which could glow in the dark.

2. REVIEW OF LITERATURE

2.1. Stocks and stock structure of a species

Stocks are arbitrary groups of fish large enough to be essentially self-reproducing, with members of each group having similar life history characteristics. To manage a fishery effectively, it is important to understand the stock structure of a species. An understanding of stock structure is vital to design appropriate management regulations in fisheries where multiple stocks are differentially exploited. In fisheries science, `stock' first referred to any group of a fish species that was available for exploitation in a given area (Milton and Shaklee, 1987). Marr (1957) explicitly differentiated stocks and subpopulations, considering the subpopulation to be a genetically self-sustaining entity, i.e., a deme, or the smallest self-perpetuating unit. The stock was defined as a population or portion of a population, all members of which are characterized by similarities which are not heritable, but are induced by the environment, and which may include members of several different subpopulations. Larkin (1972) emphasized practical aspects and considered a stock to be a production or management unit. Saila and Jones (1983) defined unit stocks as characteristic populations or sets of subpopulations within subareas of the geographic range of a species, but noted that the taxonomic status of unit stocks may vary or remain unclear. However, a stock is often taken to mean a lower category than that of the population recognized by taxonomists (Cushing, 1968). Endangered species management aims to ensure that populations that exhibit unique life history traits to particular areas are not irreversibly reduced by harvest or habitat destruction (Dizon *et al*., 1992), and is concerned with the evolutionary basis of stock definition.

2.2. Factors affecting stock structure of a species

Characteristics vary between fish stocks due to environmental and genetic factors. Environmental factors tend to influence phenotypic characteristics within stocks; phenotypic variation between stocks is usually associated with the geographical region occupied by a species throughout its range. Although

phenotypic differences do not provide direct evidence of genetic isolation between stocks, they can indicate the prolonged separation of postlarval fish in different environmental regimes (Campana *et al*., 1995). Morphometrics include the analysis of body shape, or the shape of particular morphological features of various body dimensions or parts. These data are continuous, and must be corrected for size differences among specimens. As with meristics, morphometric expression is under the simultaneous control of genetic and environmental factors.

2.3. Holistic approach to fish stock identification

Despite the need to reliably identify management units of the same species, such classification cannot be accomplished with a single technique (Edmonds *et al*., 1989). Combining the results obtained with several techniques may provide considerable insight to the possible stock structure of a species (Elliott *et al*., 1995). The ability to readily characterize the identity of an individual fish or group of fish taken in a particular area and/or time, to a particular stock remains a major challenge (Kutkuhn, 1981), and will always be probabilistic lacking a technique or protocol that unambiguously identifies the origin of individual fish (Waldman *et al*., 1988). A holistic approach to fish stock identification is highly desirable owing to the limitations and conditions associated with any particular method and the requirements of fishery management for separating units based on genotypic or phenotypic differences. Various procedures are now available that can provide a suite of information on the biology, distribution, and implied stock structure of a species. Morphometrics, meristics and life history characteristics have been used successfully for stock identification at a range of different scales (Casselman *et al*., 1981; Cadrin and Friedland, 1999), but are often limited by their possible alteration by environmental variation (Lindsey, 1964; Todd *et al*., 1981). Chemical methods enable elemental `fingerprints' to be discerned for multiple stock complexes (Begg *et al*., 1998; Thresher, 1999), although environmental variation may exist within the distribution of a single genetic stock. Molecular procedures can provide a genetic basis for stock identification (Smith, 1990;

Shaklee *et al*., 1999). Consequently, the different circumstances related to each stock identification technique, and the scale at which stocks can be detected vary depending on the situation. Overlaying all available information from a range of techniques will enable a generalized, consistent and definitive pattern of stock structure to be developed, relative to the needs of fishery management. Such an approach would enable a higher degree of confidence in a particular stock structure, rather than that for one that had been generated by a single procedure. Although, a precise determination of stock identification remains a major challenge, and will not necessarily be sufficient if fisheries occur on mixed stocks, our best opportunity at identification between these mixed catches relies on examining all available stock identification information using the most up-to-date technologies when logistically feasible. The sampling of fisheries vs. the sampling of fish is a real challenge in linking the results of laboratory intensive stock identification techniques to fisheries operations (and fisheries management), and subsequent impacts on the fish stocks. Stock identification should thus be recognized as a continuing process, evolving as management needs for stock assessment change, but always viewed against the background of a critical examination of all available data and new studies as dictated by changing resource condition and experimental technologies (Brown *et al*., 1987; Begg and Waldman, 1999).

Developments in molecular genetics had a significant impact on the fields of taxonomy and systematics, not only in terms of aiding species recognition, but also through elucidating phylogenetic relationships and mechanisms of speciation (Hillis *et al*., 1996). The increasing availability of discrete, heritable and stable protein and nucleic acid variants (Park and Moran, 1994) provides a universally applicable and objective approach for the comparative analysis of population and species identity. However, despite this objective and universal 'molecular yardstick', it is important to appreciate that there is a significant subjective element in the choice of method employed (Carvalho, 1998). The wide variety of molecular methods available allows molecular comparisons using nuclear or mitochondrial DNA genes, by targeting loci with specific mutation rates, through

direct sequencing or indirect estimation of genetic divergence using methods of differing sensitivity. The choice of method will have a significant effect on divergence estimates obtained, though such impacts can be reduced by maximizing the number of loci screened (Avise and Wollenberg, 1997) and techniques used (Carvalho and Hauser, 1999)

A foundational concept in evolutionary biology is that divergent selective regimes often generate and maintain phenotypic diversification (Smith and Skulason, 1996; Schluter, 2000). Aquatic environments can exhibit great spatial or temporal variability in both abiotic and biotic habitat parameters. Many fish species exhibit morphological differences between habitats (Jonsson and Jonsson, 2001). Divergent selection can lead to phenotypic differences through either genetic differentiation or phenotypic plasticity (Orr and Smith, 1998). Either source of divergence can drive microevolutionary change within a species, and can lead to speciation (Agrawal, 2001; Kaneko, 2002). Intraspecific polymorphism is typically believed to arise from divergent selection pressures between alternative environments. Intraspecific trophic diversification is also well known in fishes (Mittelbach *et al*., 1999; Holtmeier, 2001). The observed differences in mouth position among habitats could reflect differences in feeding, such as foraging mode or orientation, or diet composition (Langerhans *et al*., 2003).

The construction of dams for hydroelectric power is one of the most serious environmental changes for species inhabiting either land or water. Reduction of dispersion and gene flow may in time alter the relative proportions of the intra- and inter-population components of diversity (Vrijenhoek, 1998). The study of genetic variability is of prime importance for genetic approaches to fish conservation or breeding, which depend on knowledge of the amount of variation existing in a local reproductive unit (Carvalho, 1993). According to Haig (1998), the most important contribution that conservation geneticists can make to the assessment of the viability of populations is to determine the relative amounts of genetic diversity within and among the populations. Knowledge of the effects of genetic variability in species of fish is vitally important for an

understanding of how diversity is distributed among populations. On the basis of the observation that individuals that have lost heterozygosity through inbreeding usually exhibit a weakening of features of adaptation such as survival and fecundity, conservationists assume that a fall in genetic diversity in a population could adversely affect its short-term viability (Leberg, 1990). Estimating the degree of structuring in a population is a crucial step in conservation genetics work, as it allows the channeling of conservation efforts to *make* better use of available resources. Thus, if a threatened species occupying a given area is shown to be structured, the conservation strategy should endeavour to preserve its diversity in that area, since there might be local adaptations that would lose in competition with other populations introduced into the area. On the other hand, if the population of a species is homogeneous throughout its territory, then efforts to protect the species can be concentrated on just one area, and individuals from that area may be used to restock other areas when necessary. Hartl and Clark (1997) suggest that the structuring of populations occurs in almost all organisms. Many species organize themselves into natural subpopulations, such as herds, shoals, colonies and other types of aggregations. When a population is subdivided, some degree of differentiation is virtually inevitable in the acquisition of the allelic frequencies that vary in that population. What determines the structure of a population may be the system of reproduction, natural selection favouring different genotypes in different populations, degree of endogamy, gene flow and/or genetic drift within and between populations. Accordingly, in order to understand the dynamics of populations, not only must their actual structures be quantified, but it is also relevant to study the circumstances that determine them. The restocking of reservoirs or rivers with fish can have a strong impact on the genetics of the local populations (receiving stock). Competition, predation and parasitism may contribute to this, but, when assessing the implications of restocking, the importance of genetic diversity should be remembered. This refers to hereditary differences between and within the populations, and to the fact that it is vital to maintain such genetic variation, so that the species will be able to adapt to changes arising in its surroundings.

Each population of a given species possesses a different gene pool, *i.e.*, a specific set of alleles adapted to local conditions. When a locale is restocked with individuals bred in captivity which did not originate from the local population, alleles important for survival in that habitat may be diluted or lost and/or less well-adapted alleles fixed, possibly leading to the local extinction of that species (Leuzzi *et al*., 2004).

The effect of deliberate or inadvertent transfers of cultured Atlantic salmon on wild population depends on the nature and extent of genetic diversity among wild and transferred fish. Tagging and genetic studies show that salmon are divided into local, reproductively discrete populations associated with individual river systems or tributaries within systems. These are likely to be linked by historical patterns of gene flow into larger aggregations, which can be conceptualized as metapopulations, within which an evolutionary dynamic of local population formation, genetic exchange and extinction probably occurs. Diversity among populations has been documented within and between rivers, between North American and European population groups, and between Baltic and Atlantic subgroups within Europe. Diversity is in most cases associated with differences in biological performance relevant to survival and recruitment. As such, transfers have the potential to genetically alter native populations, reduce local adaptation, and negatively affect population viability and character. Transfers include escapes, both from hatcheries into fresh water and from farm cages into the sea, as well as fish planted out into rivers in attempts to re-establish extinct stocks or to enhance existing ones (Mills, 1991). The greater the adaptive differentiation between wild and transferred fish, the larger the expected decrease in survival and recruitment among hybrid offspring. This decrease can be viewed as outbreeding depression (Emlen, 1991) and results in reduced frequencies, in the population, of genotypes suited to local conditions.

Field observations have led many salmon biologists over the years (Taylor, 1991) to contend that each salmon river contains its own heritably distinct stock. Genetic diversity can be distributed within individuals, among individuals within populations, and among populations (Weir, 1996);

populations, in the genetic sense, being groups of individuals within which interbreeding is more or less random but among which interbreeding is constrained. Given that the genetic character of cultured fish is strongly influenced by their wild origins, it is important to understand the true nature of population structuring in wild salmon, and its adaptive significance. The spatial isolation of river systems and a sometimes patchy, discontinuous distribution of spawning and rearing habitat within river systems, provides the opportunity for salmon to segregate into distinct reproductive groups. It also provides the basis for the species to subdivide into local populations.

Local populations, depending on distributional discontinuities and patterns of movement, are likely to be associated into larger genetically related groupings. The sporadic occurrence of substantial amounts of gene flow among current populations, perhaps controlled by unusual environmental and demographic circumstances, cannot be ruled out. The scope for genetic diversity to evolve among Atlantic salmon populations is substantial. Genetic variation can arise in both the nuclear and mitochondrial genomes and is likely to involve in the order of 100 000 genes. The species shows heritable variation for a wide range of performance-related traits, such as growth and maturity (Tave, 1993) and there is heterogeneity among the habitat patches in relation to environmental factors which can affect such performance traits. At least some aspects of body morphology appear to be adaptive. Heritable differences in body morphology have been demonstrated between salmon from the Sabbies River and Rocky Brook tributaries of the Miramichi River, New Brunswick, which are associated with differences in tributary flow characteristics (Riddell and Leggett, 1981; Riddell *et al.,* 1981). Adaptations to water quality also appear to exist. Two tributary stocks of the Kyles of Sutherland River system in north-east Scotland show heritable differences in egg mortality associated with adaptation to low pH (Donaghy and Verspoor, 1997). Mortalities were eightfold higher for the non-native stock in the tributary where the low pH conditions occurred. Genetic differences were also found between the same two stocks in the timing of hatching in the wild, with the stock inhabiting the less extreme environment

hatching consistently earlier. Differences in growth and digestion rates have been documented between Shin salmon and salmon from the River Narcea, in northern Spain, under hatchery conditions (Nicieza *et al.,* 1994a, b). Adaptive population differences have also been observed for parasite resistance and are associated with differences in the presence or absence of pathogens in their native environments. Salmon stocks in the Baltic, where the monogenean ectoparasite *Gyrodactylus salaris* is endemic, are relatively resistant to infestation, whereas populations outside the Baltic, where the parasite has recently been introduced, are susceptible and suffer almost 100% mortality (Bakke *et al.,* 1990; Bakke and MacKenzie, 1993; Rintamakikinnunen and Valtonen, 1996). Variation at the gene is clinically associated with latitude, and more particularly temperature, in both Europe and North America as well as with temperature within river systems (Verspoor and Jordan, 1989; Verspoor *et al*., 1991). This argues strongly for the genetic changes among populations being an adaptive response mediated by natural selection.

Where salmon are taken into culture, further genetic changes relative to the source population are likely. Thus it can reasonably be assumed that transferred fish, many of which have been kept in culture, will differ genetically from native populations. As a consequence, where transferred fish mix reproductively with native fish, changes will occur to the genetic composition of the native populations. The greater the geographic distance separating native and transferred populations, the greater the potential for adaptively important genetic change. This potential is likely to be greatest where transferred fish are from a different regional population group than the native population, If adaptive differences exist between transferred and native salmon, and transferred fish survive to breed, then interbreeding will lead to an overall reduction in survival and recruitment in the native population. This reduction may or may not pose a threat to the viability and character of the native population. Where adaptively important genetic changes are small and quantitative, natural selection may be able to restore the genetic composition of the population to its previous adaptive state. However, where changes are large or qualitative, due either to the loss of

local genetic variants or to the introduction of new variation, genetic changes may not be reversible by natural selection. In such cases the induced changes could threaten the long-term viability of the population or permanently alter population character (Verspoor, 1997). The process of defining fish stocks is essential for effective fisheries management, and will continue to undergo refinement as new tools and technologies are developed (Kutkuhn, 1981).

2.4. Phenotypic variation and phenotypic plasticity

Identifying intraspecific units or stocks of a species with unique morphological characters enables a better management of these subunits of species and ensures perpetuations of the resources. Multivariate morphometry has been commonly used to investigate the discreteness and interrelationships of stocks within a species. Different types of body measurements have been traditionally used to characterize stocks. However these measurements have recently been criticized because there are several biases and weaknesses inherent in traditional characters and measurement techniques. As an alternative, a new system of morphometric measurements called The Truss Network System has been increasingly used for stock identification. This is a computer-originated approach to the collection and analysis of morphometric characteristics of different stocks. Morphological characters have been commonly used in fisheries biology to measure discreteness and relationships among various taxonomic categories. There are many well documented morphometric studies which provide evidence for stock discreteness (Bembo *et al*., 1996). However, the major limitation of morphological characters is that, at the intra-specific level phenotypic variation is not directly under genetic control but subjected to environmental modification (Clayton, 1981). Phenotypic plasticity of fish allows them to respond adaptively to environmental change by undergoing modification in their physiology and behavior which ultimately leads to changes in their morphology, reproduction or survival so as to mitigate the effects of environmental variation (Meyer, 1987). Such phenotypic adaptations do not necessarily result in genetic changes in the population and therefore, the detection

of such phenotypic differences among populations usually cannot be taken as evidence of genetic differentiation.

Geographical isolation can result in the development of different morphological features between fish populations because the interactive effects of environment, selection, and genetics on individual ontogenies produce morphometric differences within a species (Poulet *et al*., 2005; Cadrin and Friedland, 1999). The quantification of specific characteristics of an individual, or group of individual can demonstrate the degree of speciation induced by both biotic and abiotic conditions, and contribute to the definition of different stock of species (Bailey, 1997). The concept of geographical structure in fish population is fundamental for population dynamics and management of fisheries. More recently, the image analysis systems play a major role in the development of morphometric techniques, boosting the utility of morphometric research in fish population identification (Cadrin and Friedland, 1999). Data on morphometric measurements are able to identify differences between fish populations (Pinheiro *et al*., 2005), and used to describe the shape of each fish (Palma and Andrade, 2002). Multivariate techniques are widely used tools in ecological studies to assess the relationships between biological communities (Smith, 2003). These techniques can yield information complementary to that derived from biochemical, physiological and life history studies (Murta, 2000). A stepwise discriminant analysis of morphometric characters is a powerful technique to investigate the geographical variation of stocks (Pollar, 2007).

Environmentally induced phenotypic variation, however, may have advantages in the stock identification and adoption of appropriate management measures, especially when the time is insufficient for significant genetic differentiation to accumulate among populations. Genetic markers are generally oversensitive to a low level of gene flow: a relatively low level of exchange between stocks, negligible from a management perspective, may be sufficient to ensure genetic homogeneity (Carvalho and Hauser, 1994). Therefore, molecular markers may not be sufficient to detect existing genetic variation among

populations, and also only a small proportion of DNA is analysed by molecular markers.

Various stock identification techniques have been employed to elucidate the temporal and spatial discreteness of fish stocks (Ihssen *et al*., 1981; Maclean and Evans, 1981; Pawson and Jennings, 1996; Ayvazian *et al*., 2004). Morphometrics and meristics are the two types of morphologic characters that have been most frequently used to delineate stocks of a variety of exploited fish species (Murta, 2000; Silva, 2003; O'reilly and Horn, 2004; Turan, 2004; Turan *et al*., 2006). Morphometric studies are based on a set of measurements which represent size and shape variation and are continuous data, in contrast to meristic characters, which are discrete or non-continuous data. Therefore, the predictive abilities of morphometric and meristic characters differ statistically and are likely to be lower for the meristic characters. Hence, morphometric data should be analyzed separately from meristic data in multivariate analyses (Ihssen *et al*., 1981). Traditional measurements have recently been criticized because they are concentrated along the body axis with only sampling from depth and breadth, and most measurements are in the head. Furthermore, individual measurements often extend over much of the body, and some morphological landmarks such as the tip of snout and the posterior end of the vertebral column are used repeatedly as a central point for most of the measurements. The results of a morphometric analysis can depend on the particular set of measurements chosen. If the selection of distance measures does not correspond to the principal directions of shape differences, the resulting descriptions of the differences between forms will be inadequate. Thus these traditional measurements represent a biased coverage of body form (Strauss and Bookstein, 1982), and success in selecting effective characters has been attributed to a matter of chance. As an alternative, a new system of morphometric measurements called the Truss Network System has been increasingly used for species and especially for stock differentiation (Baumgartner, 1995). The Truss Network System covers the entire fish in a uniform network, and theoretically should increase the likelihood of extracting morphometric differences within and between species. It has to be noted that a

regionally unbiased network of morphometric measurements over the two-dimensional outline of a fish should give more information about local body differences than a conventional set of measurements (Strauss and Bookstein, 1982). Recent research shows that the Truss method is much more powerful in describing morphological variation between closely related fish taxa (e.g. stock) than in the case of traditional measurements (Corti *et al*., 1988).

2.5. Truss network

Truss network measurements are a series of measurements calculated between landmarks that form a regular pattern of connected quadrilaterals or cells across the body of the fish. Cells and truss characters are referenced according to the scheme of Strauss and Bookstein (1982). Measurements of specimens are made by collecting X-Y coordinate data for relevant morphological features, and followed the three-step process namely, positioning, pinning and digitizing. Morphological features or landmarks that are distinctive and homologous from specimen to specimen are selected around the outline of the fish form. Each landmark is marked by piercing the acetate sheet with a dissecting needle. Measurements should be made on one side of the each specimen throughout sampling. Additional data, such as eye diameter and head width can also be recorded and added in the truss data. The X-Y coordinate value for the positions of landmarks is digitized for each fish using statistical software. Alternatively, an X-Y coordinate digitizing pad can also be used to establish a reference set of X and Y axes to view interlandmark distances. All measurements are transferred to a spreadsheet file (e.g. Excel or Lotus), and X-Y coordinate data is transformed into linear distances by computer (using the Pythagorean Theorem) for subsequent analysis.

According to Turan (1999), the goal of this work is to take measurements (distances) between different points. These points have to be, obviously, the same in each of the fish analyzed. Therefore they must be located in places easily recognized fish after fish, these points called *landmarks*. The distances will be measured aided with an image analysis system, picture of each fish has to be

taken with a digital camera. In the next picture one can see these landmarks which are marked with the entomological pins.

2.6. Truss Analysis

When all samples for all populations have been taken from the same ages there is no need to eliminate the size effect in the data set. If otherwise, an important stage in the data preparation for morphometric analyses is to eliminate any size effect in the data set when comparing fish of different sizes. Variation should always be attributable to body shape differences, and not related to the relative size of the fish. Therefore, transformation of absolute measurements to size-independent shape variables is the first step of the analysis. Several transformation methods previously shown to be effective in removing such size-dependent variation can be compared (Turan, 1999).

Multivariate techniques simultaneously consider the variation in several characters and thereby assess the similarities between samples. PCA do not require prior grouping of individuals but combines and summarizes the variation associated with each of a number of measured variables into a smaller number of principal components (PCs) which are a linear combination of the variables that describe the shape variations in the pooled sample. Correlations between original variables and the principal components (component loading) can be used to interpret the importance of individual variables in the description of the variation of the data set. Principal components and canonical analysis can be used to produce graphs to visualize relationships among the individuals of groups by plotting population centroids of 95% confidence ellipses of first two canonical functions (CFs) and PCs. Each principal component contains the percentage of total variance of all variables. But in CVA, each function contains the percentage of the total between-groups variability. Therefore, CVA is used to describe the pattern of phenotypic differentiation among samples (Turan, 1999).

The Truss System can be successfully used to investigate stock separation within a species that allows, in a long term, a better and direct comparison of morphological evolution of stocks, while using the same set of measurements.

The particular usefulness of the Truss System as a fisheries management tool is that it is capable of examining large numbers of samples in a short time. It is also effective in identification of stocks and improving the biological basis of management, especially when they are used in conjunction with molecular genetic markers. The major drawback or requisite of this technique is that it requires computer image processing equipment to perform the analysis (Turan, 1999).

Significant morphological differentiation among samples of Tanganyika sardine *Limnothrissa miodon* was reported, indicating non-random association of fish. Such genetic patchiness may arise from biased reproductive success in localized spawning events and long-term stability of schools, resulting in genetic differentiation among schools (Hauser *et al*., 1998).

Important morphometric separation between the Nile and the Lake Victoria specimens of *Clarias* was observed after truss analysis. Nile specimens are located on the negative sector of the second component, while the Lake Victoria specimens are situated on the positive sector of this component. The second component is merely defined by the wîdth of the premaxillary toothplate, the width of the occipital process, the length of the occipital process and the dorsal fin length. Greenwood (1976) stated that although Lake Victoria is connected by river with the Nile, the Lake fauna is physically isolated by barriers that seemingly are impassable to fishes. The Lake Victoria and the Egypt populations of *Clarias gariepinus* however are both partly overlapped by the population from Chad, while that from the Senegal is intermediate between both. Possible hydrographic connections between the Chad and the Nile basins during the Pleistocene have been suggested by Roberts (1975) and are supported by the presence of taxa such as the cyprinid *Barbus apleurogramma* in Lake Victoria (Levque, 1990). Different colonization patterns, related to earlier hydrographic connections are used to explain this intraspecific variation.

Swain *et al*. (1991) used the truss system in identifying differences between the hatchery and wild populations of Coho salmon (*Oncorhynchus kisutch*). They found significant variation, which was attributed to the rearing

environment rather than to the genetic differences between hatchery or wild populations.

The fishing catch is different between the four adjacent seas of occurrence of *Pomatomus saltatrix*, being considerably high in the Black Sea (56% of the total *Pomatomus saltatrix* catch), moderate in the Marmara Sea (34%), and low in the Aegean (8%) and northeastern Mediterranean Seas (2%). Poor understanding of the fish and fishery management can lead to dramatic changes in the biological attributes and productivity of a species (Altukhov, 1981; Smith *et al*., 1991). As *P. saltatrix* is an important fishery resource in Turkey, truss analysis was done to obtain detailed knowledge on the population structure and apply such knowledge to the management applications (Turan *et al*., 2006). Morphological variation between fish populations is influenced by a mixture of environmental factors that include but are not limited to temperature, salinity, radiation, dissolved oxygen, water depth and current flow (Turan, 2000). Studies of morphologic variation between populations continue to have an important role to play in stock identification while stable differences in shape between groups of fish may reveal different growth, mortality or reproductive rates that are relevant for the definition of stocks (Swain and Foote, 1999; Cadrin, 2000).

Labeo victorianus, a fish species endemic to the Lake Victoria basin, once supported a commercial fishery. Since the 1950s landings have, however, declined due to overfishing. Recent surveys have found only two extant populations in Uganda; one in the Sio River and a second in the Kagera River. Fish from the Kagera River populations are significantly larger and exhibit differences in their reproductive biology. Using both genetic (control region sequences of mitochondrial DNA) and morphometric (truss and non-truss) characters, the level of genetic and morphological distinctiveness of the two remaining populations were investigated. Analyses of the mitochondrial control region sequence data (446 base pairs) did not show clear genetic differentiation between the populations. Principal component analysis of twenty truss and nine non-truss variables, and heteroscedastic *t*-tests on all but one measurement,

indicated that ‘size’ rather than ‘shape’ separate out fish from the two rivers (Rutaisire *et al*., 2005).

Sardine morphometric data (truss variables and landmark data) from 14 samples spanning the northeastern Atlantic and the western Mediterranean were analysed by multivariate and geometric methods. The analyses explored the homogeneity of sardine shape within the area studied, as well as its relation to that of adjacent and distant populations (Azores and northwestern Mediterranean). Principal component analysis on size-corrected truss variables and cluster analysis of mean fish shape using landmark data indicated that the shape of sardine off southern Iberia and Morocco is distinct from the shape of sardine in the rest of the area. The two groups of sardine are significantly separated by discriminant analysis, and their validity was confirmed by large percentages of correct classifications of test fish (Silva, 2003).

The truss analysis indicated a stable morphometric differentiation between anchovy's populations from Bay of Biscay and Iberian waters suggesting that the two European Atlantic anchovy stocks have distinct morphotypes. A total of 10 samples of anchovy were collected within an area from South Celtic Sea to the Gulf of Cadiz. Eye diameter and mouth length were added to the 23 distances from the truss. The pattern of correlations between principal components and the morphometric variables suggests that fish from the Iberian group had larger heads and dorsal fin base length as well as smaller body dimensions than the Bay of Biscay group (Caneco *et al*., 2005).

The analysis of the stock structure of the anchovy is carried out from a phenotypic point of view to determine the morphological differences associated with the origins of individuals from different areas with distinct environmental conditions. Samples were taken from the main fishing areas of each sea, comprising the central (Sinop) and eastern (Trabzon) Black Sea, the Aegean Sea (Üzmir) and the eastern Mediterranean (Üskenderun). Plotting discriminant functions 1 and 2, explaining 93% of between-group variability, revealed a high degree of dissimilarity among the anchovy samples, indicating that the anchovies in each sea represent different aggregations. Principal components analysis

(PCA) indicated that the observed differences were mainly from the measurements taken from the head (Turan *et al*., 2004a).

A holistic approach, combining one phenotypic and two genotypic methods, was adopted to analyze possible population differences in Indian mackerel (*Rastrelliger kanagurta*) from selected centers in the East and West coasts of India. Truss protocol system (Strauss and Bookstein 1982; Bookstein *et al*., 1985), which is more useful than the traditional morphometric methods to discriminate "phenotypic stocks" (Cadrin, 2000) has not been so far applied in the case of Indian mackerel. Principal component analysis of truss landmark variables revealed that the area encompassing depth between the origin of anal and origin of second dorsal and caudal peduncle depth has high component loadings (Jayasankar *et al*., 2004).

Liza abu stocks from the rivers Orontes, Euphrates and Tigris were investigated using genetic and morphometric data. Allozyme electrophoresis for genetic comparison and the truss network system for morphometric comparison were simultaneously applied to the same sample set. Highly significant morphological differences were observed between the three *Liza abu* stocks. The truss network system for fish body morphometrics was used to construct a network on *L. abu*. Thirteen landmarks determining 24 distances were chosen and measured on the body. Thus principal component analysis (PCA) was used to remove the size effect from the shape measures (Somers, 1986). This method extracts the first component as the isometric size factor, allowing the subsequent components to be interpreted as summarizing shape variation independent of size and random variation among the sampled individuals. The stocks seemed to be clearly distinct from each other. The pattern of phenotypic discreteness suggests a direct relationship between the extent of phenotypic divergence and geographic separation. However, genetic data do not support the detected morphometric variations. The capacity of fish populations or stocks to adapt and evolve as independent biological entities is limited by the exchange of genes among populations (Turan *et al*., 2004).

Morphologic differentiation among stocks of Mediterranean horse mackerel, *Trachurus mediterraneus*, throughout the Black, Marmara, Aegean and Eastern Mediterranean Seas, was investigated using morphometric and meristic characters. For effective fishery management and implementation of worthwhile stock rebuilding programs, knowledge of stock structure, distribution of fishing effort and mortality amongst the various components are essential (Grimes *et al*., 1987; Carvalho and Hauser, 1994; Begg *et al*., 1999). Variation in morphological and meristic characters was assumed to be entirely genetic in early studies, but is now known to have both environmental and genetic components (Robinson and Wilson, 1996; Cabral *et al*., 2003). Studies of morphologic variation among populations continue to have an important role to play in stock identification, despite the advent of biochemical and molecular genetic techniques which accumulate neutral genetic differences between groups (Swain and Foote, 1999). Examination of the contribution of each morphometric variable to canonical functions indicated that differences among samples seemed to be associated with the anterior part of the body. In meristic analyses, highest contributions to canonical functions were associated with the number of gill rakers and pectoral fin rays (Turan, 2004).

Multivariate allometric coefficients and size-adjusted shape were estimated and compared to elucidate the stock structure of spotted mackerel (*Scomber australasicus*) off Taiwan. Five samples were collected separately from the south of the East China Sea (ECS), the coastal waters of Taiwan (Tashi, Taitung and Kaohsiung) and the north of the South China Sea (SCS). Fourteen morphometric measurements were made for each individual. Morphological features of an organism are not autonomous and changes in various aspects of morphology are coordinated (Zelditch *et al*., 1992). Consequently, unless a specific morphometric character is known to have a genetic foundation, morphology is best described by multivariate techniques (Thorpe and Leamy, 1983). Dendrogram of five sampling areas was constructed by unweighted pair-group method with arithmetic means by using Manhattan *D*2 values (Sneath and Sokal, 1973) to assess the degree of similarity between the samples. Each

individual was allocated to the sample with the nearest centroid, and the proportion of individuals allocated to each sample was calculated. The proportion of individuals correctly reallocated was taken as a measure of the honesty of that sample. In such a case the proportion of individuals that are misclassified is low if all groups discriminated by cluster analysis does not derived by chance alone (Soriano *et al*., 1988). Canonical variance analysis (CVA) being a linear ordination technique was also performed to discriminate among groups. The coefficients of the linear combination obtained through maximizing the ratio of the between- to within-groups variance define canonical vectors (Owen and Chmielewski, 1985). It was concluded that there are three morphologically distinguishable stocks of spotted mackerel off Taiwan. One stock is distributed in the south of the East China Sea and the coastal waters of eastern Taiwan, another is in the coastal waters off Kaohsiung, and the other is in the north of the South China Sea (Tzeng, 2004).

The population structure of the shad, hilsa *Tenualosa ilisha*, was investigated with both allozymes and morphometric analysis. Fish samples were collected from nine sites within Bangladesh and compared with samples from four other countries that covered the entire species range. Currently, stocks are best defined with genotypic or phenotypic markers that are stable and repeatable (Booke, 1999). There is an apparent inconsistency between the results of the morphological and genetic studies that suggest multiple populations, on the one hand, and the large body of fishery biology literature from the region that demonstrate widespread seasonal movements that suggests panmixia. Fishery managers need to know whether they are dealing with single or multiple independently breeding populations in order to design sustainable management guidelines for the long term benefit of the two million fishers who participate in the hilsa fishery (Blaber, 1997). Indonesian and Indian hilsa were more distinctive than the most distant sample from Kuwait. Cross-validation of the classification revealed that sites in the middle reaches of the Meghna River in Bangladesh (Chandpur, Goalando, Bhairob Bazar) and Cox's Bazar had less than half the fish correctly classified to their origin. Fish from other countries were

much more distinctive. The morphological results reflect local environmental conditions rather than any population-level differences (Salini *et al*., 2004).

Multivariate morphometric variation was used to elucidate the stock structure of the red scad (*Decapterus kurroides*) off Taiwan. A total of seven samples from the northeastern (Keelung and Nanfangao) and southwestern (Kaohsiung and Tungkang) Taiwan were collected during spawning and non-spawning seasons. Information on stock structure is essential for any exploited species undergoing assessment and management (Ihssen *et al*., 1981). Nineteen characters for each individual were size–standardized by multiple group principal component analysis (MGPCA). The adjusted measurements were used to construct the dendrogram of seven samples by unweighted pair-group method with arithmetic means (UPGMA) method using Manhattan distance values. A randomization test was used to examine the significance of the morphometric difference between each pair of groups derived from the cluster analysis. The results obtained from cluster analyses and randomization tests indicate (1) that the morphology of red scad is significantly different between spawning and non-spawning seasons and (2) that the morphometric difference between populations from northeastern and southwestern Taiwan is significant without respect to spawning or non-spawning seasons. There appear to be at least two morphologically distinguishable stocks of this species off Taiwan (Tzeng *et al*., 2003).

Genetic variability and differentiation of the common European cyprinid *Leusciscus cephalus* was investigated within and across the drainages of the rivers Rhine, Elbe and Danube using starch gel electrophoresis and morphological characters. Morphometric characters were log transformed. For the meristic characters occurring in pairs the sum of the right and left count were calculated. Morphometric characters depend on age and size. Thus, for multivariate analysis, the residuals from a regression between head length, body height, eye diameter and distance between eyes versus body length were calculated to standardize for size. The pooled within-site slope (Thorpe, 1976) was used. Residuals and raw meristic characters were then subjected to a

discriminant analysis using sites as groups to analyse morphological differentiation between drainages. Correlation between genetic and morphological data was tested by plotting Nei's unbiased distance against Mahalanobis distance between sites using the Mantel-test within NTSYS-pc (Rohlf, 1992; Hanfling and Brandl, 1998)

To explore a method to discriminate inbred strains and evaluate inbreeding effects, morphological variation among three wild populations and three inbred stocks of *Gobiocypris rarus* was investigated by the multivariate analysis of eight meristic and 30 morphometric characters. Tiny intraspecific variations in meristic characters were found, but these were not effective for population distinction. Stepwise discriminant analysis and cluster analysis of conventional measures and truss network data showed considerable divergence among populations, especially between wild populations and inbred stocks. Thirteen characters, extracted from stepwise discriminant analysis, played important roles in morphological differentiation. These characters were mainly measures related to body depth and head size The average discriminant accuracy for all populations was 82.1% based on conventional measures and 86.4% based on truss data, whereas the discriminant accuracy for inbred strains was much higher. Morphological differences between wild populations and inbred strains appear to result from both genetic differences and environmental factors. These results suggested that multivariate analyses of morphometric characters are an effective method for discriminating inbred strains of *G. rarus*. (Shao *et al*., 2007).

Individuals of a same species living in variety of habitats may subject to different environmental conditions. Aquatic environments exhibit great spatial and temporal variability in both abiotic and biotic habitat parameters (Lowe-McConnell, 1987) and intraspecific diversification is well documented in fishes (Robinson and Wilson, 1994, Smith and Skulason, 1996). Three freshwater fish species namely *Puntius dorsalis*, *P. vitatus* and *P. bimaculatus* were sampled from different locations in four different altitudes of five major rivers of Sri Lanka to study phenotypic plasticity. Twenty three length measurements and fifteen meristic characters were recorded from each individual and fourteen

physicochemical parameters of each location were also measured. Relationship between altitude range and the species morphology was analysed by discriminant analysis and hierarchical cluster analysis. Results indicated that length characters which determine the shape of the individual mainly contribute to the discrimination of individuals according to their respective altitude range. Ratios of maximum body width to standard length, pre ventral length to post ventral length and fork length to standard length in *P. dorsalis*, *P. vitatus* and *P. bimaculatus* respectively were the main discrimination characters that grouped the individuals according to the respective altitudes. Variations in length characters were found to be adaptations to their habitat. In *P. vitatus* ratio of pre ventral length and post ventral length contribute totally for discrimination (100%). Fork length mainly contributes to discriminate *P. bimaculatus* (83.8%). As the percentage variance for post anal length and post ventral length is low, maximum body depth is the major discriminating factor for *P. dorsalis*. Many organisms can modulate their morphology in response to environmental cues. Such plasticity is thought to be an important adaptive strategy for populations experiencing variable environmental conditions (Scheiner, 1993) and it is likely that phenotypic plasticity plays an important role in diversification (West-Eberhard, 1989). These populations subject to local selection pressures leading ultimately to increased fitness termed local adaptations (Carvalho, 1993). This could even result in genetic divergence of populations (Silvia *et al*., 2006).

The truss morphometric was examined by canonical discriminant analysis with two steps to study the separation between species of a striped *Puntius*. First step was by examining all characters, and second step only examined selected characters (6 characters). The selection of characters followed value of Wilk's Lambda, and the important characters such are ED (eye diameter), PBVB (pectoral fin base – ventral fin base), CPL (caudal peduncle length), DBVB (anterior dorsal fin base – ventral fin base), PDUCP (posterior dorsal fin base – upper caudal peduncle), and CPD (caudal peduncle depth). A plotting of selected characters by function 1, 2, and 3 explained variation 58.24%, 25.43%, and 12.63% each, and when joined these function explained 93.48% variation. This

result showed separation between species of a striped *Puntius*. A plotting between function 1, 2, and function 3 shows that *Puntius* sp. is distinct from *P. johorensis, P. gemellus, P. trifasciatus*, and *P. lineatus* and it is closer with *P. trifasciatus* and *P. gemellus* (Haryono, 2005).

Fourteen classical morphometric characters were studied in different populations of *Puntius filamentosus*. The morphometric characters did not vary much among the population from Tamiraparani River basin; however, the population from Alancholai had significant difference in some morphometric characters. It distinguished from Tirunelveli and Gadana population in body width, body depth, head length, eye diameter, inter orbital width, pectoral fin length and pelvic fin length. It also differed from Papanasam population in predorsal length, length of caudal peduncle and length of anal fin. Most of the morphometric characters of fishes are similar and often overlap within the population. This morphometric data are not enough to support the established genetic structure of the population often, which leads to taxonomic uncertainty (Ponniah and Gopalakrishnan, 2000; Johnson *et al*., 2007)

The morphologic and genetic variations of *Engraulis encrasicolus* (L. 1758) were studied based on morphometric, meristic and allozyme analyses. Samples were collected throughout the Black, Marmara and Aegean Seas. Morphometric data were collected using the "Truss network system". Data points were arranged in "trusses" around the fish, a layout which maximises the number of measurements and increases the sensitivity of the analysis (Strauss and Bookstein, 1982). Co-variation of characters was investigated by multivariate analysis of variance (MANOVA). In discriminant function analysis, the first canonical function accounted for the largest amount of between–group variability (46%) while the second and third accounted for 23% and 12% respectively. Plotting DF1 and DF2 explained 69% of the between-group variation and revealed clear between-population differences. The Aegean Sea samples (AS1 & AS2) were the most isolated from each other and from all other samples. The western Black Sea sample (BS3) was also clearly separated from the other Black Sea samples, but was closer to the Marmara Sea sample (MS). The middle (BS2)

and eastern Black (BS1) sea samples were overlapping together. (Erdogan *et al*., 2009).

The morphological variability among populations of *Galaxias platei* from 20 postglacial lakes in southern South America was studied. In total, 23 morphological characters were measured in all adult (> 103.7 mm) specimens (N = 380), using a digital caliper and following Milano *et al*. (2002). The PCA was based on the 23 morphological variables obtained for 370 individuals (10 individuals eliminated because of missing values). The first five PCs explained 72% of the total variance. This could be the result of different colonization routes and histories and/or different ecological conditions and selection pressures (Milano *et al*., 2006).

The morphometric differentiations between two populations and sexes of shemaya fishes (*Chalcalburnus chalcoides*) were studied using a truss network. Truss distances between 15 landmarks of 66 specimens were measured. Size adjustment transformations were assessed by dividing characters (truss distances) by centroid size of specimen. Multivariate analysis of variance (MANOVA), principal component analysis and discrimination analysis were performed to investigate distinction and patterns of morphological variations between populations and sexes. The MANOVA (Wilks test) indicated a significant difference for mean vectors between populations (Λ = 0.136; F = 47.76; P = 0.001) and sexes (Λ = 0.120; F = 45.32; $P < 0.001$). Discrimination analysis correctly classified 97% and 89.4% samples to their original groups for population and sex, respectively. The findings support the use of the truss network to study morphological variation among populations remarkably better than traditional morphometric measurements as it provides interesting perspectives for the study of biodiversity patterns (Bagherian and Rahmani, 2009).

2.7. Isolation and quantification of DNA

Fish fins and scales are a reliable non-destructive source of DNA and these materials have been used to isolate DNA from many species (Estoup *et al*.,

1996; Nielsen *et al*., 1999; Adcock *et al*., 2000). However, there are some difficulties due to the consistency and small size of these tissues which can lead to a low amount and poor quality of total recovered DNA. After tissue digestion, a phenol-chloroform isoamyl alcohol purification step was utilized, as suggested by Taggart *et al*. (1992) and Sambrook and Russell (2001). The use of phenol-chloroform proved to be essential to obtaining pure DNA samples from fish fins and scales. Crude extractions could result in a DNA contaminated with proteins that may not be stable for long-term storage. Repeated DNA extractions with phenol-chloroform were not necessary. Single and double washes gave same results, avoiding protein residues. The DNA amount isolated from fins was high, when compared to the amount obtained from liver or muscle. Therefore, the extraction of DNA from fish fins or scales offers an extremely positive alternative to conventional DNA isolation techniques, representing a minimally destructive sampling approach. Samples of fins and scales preserved on ethanol/EDTA proved to be more suitable as a DNA source, when compared to air-dried samples. Preservation of nucleic acids depends primarily on the inhibition of tissue nucleases and denaturation, which can be achieved with EDTA and ethanol, respectively (Dessauer *et al*., 1996). The ethanol/EDTA storage solution also permits to maintain the softness of the tissue, facilitating its further dissociation in the digestion buffer. DNA samples obtained from fins were successfully used as templates for RAPD and specific nuclear and mitochondrial DNA sequences, as the 5S rDNA and the mitochondrial D-loop region, respectively (Wasko *et al*., 2003).

2.8. Molecular methods to identify genetic variation

The freshwater resources of India are currently experiencing an alarming decline in fish biodiversity due to several factors and a result, a sizeable portion of freshwater fishes have been categorized as threatened. Erosion of embankments of rivers, extraction of river water for irrigation, siltation of riverbeds and water pollution has drastically reduced fish population of the natural water bodies. Several species of fish have declined and some have become endangered. Genetic markers are important instruments for the study of

fish populations and fish lots. The development of molecular methods has permitted genomic analysis and made it possible to analyze existing variations, both in the regions which encode genes, as well as regions with unknown functions (Jayme *et al*., 2008).

RAPD (Random Amplified Polymorphic DNA) procedures allow for identification of parentage, more distant relatives, founders to new populations, unidentified individuals, population structure, effective population size, population-specific markers, etc. In recent years, genetic issues have gone from relative obscurity to a significant emphasis in conservation research as modern molecular techniques revolutionize our ability to delineate relationships among individuals, populations, and species. Maintaining adequate levels of genetic diversity within and among populations is one critical aspect to consider, yet in some cases it may be futile to be concerned with long-term goals when habitat is being destroyed at a rate such that even short-term population survival cannot be assured. Further, while combining genetic results with extensive field data is the best way to evaluate population viability, at times field data collection may be too expensive or time-consuming within a defined timeframe. In this situation, molecular analyses can provide at least a rough evaluation of some of the issues at hand in a timely fashion (Haig, 1998).

There are situations in which it may be desirable to electrophoretically estimate genomic variability and then to use these data as a base-line for comparing the genetic effects of a particular pattern of stock breeding or exploitation. For example, when exploitation of a species can be anticipated, base-line information on the genetic variation of pre-exploited stocks would be desirable. This would allow some direct assessment of the genetic consequences of exploitation by continuous monitoring of exploited stocks, or populations. Fishes probably surpass all vertebrate groups in their variety of social structures and kinds of life histories. As a consequence of this diversity, it is hazardous to generalize about demographic, geographic and genetic structural characteristics of fish (Allendorf and Utter, 1979). Each species must be examined as a unique case, even recognizing the possibility for intra-specific variation in population

structure. It is a moot point whether a naturally or artificially recolonized stock becomes as well adapted as the original stock. The point is to maximize the chances of a successful restocking or recolonization by taking into account all of the relevant genetic and ecological variables.

2.9. Random Amplified Polymorphic DNA (RAPD) technique

Simple and reproducible fingerprints of complex genomes can be generated using single random primers and the polymerase chain reaction (PCR). This method is simple and fast, and can be applied to any species for which DNA can be prepared. This method, which is called Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) 'fingerprinting' has the further merit of requiring little knowledge of the biochemistry or molecular biology of the species being studied. RAPD-PCR does not require a particular set of primers. Instead, this method uses primers chosen without regard to the sequence of the genome to be fingerprinted. Each primer gives a different pattern of PCR products, each with the potential of detecting polymorphisms between strains. Thus, the data produced allows the differentiation of even closely related strains of the same species (Welsh and McClelland, 1990)

PCR-RAPD applies the PCR technique to genomic DNA samples, using randomly constructed oligonucleotides as primers. A single short primer (10 base pairs) and low annealing temperature are combined to obtain specific amplification patterns from individual genomes. These anonymous DNA fragments are separated by size on a standard agarose gel and visualised by ethidium bromide staining. PCR-RAPD is simpler, considerably faster and less expensive than other methods for detecting DNA sequence variation (Caetano-Anolles *et al*., 1991a,b). Since RAPD can be used over numerous loci in the genome, the method is generally attractive for analysis of genetic distance, genetic structure at the population level, breeding applications, and phylogenetic reconstruction (Clark and Lanigan, 1993). Practical problems in RAPD applications include the difficulty in distinguishing products of different loci with similar molecular weights (co-migration) (Grosberg *et al*., 1996). Polymorphism is detected as band presence versus absence after PCR amplification, since RAPD

markers are usually dominant characters (Williams *et al*., 1990). These phenotypically dominant markers in diploid organisms make it impossible to distinguish between homozygotes and heterozygotes for the dominant allele. This problem does not preclude the estimation of allele frequencies necessary for population genetic analysis, but it does reduce the accuracy of such estimates compared to analysis with co-dominant markers, i.e. allozymes (Lynch and Milligan, 1994). Each RAPD locus is treated as a 2 allele system, with the assumption that only 1 of the alleles per locus is amplifiable by the PCR. The existence of multiple amplifiable alleles at a locus is reported to be relatively rare (Clark and Lanigan, 1993), but evidence to the contrary has also been reported (Grosberg *et al*., 1996). RAPD markers are usually dominant, and more loci need to be screened to obtain the same accuracy of estimates of genetic variation levels (Athanasios *et al*., 2003).

By applying molecular markers to issues such as habitat fragmentation, geographic genetic structure and conservation of genomic biodiversity, conservation genetics can contribute to land use planning decisions and ecosystem management. By linking high-resolution genetic data to geographic databases, it is possible to relate genomic diversity and geographic distribution to environmental parameters such as land use practices, hydrology and climate data. In addition, gene flow among populations provides an indirect measure of historical connectivity among streams. Molecular genetic techniques appear to be robust tools in conservation biology for identifying genetic isolation between stocks, permitting delineation of management units, and allowing assessment of conservation priorities from an evolutionary perspective (Menezes *et al*., 2006).

RAPD can be an efficient tool to differentiate geographically and genetically isolated populations, and has been used to verify the existence of locally adapted populations within a species that may have arisen either through genetic selection under different environmental conditions or as a result of genetic drift (Fuchs *et al*., 1998). The practice of releasing cultured fish into the wild is widespread, but without a careful genetic analysis, it may damage the goals of preservation, leading to the homogenization of populations and

decreasing species diversity (Taggart and Ferguson, 1986; Fritzner *et al*., 2001). Hence, the aim of conservation programs should be to develop an integrated strategy that conserves as much genetic diversity within the species as possible, and ensures the presence of utilizable fish resources (Hansen and Loeschcke, 1994). Since homozygotes cannot be distinguished from heterozygotes by the RAPD technique, the absence of amplification of a band in two genotypes does not necessarily represent genetic similarity between them (Duarte *et al*., 1999; Hatanaka and Galetti Jr., 2003)

Genetic diversity or variation and its measurement have vital importance in interpretation, understanding and management of populations and individuals. Population genetics in itself can be defined as the science of how genetic variation is distributed among species, populations and individuals, and fundamentally, it is concerned with how the evolutionary forces of mutation, selection, random genetic drift and migration affect the distribution of genetic variability (Hansen, 2003). With the advent of genetic methods, stock identification based solely upon morphological and meristic differences has become rare. Instead, these data are used in conjunction with genetic data. Because morphological and meristic characters can be influenced by the environment, variation in these characters may not have a genetic basis, and these characters do not necessarily provide information on genetic and evolutionary relationships (Gall and Loudenslager, 1981).

These molecular markers combined with new statistical developments enable the determination of differences and similarities between stocks and individuals, and the population of origin of single fish, resulting in numerous new research possibilities and applications in practical fisheries and aquaculture stock management. Many of the marker systems are codominant, reducing the population sizes necessary to draw statistically significant conclusions. Molecular markers for mapping applications should be reliable and easy to detect, with a high throughput of samples being possible. The use of molecular methods such as RAPD technique (Almeida and Sodre, 2002; Qiubai *et al*., 2006), AFLP technique and microsatellite DNA has been widely applied in fish

characterization. RAPD markers are type II markers because RAPD bands are amplified from anonymous genomic regions via the polymerase chain reaction (PCR). The complete genome is screened, genetic polymorphisms in the respective primer sites of the genome can be visualized within 24 h from extraction of genomic DNA and the storage of material is easy. Because the primers are short and relatively low annealing temperatures (often 36–40^{0}C) are used, the likelihood of amplifying multiple products is great, with each product (presumably) representing a different locus.

The main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterisation of the genome of the species in question. Because most of the nuclear genome in vertebrates is non-coding, it is presumed that most of the amplified loci will be selectively neutral. Genetic variation and divergence within the taxa of interest are assessed by the presence or absence of each product, which is dictated by changes in the DNA sequence at each locus (Perez *et al*., 1998). At an appropriate annealing temperature during the thermal cycle, oligonucleotide primers of random sequence bind several priming sites on the complementary sequences in the template genomic DNA and produce discrete DNA products if these priming sites are within an amplifiable distance of each other. The drawbacks of using short oligonucleotides as random primers are the low stringency necessary for DNA amplification, which can lead to non-reproducible results. The resulting pattern of bands is very sensitive to variations in reaction conditions, DNA quality, and the PCR temperature profile (Hoelzel and Green, 1998). The presence of paralogous PCR product (different DNA regions which have the same lengths and thus appear to be a single locus) limits the use of this marker. Each primer directs amplification of several discrete loci in the genome so that allelism is not distinguishable in RAPD patterns. In other words, it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous. RAPD markers are therefore dominant. Lynch and Milligan (1994) estimated that 2–10 times more individuals

need to be sampled per locus for dominant markers compared to co-dominant markers. Krauss and Peakall (1998) suggested that this disadvantage may be overcome because of the large number of available polymorphisms; typically over 100 polymorphisms per gel-lane are possible.

In fishes, RAPD has been successfully used to supplement systematic and phylogenetic studies (Jayasankar and Dharmalingan, 1997; Callejas and Ochando, 1998; Almeida *et al*., 2001; Prioli *et al.,* 2002; Barman *et al.,* 2003), in population structure analysis (Dergam *et al.,* 1998; Sekine *et al.,* 2002; Hatanaka and Galetti Jr., 2003; Almeida *et al*., 2003), in fishery management and conservation genetics of wild populations (Hatanaka and Galetti Jr., 2003; Bartfai *et al.,* 2003). RAPD technique has been applied to the study of phylogenetic relationships in tilapia and cichlid species (Bardakci and Skibinski, 1994). The technique was found to be useful in reconstructing cichlid fish phylogeny (Sultmann *et al*., 1995). RAPD has been used in the identification of largemouth bass subspecies and its intergrades (Williams *et al*., 1990; Welsh and McClelland, 1990), and for the study of Atlantic coast striped bass (Bielawski *et al*., 1995). The RAPD technique demonstrated low levels of polymorphism among strains of channel catfish, *Ictalurus punctatus* and Blue catfish *Ictalurus furcatus*, but polymorphism between them was shown to be higher (Liu *et al*., 1999). Genetic differentiation within and among sea bass *Dicentrarchus labrax* from different locations using RAPD showed high levels of polymorphism (Caccone *et al*., 1997).

RAPDs have gained considerable attention particularly in population genetics (Lu and Rank, 1996), species and subspecies identification (Bardakci and Skibinski, 1994), phylogenetics, linkage group identification, chromosome and genome mapping, analysis of interspecific gene flow and hybrid speciation, and analysis of mixed genome samples (Hadrys *et al*., 1992), breeding analysis and as a potential source for single-locus genetic fingerprints (Brown and Epifanio, 2003). RAPD analysis has been used to evaluate genetic diversity for species, subspecies and population/stock identification in guppy (Foo *et al*., 1995), tilapia (Bardakci and Skibinski, 1994), brown trout and Atlantic salmon

(Elo *et al*., 1997), largemouth bass (Williams *et al*., 1998), Ictalurid catfishes (Liu and Dunham, 1998), common carp (Bártfai *et al*., 2003) and Indian major carps (Barman *et al*., 2003). Naish *et al*., (1995) found the technique useful in detecting diversity within and between strains of *Oreochromis niloticus*. RAPD markers have been used for species identification in fishes (Partis and Wells, 1996) and molluscs (Klinbunga *et al*., 2000; Crossland *et al*., 1993), analysis of population structure in black tiger shrimp (Tassanakajon *et al*., 1998) and marine algae (Van Oppen *et al*., 1996), analysis of genetic impact of environmental stressors (Bagley *et al*., 2001), and analysis of genetic diversity (Wolfus *et al*., 1997; Hirschfeld *et al*., 1999; Yue *et al*., 2002). 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).

RAPD markers were used to estimate the population structure and phylogenetic relationships among the eight species of the genus *Barbus* that inhabit the Iberian Peninsula. Most diploid organisms are polymorphic at thousands of structural gene loci and that every individual has a unique protein complement. Ten random oligodecamers were used to amplify DNA from 232 fish sampled from 15 populations. A total of 270 markers were detected that revealed low levels of genetic variability. The conclusions of cluster analysis indicate two main branches and three well-differentiated groups: north-eastern, Mediterranean and Atlantic (Callejas and Ochando, 2002)

To assess the genetic variation in three rivers: the Halda, the Jamuna and the Padma as well as in one hatchery population of *Labeo rohita,* RAPD assay was used. RAPD markers were amplified from DNA samples of 35 fish from each of the four populations using six decamer random primers. The polymorphic loci proportions were 0.33, 0.28, 0.28 and 0.26 and Nei's gene diversity values were 0.06, 0.07, 0.06 and 0.05 for the Halda, the Jamuna, the Padma and the hatchery populations, respectively. The pairwise population differentiation (F_{ST}) values indicated a low level of genetic differentiation between the population pairs. From the unweighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei's genetic distances a correlation between genetic

affinities and geographical area was found. The populations were segregated into two groups: the Halda in one group and the Jamuna, the Padma and the hatchery in another group. The RAPD technique can be introduced as a tool in the population genetics of the rohu fish to provide information on their genetic stock structure (Islam and Alam, 2004)

Random amplified polymorphic DNA fingerprinting assay was applied to examine genetic variability in the freshwater mud eel, *Monopterus cuchia*, a vulnerable species in Bangladesh. Recently abundance of this species in nature has declined due to heavy fishing pressure, habitat destruction, aquatic pollution and indiscriminate uses of pesticides. Also, the species is near threatened (lower risk) in the freshwater bodies in India based on the IUCN red list categories (Dahanukar *et al*., 2004).

Upon testing 40 random decamer primers four primers giving reproducible results were selected and used for generating DNA fingerprints from a total of 51 fish collected from the district of Mymensingh, Bangladesh. A total of 39 loci were scored, 30 of which were found to be polymorphic in nature. The average values of Nei's gene diversity, Shannon's Information Index and band sharing based similarity index were 0.285, 0.423 and 88.33% respectively indicating a substantial level of genetic diversity in the studied samples of the vulnerable species. Due to the scarcity of *M. cuchia* in Bangladesh only 51 individuals were examined in the study however the PCR RAPD fingerprinting technique has been found to be suitable for assessing the genetic structure of the freshwater mud eel that could be used as a baseline study for taking any program on conservation and stock improvement of this species (Alam *et al*., 2010).

A study conducted by Nesbo *et al* 1998 reported four different Perch populations in the Baltic sea region when compared from the northern part of the Gulf of Bothnia (Sweden), representing two anadromous populations, one lake population and one brackish sea population. Using both the mtDNA and the RAPD, early stages of population differentiation was studied as the lake was isolated from the Gulf of Bothnia due to elevation of shorelines during the last 100 years. A holistic approach, combining one phenotypic and two genotypic

methods, was adopted to analyze possible population differences in Indian mackerel (*Rastrelliger kanagurta*) from selected centers in the East and West coasts of India. Clustering pattern of polypeptide markers revealed relatively greater population homogeneity among Mandapam fish (58%) than Kochi samples (33%). The three random amplified polymorphic DNA (RAPD) primers used in the present study have generated a total of 59 loci varying in size from 560 to 4500 bp. None of the populations from Mandapam, Kochi and Karwar showed RAPD fragments of fixed frequencies, to be treated as population-specific markers. No significant differences were found among the three populations (Jayasankar *et al*., 2004).

The genetic diversity of *Heteropneustes fossilis* based on RAPD markers were studied from 6 individuals belonging to two ecological habitats i.e., upper lake and lower lake of Bhopal. 128 scorable DNA fragments were found, of which 24 (18.75%) were polymorphic. By comparing RAPD banding patterns, small variations were found between and within the populations. The present study yielded data elucidating the usefulness of complementary approaches to make diversity analysis more explanatory and purposeful for optimum genetic amelioration and effective conservation of its genotypic variability (Garg *et al*., 2009b).

PCR-RAPD analysis was used to estimate genetic variation within and between 6 Northeast Atlantic populations of Dover sole *Solea solea* (L.). A total of 16 fish were randomly selected from each population, and the results were compared with allozyme variation within and between populations. Results from both methods were in general agreement, but the RAPD technique detected higher levels of genetic variation. Divergence between populations is indicated by cluster analyses of both allozymes and RAPD data and both allozyme and RAPD data indicate that the samples can be clustered into two groups (Athanasios *et al.*, 2003).

Wild and captive populations of a commercially important neotropical freshwater fish, *Brycon cephalus* (Amazonian matrincha), were analyzed by Wasko *et al*. (2004) in order to evaluate the levels of genetic diversity in a

breeding programme. RAPD fingerprinting was used to assess the genetic variability of a wild stock from the Amazon River and of three captive stocks that correspond to consecutive generations from the fishery culture. Although farmed stocks showed considerably lower genetic variation than the wild population, a significantly higher level of polymorphism was detected in the third hatchery generation reflecting a common breeding practice on several hatchery fish programmes that use a small number of parents as broodstocks, obtaining reproductive success with few non-identified mating couple. The synthesis of the data derived from genetic studies, together with those of other disciplines, can be applied directly to species management plans (O'Brien, 1994).

The genetic variability within and among the different stocks of *Brycon cephalus* was evaluated through the use of RAPD markers. Molecular markers are currently used in order to identify the levels of genetic variation within and between stocks of *B. cephalus* and also for individual genetic characterization. Therefore, the present study intended to contribute to a practical and theoretical framework to the fishery management and genetic conservation of the species. A reduction in the number of polymorphic loci can also be correlated to the limited number of wild specimens and/or populations used to settle hatchery stocks. Numerous studies using genetic markers correlated the domestication process in fish species with a decrease in genetic variability (Ferguson *et al*., 1991; Kohl *et al*., 1999; Winkler *et al*., 1999). This fact can be associated with several events, such as the use of few animals to establish hatchery stocks, employment of inappropriate reproductive strategies, and even genetic drift and inbreeding (Wasko *et al*., 2004).

RAPD technique was used to examine the genetic variability of *Brycon lundii,* collected on two regions with distinct environmental conditions in the Silo Francisco River (Brazil), downstream from a hydroelectric station (Wasko and Galetti Jr., 2002). Using decamer oligonucleotides as single primers in Polymerase Chain Reaction (PCR), genetic similarity index, mean allele frequency and mean heterozygosity were estimated, revealing variations between samples from the two regions. Moreover, a fragment of about 1200 base pairs

was found in 100% of the examined animals collected at the region closer to the hydroelectric dam, while its frequency was much lower (27.3%) within the sample from the second collecting site, 30 km downstream from the dam, indicating a possible correlation between genetic variation and geographical area. A dendogram representing the relationships among genotypes was obtained, demonstrating at least two major clusters of animals. The obtained results showed that some of the analyzed decamer primers permitted the detection of polymorphic fragments in *Brycon lundii,* revealing different levels of genetic variability within and between region A and region B. Band-sharing-based similarity indices, mean allele frequency and heterozygosity values were sufficient to distinct these two sampling localities, even with the limited survey of individuals and primers used in this study. The careful selection of appropriate natural stocks, based on genetic criteria, can offer greater potential for success in species-recovery and maintenance programs (Quattro and Vrijenhoek, 1989). The results described holds great promise for further analyses and gives support to biodiversity maintenance and recovery efforts of *B. lundii.*

The PCR RAPD technique was used to examine genetic variability and population structuring in the four wing flyingfish, *Hirundichthys affinis* within the central western Atlantic. Three random decamer primers and pairs of these primers were used to amplify nuclear DNA from 360 fish sampled from six populations (at five locations) across the region. A total of 58 polymorphic RAPD markers were identified, 20 of which were population-specific and six of which were subregional or stock-specific markers. Cluster analysis of similarity indices indicated the presence of three genetically distinct subregional stocks located in the eastern Caribbean, southern Netherlands Antilles and Brazil, respectively. Estimates of gene diversity (f) and gene flow (*Nm*) are consistent with this three-stock hypothesis. Results show that PCR RAPD technique is suitable for determining population stock structure in this species and a three-stock approach to managing *H. affinis* within the central western Atlantic is appropriate (Gomes *et al*., 1998).

Random amplified polymorphic DNA (RAPD) markers were used to analyze genetic variability and structure of populations assigned to the Neotropical fish species *Astyanax scabripinnis* from an urban stream located in Londrina, Parana State, southern Brazil. In urban areas, where aquatic ecosystems are constantly suffering the discharges of effluents produced by human activities, populations of fish have to adapt frequently to environmental changes. Thirty individuals of *Astyanax scabripinnis* were collected from three sites throughout the upper Cambe stream. A total of 10 primers amplified 159 loci, of which 128 (80.5%) were polymorphic. Each of the three populations showed very similar proportions of polymorphic loci, which ranged from 63.5 to 64.8%. Unbiased genetic distances varied from 0.0612 to 0.0646. The genetic similarity among all individuals studied ranged from 0.424 to 0.848 (Silvia *et al*., 2006).

10 gizani populations were recorded inhabiting different natural intermittent stream systems and one living in a small reservoir. Determining the units of an endangered species that have to be managed and conserved, it was considered essential to obtain genetic information combined with other ecological and biological information about the major gizani populations, prior to the implementation of conservation actions, such as artificial breeding, maintenance of populations and planned stocking. The RAPD method (Welsh and McClelland, 1990; Williams *et al*., 1990) was used successfully to screen randomly the nuclear genome, providing useful markers to discriminate between closely related species, subspecies and populations, to determine their genetic affinities, for estimating heterozygosities within populations and gene identities within and between populations (Mamuris *et al*., 2005)

RAPD was used to delineate the hilsa populations sampled from the Ganga, Yamuna, Hooghly, and Narmada Rivers at six different locations using six degenerate primers to generate the fragment patterns. All primers were highly polymorphic and generated high numbers of amplification products. Nei's genetic distances were calculated between locations. The overall average genetic distance among all the six locations was 0.295. The Fst value within the Ganga

was 0.469 and within the Hooghly it was 0.546. The overall Fst value for the six populations analyzed was 0.590. The UPGMA dendrogram clustered the hilsa into two distinct clusters: Ganga and Yamuna populations and the Hooghly and Narmada populations. Thus, RAPD has been used in population studies in fisheries, and can be used efficiently for geographic analysis of populations with differential degrees of geographic isolation (Brahmane *et al*., 2006)

Many factors such as hydroelectric dams, water pollution and other environmental changes have contributed to the destruction of fish habitats and the eradication of natural stocks. Using the RAPD technique, the genetic variation in *Prochilodus marggravii* from three collection sites in the area of influence of the Três Marias dam (MG) on the São Francisco river (Brazil), was analysed. The results revealed that the fish in the downstream region nearest the dam have a higher similarity coefficient than those from the other sampling sites, which may be related to differences in environmental characteristics in these regions. The results are useful for the fishery management, aquaculture and stock conservation of this species (Hatanaka and Galetti Jr., 2003).

Atlantic Coast striped bass exhibit exceptionally low levels of genetic variation. Reproductive fidelity of migrating striped bass for their natal tributary suggests reproductive isolation exists between river systems. A major issue in the management of Mid-Atlantic striped bass populations is identification and characterization of genetically distinct populations. The ability of RAPD method to uncover genetic variation in this highly conserved species was investigated. Sufficient levels of variation were detected to allow a population genetic analysis of the four migratory populations of Atlantic Coast striped bass. Lynch's analogue of Wright's F_{ST} suggests that Atlantic Coast striped bass are genetically subdivided (F_{pST} for pooled Atlantic samples=0.44). Significant heterogeneity was detected in the frequencies of 32% of surveyed RAPD markers. (Bielawski and Pumo, 1997).

RAPD-PCR assays were used to identify DNA polymorphisms in the European sea bass *(Dicentrarchus labrax).* Samples from eight Mediterranean localities and one Atlantic sample from Portugal were studied. A sample from a

congeneric species *(D. punctatus)* was included for comparison. The study included 107 RAPD markers and 260 individuals. Within-population RAPD variation was high, but between-population genetic differentiation was quite low showing that RAPD polymorphisms can be powerful tools to study intraspecific genetic differentiation. The data are consistent with other molecular studies in revealing high levels of intrapopulation variation and low levels of genetic differentiation between samples (Caccone *et al*., 1997).

RAPD analysis was conducted for the assessment of the genetic relationship between cultured populations of the Pacific oyster *Crassostrea gigas* Thunberg in Hiroshima and Goseong, the largest oyster farming areas in Japan and Korea, respectively. Of 25 arbitrary primers comprising decamer nucleotides of random sequences, polymerase chain reaction amplifications with 5 different primers gave reproducible electrophoretic patterns. A total of 49 RAPD markers were clearly identified for the Hiroshima and Goseong populations, and 46 markers were polymorphic presenting mean polymorphism rates of the respective populations at 92.29% and 93.32%. Pairwise genetic distances of each 20 individuals from these populations served to produce a UPGMA dendrogram. The dendrogram comprised two main clusters, one of which was a nested cluster including all individuals of the Hiroshima population along with 12 individuals of the Goseong population, and the other cluster included the remaining individuals of the Goseong population. Results indicate that RAPD markers are useful for the assessment of the genetic relationships between populations of the Pacific oyster (Aranishi and Okimoto, 2004).

Genetic diversity of the Nile tilapia *Oreochromis niloticus* collected from the river Nile (Cairo, Assuit and Qena) and two Delta lakes (Burullus and Manzalla) in Egypt was examined by randomly amplified polymorphic DNA analysis (RAPD). Of 25 primers examined, 21 primers produced 230 RAPD bands. The percentage of polymorphic bands in Manzalla (29.4%) and Burullus (24%) populations was low compared with Assuit (30.54%), Cairo (33.5%) and Qena (44.84%) populations. The highest percentage of polymorphic bands was observed in the Qena population, suggesting a greater potential for use in

breeding programs. The molecular phylogenetic tree constructed by unweighted pair-group method of analysis shows Manzalla and Burullus populations strongly linked and separate from the Assuit and Cairo populations, with Qena population as outgroup (Hassanien *et al*., 2004).

RAPD analysis has been used to discriminate between the 2 different populations of *M. vittatus* of Madhya Pradesh. This technique is more sensitive than the mt-DNA analysis, which failed to reveal variations within the tilapia populations (Seyoum and Kornfield, 1992). Genetic variation was studied between 4 different populations of Hilsa Shad from Ganga, Yamuna, Hoogly and Narmada rivers of India using RAPD technique (Brahmane *et al*., 2006). RAPD fragments observed in the 20 individuals, showed a reasonable degree of genetic diversity within and between the populations. The percentages of polymorphism, monomorphism and uniqueness were 64.98, 46.90 and 3.61, respectively in all the bands obtained from the 5 primers. The population specific bands could not be discerned from the fragment patterns generated. The populations of *M. vittatus* from Mohinisagar reservoir Gwalior were in one cluster while rests of the individuals of Bhadwada reservoir, Bhopal were in another cluster except an individual. This observation clearly indicated that, both the populations of *M. vittatus* have separate gene pool. The intra-specific genetic similarity between individuals of Mohinisagar reservoir was higher than the individuals of Bhadwada which may be due to geological variations or changes in aquatic environments. All values of morphometric parameters of individuals of Mohinisagar reservoir were higher than the fishes of Bhadwada reservoir. These morphometric observations also indicated that, the aquatic environment of Mohinisagar reservoir may be more favorable than the Bhadwada reservoir of Bhopal (Garg *et al*., 2009a).

Genetic variations (revealed from RAPD markers) between the two sexes of *S. galilaeus* were analyzed using POPGENE (version 1.32) to spot light on the genetic diversity within and between both of them. Manzala Lake (large, shallow and brackish water) is one of the Deltaic Mediterranean lakes in Egypt. This lake is exposed to different levels of pollutants. Devlin and Nagahama (2002) reported

that, sex determination and differentiation are fundamental components of the genetic information passed on from generation to generation. Ten RAPD primers were tested on 20 individuals of each *S. galilaeus* sex separately. A total of 151 RAPD bands were examined in a total number of 40 fish individuals (20 males and 20 females). The percentages of polymorphic bands were 96.6% and 96% in males and females respectively. Results showed that the highest mean value of gene diversity (h = 0.35) and Shannon's Information index (I = 0.52) were observed in investigated males compared to females. The average of actual number of alleles (na) across studied loci was 1.96 for both two sexes. The analysis of RAPD data using POPGENE succeeded in screening the biodiversity among and within the applied *S. galilaeus* populations (Saad, 2009).

Molecular (Randomly Amplified Polymorphic DNA- Polymerase Chain Reaction; RAPD-PCR) and biochemical (Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis; SDS-PAGE) genetic markers of six *Clarias gariepinus* populations collected from different Egyptian locations were used to infer the genetic signature of each studied fish population. Almeida *et al.* (2001) successfully used the RAPD technique to distinguish between two species *(Pimelodus cf absconditus and Iheringichthys labrosus)* that presented very similar external morphology. A total of 95 amplified bands were detected of which 66 (69.5%) were polymorphic. The molecular data revealed from these genetic markers were used to reconstruct the dendrogram and calculate the genetic distance among the applied fish populations. The Unweighted Pair-Group Method (UPGMA) cluster analysis of the similarity matrix based on RAPD analysis separated the studied populations into two clusters. Some specific genetic markers were detected for each studied fish population. The study revealed a relatively high level of genetic diversity which is required for populations to be more adaptive with the environmental changes. The results are useful, especially in *Clarias gariepinus* breeding programs which use genetic markers as a marker assisted selection to improve the fish performance (Saad *et al*., 2009).

In the present study, the RAPD markers were analyzed to investigate whether *A. seenghala,* populations those were sampled from the two major reservoirs (Bhadwada reservoir at Bhopal and Mohinisagar reservoir at Gwalior) are genetically different. Genetic monitoring is ideal for use in a reproduction program with the aim of genetic conservation (i.e., stocking). Genetic variations in two populations of Hilsa Shad from Padma and Meghna Rivers of Bangladesh showed the populations from Padma were genetically different than those from the populations of Meghna (Shifat and Begum, 2003). Ten RAPD primers were primarily screened of which five primers gave polymorphism, were selected for the final RAPD analysis. Using these five primers, 659 scorable DNA fragments were found, of which 513 (77.85%) were polymorphic, 140 (21.24%) were monomorphic and 6 (0.91 %) were unique. By comparing RAPD banding patterns, variations were found between and within the populations. The unweighted pair group method with averages (UPGMA) dendrogram showed two clusters, the Mohinisagar reservoir (Gwalior) population formed one cluster and the other cluster was represented by the population of Bhadwada reservoir (Bhopal). Although the morphological differences were negligible using these techniques a little but significant difference was detected in genetic diversity among two populations of *Ariochthys seenghala.* The present study may serve as a reference point for future examinations of genetic variations within the populations of fishes, which are commercially important (Garg *et al*., 2009).

Rahman *et al*. (2009) used RAPD markers to assess genetic variation in three wild population of catla in the Halda, Jamuna and Padma rivers and one hatchery population in Bangladesh. Genetic variation is a key component for improving a stock through selective breeding programs. The RAPD technique is useful for studying variation in species with low genetic variability when other techniques such as isozyme analysis and mtDNA control region sequencing fail to reveal differences between individuals (Bowditch *et al.,* 1994). Five decamer random primers were used to amplify RAPD markers from 30 fish from each population. Thirty of the 55 scorable bands were polymorphic, indicating some degree of genetic variation in all the populations. All distinct fragments were

given identification numbers according to their size as measured by the DNAfrag software version 3.03 (Nash, 1991) and scored separately for each sample and primer on the basis of their presence (1) or absence (0). The scores obtained for all primers were then used to create a single data matrix for estimating the proportion of polymorphic loci, Nei' s (1973) gene diversity *(fl),* gene flow *(N_m)* and homogeneity test at different loci using the POPGENE software version 1.31 (Yeh *et al.,* 1999). The same software was also used to construct unweighted pair-group method with averages (UPGMA) dendrograms (Sneath and Sokal, 1973) based on the estimated genetic distances. The proportion of polymorphic loci and gene diversity values reflected a relatively higher level of genetic variation in the Halda population. Estimated genetic distances between populations were directly correlated with geographical distances. The UPGMA dendrogram showed two clusters, the Halda population forming one cluster and the other populations the second cluster. The study revealed 30 polymorphic loci, indicating a high level of polymorphism in the four C. *catla* populations studied and supporting the suitability of RAPD markers for effectively discriminating different populations (Rahman *et al*., 2009).

RAPD markers were applied to assess genetic diversity and population structure of wild and hatchery populations of the white seabream *Diplodus sargus* and the common two-banded seabream *D. vulgaris* (Sparidae). The RAPD method (Williams *et al*., 1990) has been widely used in molecular biology laboratories and frequently applied to reveal population-genetic variation, divergence, and biogeography (Schaal and Leverich, 2001). In aquaculture fish species, it has already been successfully applied to catfish (Liu *et al*., 1998), red seabream (Jiang *et al*., 2004), carp (Wang and Li, 2004), gilthead seabream (Bilgen *et al*., 2007), and flounder (Liu *et al*., 2007). The percentage of genetic variation within *D. sargus* and *D. vulgaris* populations, based on coefficient of gene differentiation, reached 82.5% and 90% of the total genetic variation, respectively. An undeniable decrease in genetic variation was found in both hatchery populations, particularly in *D. sargus,* compared to the wild ones. However, the high values of variation within all populations and the low levels of

genetic variation among populations did not indicate inbreeding or depression effects, thus indicating a fairly proper hatchery management. The creation of a genetic baseline database will contribute to a more efficient conservation management and to the design of genetically sustainable restocking programs. The disadvantage of its low reproducibility can be overcome by replicating exactly the same laboratory conditions and assaying 2 or more times to ensure reproducibility. Consequently, RAPD is one of the best methods for the assessment of genetic variation among populations in species where little molecular genetic information is available (Pereira *et al*., 2010).

2.10.Controlled Breeding

The demand for fish for food, recreation, and ornamental aquaria is steadily increasing. Natural fish populations have declined during the last several decades because of environmental degradation and over-fishing. India is a rich country blessed with enormous wealth of natural resources but unfortunately both terrestrial and aquatic resources are declining due to various anthropogenic stresses. On one hand our aquatic resources are dwindling and we are losing precious genetic resources and on the other hand neither do we have a contingency plan nor do we have an appropriate technology for seed production and culture of most of the endemic cultivable food and ornamental species (Abidi, 2003).

Tropical-fish fanciers consider breeding of any recently collected species a challenge, and the huge demand for young of all popular tropical aquarium fishes has resulted in large and specialized fish breeding industries in many countries like Hong Kong, Singapore, and the USA. Tropical aquarium fish bred in captivity cover as wide a range of species as are farmed for food and breeding technology (Axelrod, 1987). Aquarists return time and again to the vital importance of diet, water quality, and breeding site. The factors interact, of course, and vary in importance with the species, but they determine success or failure. More effort should be put into improving broodstock diet during conditioning. Obtaining high-quality food, even for a limited conditioning period that may last only a few weeks, is an obvious problem for many culturists, but if

they are successful in this single most crucial area of broodstock management, the dividends will be great. Successful aquarium-fish breeders are scrupulous about finding and maintaining the right levels of pH, temperature, and dissolved solids ("hardness") for their fish breeding (Harvey and Caroisfeld, 1993).

This has resulted in an increased effort in the development of techniques for hatchery production of fish. However, a number of fish species that have or potentially have great economic significance for aquaculture do not reproduce spontaneously in captivity. Many of these fish spawn in environments that are nearly impossible to simulate in a hatchery. Hormone-induced spawning is the only reliable method to induce reproduction in these fishes. It has been used in controlled breeding for almost 60 years. Surprisingly, the same procedures, with only minor modifications, have been used to spawn an entire range of fishes. Induced spawning can be used to: synchronize reproduction of large numbers of fish for simultaneous spawning, thereby simplifying production and marketing of the fish; produce fry outside the normal spawning season for maximum hatchery production and to provide fish when the price and market demand is greatest; produce hybrids that are different from the parent species; produce sterile polyploid fish (for example, sterile triploid grass carp for aquatic weed control); and maximize survival of fry under controlled hatchery conditions (Rottmann *et al*., 1991b).

The physical injury and physiological stress of capturing, handling, transporting, injecting, and holding brood fish can have a greater detrimental effect on spawning success than almost any other factor (Haydock, 1971). Fish must be handled carefully and optimum water conditions must be maintained to minimize stress. Female brood fish ready for spawning are in a particularly delicate condition. When female fish are stressed or injured, they may undergo rapid physiological changes that can result in the breakdown (resorption) of eggs in the ovary. Crowding, dissolved oxygen depletion, rapid changes in temperature, and osmotic imbalance are well known causes of stress and must be avoided. Secondary female sex characteristics such as plumpness of the abdomen and redness of the vent are extremely subjective and can be misleading. Sampling

the eggs and sperm of the brood fish eliminates the guesswork in determining the stage of sexual development. The diameter and appearance of the egg and the position of the nucleus in the egg are visual indicators of development. An understanding of sperm viability and egg stage development will greatly improve the success of hormone-induced spawning of fish. The internal mechanisms that regulate spawning are similar for most fishes (Devlin and Nagahama, 2002). The external environmental factors that control reproduction, however, vary considerably among species. Environmental factors that have been shown to play a significant role in the reproductive cycle are: photoperiod; water temperature; water quality (e.g., dissolved oxygen, pH, hardness, salinity, alkalinity); flooding and water current; tides and cycles of the moon; weather cycles (e.g., atmospheric pressure, rainfall); spawning substrate (e.g., aquatic plants, sticks, gravel, spawning caverns); nutrition; disease and parasites; and presence of other fish. These factors do not function independently of each other, but are interrelated.

2.10.1.Hormone induced spawning

The internal mechanism that regulates the process of reproduction in fish is the brain-hypothalamus pituitary-gonad chain. Hormone-induced spawning techniques influence this sequential mechanism at several levels, by either promoting or inhibiting the process. The primary substances used for hormone-induced spawning have been: (1) pituitary extracts and (2) purified gonadotropin to stimulate the ovaries and testes; (3) LHRH analogs (LHRHa) alone or in combination with (4) dopamine blockers which enhance the potency of LHRHa to stimulate the pituitary; or (5) steroids to stimulate the gametes directly. The appropriate hormone preparation should be selected on the basis of the species to be spawned and the availability of the hormones. The hormones must be mixed and stored properly to prevent contamination and preserve potency. The proper dosage must be calculated for the brood fish, and the optimum injection schedule must be used for best results. To calculate the proper dosage, (1) the recommended dose, (2) approximate weight of the brood fish, and (3) the desired volume of the injection must be determined. The quantity of hormone to be injected can then be calculated from the weight of each individual brood fish and

the appropriate injection schedule.

Fish brain (hypothalamic portion) secretes gonadotropin releasing hormone (GnRH): the key regulator of reproductive events in all vertebrates. It is a decapeptide and four different forms of it, so far isolated from various piscine sources, vary from mammalian GnRH (Millar *et al*., 1997). GnRH reaches pituitary via portal circulation and binds to its receptor in the pituitary gonadotroph cell membrane. This causes release of gonadotropic hormone (GTH) from gonadotroph cells. GTH then travels through circulation and finds its target organ, ovary or testis, where it occupies specific receptor in the cell membrane of theca and granulosa cell of ovary or Leydig cell of testis. Reproduction in majority of fish is seasonal. Hormonal regulation for gonadal recrudescence, vitellogenic event and final maturation occur at certain specific times of the year depending on the geographical location and environment of a particular place.

After the growth phase, oocytes enter into the stage of maturation. Full-grown post-vitellogenic oocytes in the ovary of teleostean fish are physiologically arrested at the first meiotic prophase and await final maturation, which is hormonally controlled. According to Kucharczyk *et al*. (2005), this involves germinal vesicle breakdown (GVBD), chromosome condensation, assembly of the first meiotic spindle and extrusion of the first polar body. The germinal vesicle migrates toward the micropyle of animal pole, the membrane of the germinal vescicle then breaks down and its contents mix with the surrounding cytoplasm. At this time, chromosomes condense, align on the first metaphase spindle, complete meiosis I, and then realign on the second metaphase spindle, when they are considered matured egg and again remain arrested until fertilization. Over and above these nuclear changes, there are certain remarkable alterations in cytoplasm during oocyte maturation, which include the coalescence of lipid droplets and yolk globules, rapid increase of oocyte size due to hydration and a visible increase in oocyte translucency.

In present day aquaculture practice, GnRH or its chemical analogues are used for induced spawning of culturable fish. This is not only cheaper but also extremely dependable as failure rarely occurs. Being a decapeptide, GnRH is

comparatively more stable than GTH or pituitary extract and can be stored for a long time in an ordinary refrigerator. To spawn (i.e. to release the finally matured germ cells, oocytes and sperms), a sudden rise of endogenous GTH level is essential which may be three- to five-fold in excess of the basal level. There is one problem with GnRH use; its activity is inhibited by endogenous dopamine. Dopamine occupies GnRH-receptor and thus blocks its action on pituitary gonadotroph cells. Use of domperidone (or pimozide), a dopamine antagonist, increases GnRH-receptor capacity, thus enhancing GnRH responsiveness. Therefore, for induced ovulation, together with salmon GnRH-analogue, pimozide/domperidone (anti-dopamine) has been used. On the basis of this information, Ovaprim, a commercial product has been prepared by the Syndel Laboratories, Canada, which is now marketed by Glaxo Laboratories Ltd for induced breeding of fish. The first successful report in using synthetic GnRH analogue for induction of ovulation and spawning of cultured carps is available from a research group in China. This successful application of GnRH analogue has been later followed for induced breeding of various fishes including common carp, rainbow trout, Atlantic salmon, milk fish and grey mullet (Bhattacharya, 1999).

Time of ovulation, fecundity, and egg size were recorded in mature females, and sperm counts were carried out on the milt from the male fish, from both the stressed and control groups to study the effect of stress on the quality of gametes produced by rainbow trout (Campbell *et al*., 1992). Eggs from ovulated females were fertilized with milt from males subjected to the same treatment regime. Subsequent development of the fertilized eggs was then monitored. There were no differences in somatic weight or length between the two groups at the end of the experiment, but exposure of rainbow trout to repeated acute stress during reproductive development resulted in a significant delay in ovulation and reduced egg size in females, significantly lower sperm counts in males, and, perhaps most importantly, significantly lower survival rates for progeny from stressed fish compared to progeny from unstressed control fish. There is considerable evidence that corticosteroids, acting at the level of the hy-

pothalamus, pituitary gland, and the gonads mediate the suppressive effects of stress on reproduction.

Induced breeding of fish in India is being practised ever since the advent of the technique of hypophysation of the Indian major carps in the late 1950s (Chaudhuri and Alikunhi, 1957) and the exotic carps in the early 1960s (Alikunhi *et al*., 1963). India produced about 17,000 million fish fry in the year 2003 and the lion's share of India's fish seed production came from induced breeding of carps (Basavaraja, 2006). The use of fish pituitaries or human chorionic gonadotropin (HCG) is now limited, owing to inconsistent results. The Linpe method of breeding fish with injection of the combination of GnRH agonist analogues and domperidone (DOM) antagonist drugs has conclusively proved to be a highly effective means of inducing spawning in several cultured freshwater fish (Lin *et al*., 1986; Peter *et al*., 1988). The country-wide field trials on the induced breeding of Indian major and Chinese carps using Ovaprim by Syndel Laboratories Inc., Vancouver, BC, Canada, have shown consistently better results than fish pituitaries (Nandeesha *et al*., 1990; 1991). Subsequently, ovaprim revolutionized carp seed production activities in India, replacing the traditionally used fish pituitaries (Nandeesha *et al*., 1991). Hormones injected for induced spawning of fish do not produce eggs and sperm (gametes); they only trigger the release of fully developed gametes. Fish must not only be sexually mature but also in the advanced stage of sexual development before induced spawning will be successful.

Artificial fertilization has unique advantages and will continue to be used in many situations. Some of these advantages are that: spawning enclosures are not required; handling of fertilized eggs is easier; milt can be used efficiently when it is scarce (by dilution or preservation); mixing of gametes for genetic improvement is easier; and interspecific or intergeneric hybrids can be produced. Spermatozoa are kept immotile (unmoving) in the testis by high concentrations of potassium. When they are shed into the surrounding water, the potassium is diluted and the sperm cells are activated (start to swim or become motile). Motility usually lasts less than 1 minute, and fertilization itself requires only a

few seconds of contact between eggs and spermatozoa. To complicate matters further, the eggs of many species are activated by contact with water and must be fertilized immediately. The dry method is the best basis for a fertilization technique, because it takes advantage of these aspects of gamete physiology.

Two common methods of selecting the female broodstock are: 1) egg appearance; and 2) physiological. For species that reproduce during a precise spawning season, only a small number of females need be sampled to get an indication of their stage of development. The diameter and general appearance of the egg are indicators of development. Immature eggs are much smaller than ripe eggs and are usually nearly clear or opaque white or yellow, depending on the fish species. Eggs that have begun to break down (resorb) in the ovary appear whitish in color. Those eggs that have begun to resorb appear irregular in composition, and the egg contents appear to have pulled away from the cell membrane. They often appear soft or partially deflated rather than firm and round. Movement of the nucleus (germinal vesicle) from the center of the egg to the edge (germinal vesicle migration) is a preliminary step to ovulation. An understanding of sperm viability and egg stage development will greatly improve the success of hormone-induced spawning of fish (Rottmann *et al*., 1991b).

The eggs and milt of fish can be taken by several different methods: (1) tank spawning; (2) hand stripping, and (3) surgically removing the eggs. The method of choice depends on the fish species hatchery facilities, experience and skill of the hatchery staff, and the desired manipulations of eggs, sperm, or fertilized eggs. Tank spawning is the simplest method for obtaining a hormone induced spawn. Brood fish of both sexes are placed together in the spawning tank following injection(s). The female ovulates when she is physiologically ready. The males stimulate the female to release the eggs and fertilize the spawn.

Hand stripping is commonly used for taking the spawn of many species of fish. Brood fish are separated by sex prior to hormone injection to prevent spawning in the holding tank. It is important to determine the exact time of ovulation when hand stripping. In many species, egg quality can deteriorate rapidly if the eggs are not taken shortly after ovulation. For most species,

ovulation can best be verified by checking the female to determine when eggs flow freely from the vent. To strip the eggs, the fish is held slightly on her side, tail down; gentle hand pressure is applied to the abdomen, moving toward the vent. The stream of eggs is directed into a clean, dry bowl positioned so that water from the fish does not drip onto the eggs. It is important to insure that no water comes in contact with the eggs until after the milt is added and mixed. Water activates the sperm and also causes the opening through which the sperm enters the egg to close. For delicate species that seldom survive the rigors of hand stripping, humanely killing them and surgically removing the eggs may be the best option. In addition, more eggs can usually be obtained by this method than by hand stripping. The eggs obtained by hand stripping or surgical removal are usually fertilized with fresh milt. Males are captured, wiped off, and held belly down over the bowl containing the eggs. The portion of the abdomen posterior to the pelvic fins is gently massaged to extrude the milt onto the eggs. Milt can be collected from males and stored up to three weeks prior to stripping eggs. In the hatchery, stickiness of eggs causes problems during incubation. Siltclay, Fuller's earth, or bentonite suspension, urea and salt solution, and tannic acid solution are preparations commonly used to deactivate the sticky layer of fish eggs. Induced hatchery spawning of fish requires a continuous series of decisions, any of which if improperly made, can diminish or completely obliterate the success of the project. There are many ways to fail at each step and only a very few that are productive. Consistent performance requires strict attention to detail (Rottmann *et al*., 1991a).

The breeding performance and nursery practices of the threatened indigenous fish species *Labeo bata* in Jessore, Bangladesh during 1998 to 2002 were studied. Eight trial doses of PG (Pituitary Gland) used for induced breeding of *L. bata* were 1.0 mg PG/kg in the first dose and 1.0 to 8.0 mg PG/kg in second dose for female. On the other hand, five trial doses were administered for the male to identify the suitable single dose (1.5mg PG/kg) only. The best performance was obtained with 5.0 mg of PG/kg body weight in the second dose for female of wild and successive three generations (Hossain *et al*., 2007).

Wild spawners of common bream, *Abramis brama,* were caught in the Kortowskie Lake (North Poland) and transported to a hatchery for artificial spawning. One of the most important problems in cyprinid aquaculture is to obtain good-quality gametes (Kulikovsky *et al*., 1996; Horvath *et al*., 1997). For this reason many hormonal treatments are used to stimulate gamete maturation in commercial cyprinids. One of the most commonly applied spawning agents is carp pituitary extract (CPE) (Yaron *et al*., 1984; Thalathiah *et al*., 1988), in some cases with addition of human chorionic gonadotropin (HCG) (Kucharczyk *et al*., 1997a). The good results of induced ovulation in cyprinid fish were also obtained after hormonal stimulation by a synthetic analogue of gonadotropin-releasing hormone (GnRH), frequently with strong dopamine antagonists (Yaron, 1995; Barth *et al.,* 1997). Injections of HCG were intramuscular in the dorsal region of the body (Thalathiah *et al*., 1988). The above-mentioned problem of artificial spawning is much better visible in the case of wild cyprinids, mostly captured from natural populations. The development of controlled reproduction of wild cyprinids is still needed as an integral component of ongoing conservation efforts (Wildt *et al*., 1993). Artificial spawning is necessary to develop techniques of genome engineering and banking in bream (Kucharczyk, 2002). The fish was weighed, and oocytes were taken from females. Oocytes were sampled *in vivo* and placed in Serra's solution for clarification of the cytoplasm (Kozlowski, 1994).

Fish were hormonally induced using GnRH analogue combined with metoclopramide (ovopel). The results of bream reproduction in captivity were compared with fish treated with the combination of HCG and CPE and with control group injected saline. Females from the control group did not spawn whereas those from hormonally induced groups ovulated: 62% after CPE treatment and 100% after GnRHa treatment. Generally, the fish after ovopel stimulation showed the best hatchery parameters.

Eggs were stripped into a plastic vessel and were fertilized using a "dry method" (Kucharczyk *et al*., 1997a). Only those sperms were taken for egg fertilization that showed the motility of more than 80% of spermatozoa. It is well

known from hatchery practice of cyprinids, especially from wild stocks or populations, that one of the main problems is not ovulation but a small volume of sampled milt, in many cases with low spermatozoon concentration and bad spermatozoa motility (Kucharczyk, 2002; Kucharczyk *et al*., 2005).

Historically, the white sturgeon supported a major fishery in North America, yet overharvesting at the turn of the century resulted in a drastic decline in its abundance. It appears that the only means of ensuring further commercial exploitation of sturgeon is through hatchery production, stock replenishment, and domestication of sturgeon species. Ovulation and spermiation were induced in wild caught broodstock with injections of pituitary glands from sturgeon and common carp. Eggs were incubated and hatched in MacDonald jars. Larvae and fingerlings were reared in large numbers using a variety of intensive and extensive techniques (Doroshov *et al*., 1983).

Catadromous female eels were administered once a week with commercial gonadotropins, DES-Na and Vitamin E for a total of six occasions, and then with the acetone-dried pituitary glands of fishes several times over a suitably short period. The administration of LHRH analogue was also tried at the later stage of maturation. The matured female and male eels were then kept in the same tank (1.6 x 3.5 x 0.9m, water depth 0.6 m) and the above-mentioned hormone treatments were continued. When the water temperature was raised to 21-22°C, spawning behaviour was observed at night and the spawning took place in the early morning. The fertilized eggs from this spawning hatched normally. The abdomen of the spawned eels swelled up once again over a considerably short duration in almost all cases, so it can be presumed that spawning can take place at least twice. Maturation of the Japanese eel *Anguilla japonica* was achieved with hormone treatment. Along with improvement of techniques, the number of eels with conspicuously large bellies increased yearly. Fertilizable eggs were obtained in tanks with the administration of natural LHRH at the later stage of maturation, for the first time but they did not hatch. With years, the periodicity as well as the quantity of the fertilized eggs obtained has increased. Even if the maturation process of female eels can be achieved, the spawning of

good quality eggs cannot be obtained if water temperature and the other circumstances are not suitable (Hideo Satoh *et al*., 1994).

The initiation and modulation of reproductive development is timed with reference to earlier predictive environmental cues (proximate factors). In fish, both photoperiod and temperature have been identified as important proximate cues, but in salmonids, the primary environmental influence on reproductive timing is the seasonally changing daylength (Bromage and Cumaranatunga, 1988; Bromage *et al*., 1993*).* These cues act as a trigger to start an endogenous seasonal rhythm of reproductive functions (Gwinner, 1986). The ability to use periods of continuous light to manipulate spawning time could provide fish farmers with an extremely simple method for spreading the production of eggs. Periods of continuous light, applied in advance of the significant increase in daylength that occurs under the natural seasonal cycle, advanced maturation, whereas periods of continuous light applied after the natural occurrence of long days delayed maturation (Randall *et al*., 1998).

Successful induced breeding of fish using different substances with direct or indirect gonadotropic action has been reported for several species and has been reviewed by Fontaine (1976), Harvey and Hoar (1980) and Lam (1982). Even for fish species which can undergo natural spawning in captivity, induced ovulation followed by artificial fertilization is generally preferred in order to achieve a greater control over fry production. The problem in a number of cases is that the procedures in use still suffer from a lack of standardization and thus may prove difficult to repeat in different contexts because the various parameters affecting the results are not carefully defined (Legendre, 1986).

Seed production is one of three key biological areas that must be addressed in the development of a viable aquaculture system - the others are diet and fish health. Induced spawning of the spotted murrel *(Channa punctatus)* and catfish *(Heteropneustes fossilis)* was successfully carried out using ovaprim and human chorionic gonadotropin (HCG). Breeders were administered a single intramuscular injection of the hormones at varying dosages. Successful spawning of *C. punctatus* was observed at 0.3 and 0.5 ml/kg body mass for ovaprim and at

2000 and 3000 IU/ kg body mass for HCG. For *H. fossilis* successful spawning was observed at 0.3, 0.5 and 0.7 ml/ kg body mass for ovaprim and 1000, 2000, and 3000 IU/kg body mass for HCG. The ever increasing cost of donor pituitary and the cumbersome process obliged experts to test alternative hormones such as human chorionic gonadotropin (HCG) (Zairin and Furukawa, 1992; Inyang and Hettiarachchi, 1994), luteinizing hormone-releasing hormone (Fermin, 1992) and ovaprim (Alok *et al*., 1993; Haniffa *et al*., 1996). Breeders were selected by external morphological characteristics and hand stripping (Billard *et al*., 1984). The dosage of ovaprim selected by previous authors for induced spawning in carp and murrel ranged between 0.3 ml/kg and 0.6 ml/kg body mass (Nandeesha *et al*., 1993; Francis, 1996; Haniffa *et al*., 1996). The latency periods reported in the literature are 6-25h for *C. punctatus* (Banerji, 1974), 22-25 h for *H. fossilis*, and 16-20h for *Clarias gariepinus*. A latency period of 28-34h was noticed for *C. punctatus* irrespective of the hormones. *H. fossilis* spawned after a latency period of 18-24h for both hormones (Haniffa and Sridhar, 2002).

Ovaprim is effective in inducing ovulation in *O. bimaculatus*. The time taken for response (5–6 h) is the lowest recorded for any catfish. After spawning, fertilized eggs were collected, counted and the percentage of fertilization was determined (Lagler, 1982). The fertilization rate (75%) is comparable to earlier reports in *Heteropneustes fossilis* (50% and above) using D- Lys6 sGnRH –A (Alok *et al*., 1993), *Clarias macrocephalus* (60 – 80%) using LHRH-a + PIM, *Silurus asotus* (81.5– 98.0%), *Ictalurus punctatus* (78.6%) and *Mystus punctatus* (85%) using dried carp pituitary extract. The present dosage of 0.5 ml/kg body weight of ovaprim may be used as a standard in future breeding of *O. bimaculatus* (Sridhar *et al*., 1998).

Controlled reproduction of pangasiid fish has been assessed mainly for *P. hypophthalmus* (Thalathiah *et al*., 1988), and the artificial propagation of *P. bocourti* has not been reported so far. Milt was collected 12 h after injection and was drawn directly into a syringe containing immobilizing solution (NaCl: 155 mM, Tris: 20 mM, pH 7). Milt was diluted with two volumes of immobilizing solution and put into an open flask for 1 day storage at 4-6 °C. Milt solution was

stirred before use and motility of spermatozoa in water was checked under a microscope (100X). Since the eggs are sticky, they were placed directly on a net screen for incubation. Incubation can also be done in a Zuger funnel after fertilization in urea and NaCI solution (4 and 3 $g1^{-1}$, respectively), followed by rinsing eggs in tannic acid solution (0.8 $g1^{-1}$) according to the method used in common carp (Woynarovitch and Horvath, 1980). Successive HCG injections at low dose induced the completion of vitellogenesis in *P. bocourti.* Such preparatory treatment has also been reported in *Colossoma oculus* with pituitary gland extracts, *Epinephelus fario* and *E. salmonoides* with HCG, and *Anguilla japonica* with sGTH. Successive injections seem to mimic an endogenous and gradual rise of gonadotropin as in the case of implants containing GnRHa, which were successfully used in *Lates calcarifer* and *Pagrus major* (Matsuyama *et al*., 1995). The releasing rate is dependent on the ratio between the constituents, cholesterol and cellulose and requirement of dopaminergic antagonist like pimozide was also determined. In *Clarias batrachus,* pimozide is necessary to have a response to the LHRHa ovulation treatment (Manickam and Joy, 1989). The lower fecundity of *P. bocourti* raised in cages could have resulted from stress factors such as confinement, density and handling of brooders, known to affect sexual maturation in other species (Billard *et al*., 1980). In contrast, the fecundity of *P. hypophthalmus* did not differ between cages and ponds, suggesting a greater tolerance to the rearing conditions for this species (Cacot *et al*., 2002).

Whitefish, *Coregonus lavaretus* (L.), is one of the most valuable species of Polish ichthyofauna. This species has high environmental requirements, and it inhabits clean, well-oxygenated lakes. Whitefish is classified as an endangered species (Witkowski *et al*., 1999), and catches currently do not exceed 10 tons annually (Wolos and Mickiewicz, 2006). Decreases in the numbers of this fish that are noted in many areas might be linked to overfishing, deteriorating environmental conditions, and limited spawning sites and natural recruitment (Winfield *et al*., 2004, COSEWIC, 2005). Creating a brood stock is a long-term and costly process, which is why any efforts aimed at producing high quality, sexually mature fish should be preceded by gathering information regarding what

is possible to achieve. It must be kept in mind that such results might differ from those obtained in the wild. This refers in particular to the time required by the fish to achieve sexual maturity and the quantity and quality of the reproductive products that are obtained. Working with fish under controlled conditions presents a range of difficulties including environmental conditions that differ from those in the wild, and fish sensitivity to manipulation. Another issue is the selection of a suitable diet, which is usually different from that of fish in the wild. Subjecting the fish to inappropriate rearing conditions and diets can result in disturbed gametogenesis (Bogdanova, 2004) and less effective fish reproduction (Miroslaw *et al*., 2010).

The external environmental factors that control reproduction vary considerably among species. Abiotic and microbial factors play a significant role in fish breeding, early ontogeny and survival of spawn in high-density culture. A large-scale mortality of developing embryos and spawn of culturable carps in fish hatcheries due to unfavourable environmental factors is of serious concern to fishery scientists and fish farmers. The conditions considered optimal for survival and growth of older fish may be a limiting factor for embryo or spawn. A natural variation in some abiotic and microbial factors of water occurs during fish spawning and become even more serious in a closed hatchery system or in places where the quality water for fish spawning is limited. Dwivedi *et al*. (1988) observed the optimum range of factors like temperature, pH and dissolved oxygen for carp breeding and spawn rearing. Singh and Das (2006) reported that the reproduction, mortality and growth of *Cyprinus carpio* are influenced by some abiotic factors. Mohan (2000) studied the effects of some of the abiotic factors on the breeding of some Indian and exotic carps. The proper environmental conditions stimulate the reproduction process, while unsuitable conditions can override any attempt of induced spawning.

Experiments with fishes suggest that offspring survival varies positively with the age, size and condition of parents, as well as the size and content of gametes (Heath and Blouw, 1998; Berkeley *et al*., 2004; Rideout *et al*., 2004; Kamler, 2005), and recent evidence has suggested that these relationships are

relatively consistent across years (Seamons *et al*., 2007; Venturelli *et al*., 2009). The survival and quality of progeny can be strongly influenced by nongenetic effects derived from the physiological condition of the mother during gametogenesis. The influence of maternal condition on the size and quality of larvae at dispersal was examined for the tropical damselfish, *Pomacentrus amboinensis*. Much of the variability in progeny size was explained by levels of the stress-associated steroid hormone, cortisol, in the female. Density-dependent processes, such as competition, can act to emphasize phenotypic differences among individuals in the breeding population (Ostfeld and Canham, 1995) and determine reproductive output (Kerrigan, 1996; Mccormick, 1998).

Water pollution due to rapid industrial development and the increasing use of insecticides may, in not too distant future, deplete natural spawning stocks and cause heavy mortality of larvae and fry (Dupree and Huner, 1984; Piper, 1989). Although *C. batrachus* breeds naturally in ponds, the efficiency and rate of induced spawning of catfish with ovaprim has been found to be less than 50% (Thalathiah *et al*., 1988; Ngamvongchon *et al*., 1988), which is very low compared to that of carps. Treatment of fish with spawning agents like ovaprim has been successful in carps, goldfish and others. However, the effect of ovaprim has not been as pronounced in the Indian catfish. Studies have demonstrated that this decrease in spawning efficiency may be attributed to a decrease in the expression of GnRH-receptor II in the catfish ovary, leading to a concomitant decrease in the development and maturation of primary follicles and subsequent ovulation (Munmun *et al*., 2010).

Induced spawning of African giant catfish *(Heterobranchus bidorsalis)* was successfully carried out using synthetic hormone (Ovaprim) and natural hormone (homoplastic hormone-pituitary extract from *H. bidorsalis*. The results showed that ovaprim performed significantly better ($P < 0.01$) in almost all the parameters investigated. The two hormonal materials gave slightly different results in terms of pre and post hormonal induced spawning mean somatic weight loss of 423.83 ± 14.19 g and 446.00 ± 13.37 g, mean number of dead eggs of 396.10 ± 19.15 and 194.90 ± 11.00, hatchability of 9,180.13 ± 343.37 and

11,162.27 ± 362.00 hatched larvae, 35.80 ± 1.11 and 12.37 ± 1.54 deformed larvae, and 99.61 and 99.88% survival were recorded respectively for homoplastic hormone and ovaprim, respectively. Comparative cost benefit analysis showed that ovaprim which recorded better results, was also relatively cheaper. Because of its relatively cheap cost, ease of handling and better survival of hatchlings from *H. bidorsalis*, ovaprim is highly recommended for hatchery users (Nwokoye *et al*., 2007).

Three areas contributing to the advancement of hatchery management are: broodstock management, induced spawning and larval feeding. The use of gonadotropin-releasing hormone agonists (GnRHa) given as an injection or a slow release implant for induced spawning is becoming more widely used. The addition of dopamine antagonists with GnRHa may not be necessary to successfully induce spawn some species of fish. The use of GnRHa can advance the maturation of oocytes allowing such fish to be successfully induced spawned. The mode of GnRHa application was investigated by Fornies *et al*. (2001). Mature female sea bass (*Dicentrarchus labrax*) were treated with GnRHa-loaded microspheres, GnRHa-loaded implants, or given a single injection of GnRHa. Microspheres and implants induced multiple spawns in female, whereas GnRHa-injected fish spawned only once. Determining proper dose rate using implants was studied by Ibarra-Castro and Duncan (2007) working with recently caught wild spotted rose snapper (*Lutjanus guttatus*).

Nayak *et al*. (2001) evaluated the efficacy of (LHRHa), the dopamine antagonist pimozide (PIM) and a commercial spawning product (ovaprim, containing both GnRHa and a dopamine antagonist) on oocyte maturation, ovulation and spawning of the catfish *Heteropneustes fossilis*. Both LHRHa and pimozide when given alone failed to induce ovulation, but both drugs did advance oocyte maturation. Ovaprim has been successfully injected into more than 15 fish species in the last 20 years. Dagin, another commercial product, combines an analog of sGnRH (10 mg/kg), with a dopamine antagonist, metoclopramide (20 mg/kg). Yaron *et al*. (2009) tested common carp pituitary and Dagin to induce common carp reproduction. The spawning ratio and embryo

viability were similar, but the latency between injection and ovulation was considerably longer and more variable in Dagin-treated than in CCPE-treated carp. They recommended the use of carp pituitary at the beginning and end of the spawning season when the LH content in the pituitary is low, and Dagin in mid-season. A commercial induce-spawning agent (Ovatide), was evaluated by Marimuthu *et al*. (2007). Ovatide, which is a mixture of a synthetic GnRH analog and a dopamine antagonist, was used to induce spawn striped snakehead, *Channa striatus*. The authors found that a dose of 0.4 ml/kg was effective for inducing spawning. Gonazon, which contains the GnRH analogue, azagly-nafarelin but not a dopamine antagonist, is approved in EU for salmonids (Phelps, 2010).

With respect to pompano, preliminary work indicated that pompano can be conditioned to spawn (by strip and volitional spawning methods) under varying photothermal conditions via administration of human chorionic gonadotropin (HCG). Although eggs were produced, fertilization rates were highly variable and many eggs exhibited abnormal development. To initiate spawning, ripe females (mean oocyte diameter> 500 μm) and males were implanted with a 75 μg GnRHa pellet. Fish spawned approximately 36 hours post-implantation and eggs were collected and stocked into incubation tanks (24-26°C) with hatching occurring approximately 30-36 hours post-fertilization. Hatching rate of fertilized eggs in trials conducted ranged from 73.1-95.4%. Further, the development of sustained-release GnRHa formulations (Sherwood *et al*., 1988; Powell *et al*., 1998) have proven effective in the promotion of extended captive reproduction of fish that spawn serially over a protracted season such as Florida pompano (Watanabe *et al*., 1998; Weirich and Riley, 2007).

Until now the fry of *R. rita* can only be collected from the river systems where they breed naturally. Mollah *et al*. (2008) reports the first incidence of successful induction of breeding in the riverine catfish *Rita rita* using carp pituitary gland (PG) extract. A breeding trial using four PG doses viz. 80, 100, 120 and 140mg/kg body weight of fish was conducted to optimize the dose of pituitary gland (PG) extract in terms of induction of ovulation in female. The male received a dose of 40mg PG/kg body weight in all cases and was sacrificed

for collection of milt. The best performance was shown by the fish treated with 100mg PG/kg body weight in respect of inducing ovulation in females and fertilization and hatching rates of eggs.

2.10.2.Early embryonic development

Controlled reproduction of fish makes it possible to develop method for selective breeding of superior strains and for furnishing parasite- and disease-free fish seeds. Artificial fertilization of the hand-stripped eggs was made by the dry method in plastic pans. In the case of mullets, the fertilized eggs were hatched in aquaria with circulation of sea water as well as in natural condition in shallow sea (Tang, 1964). The embryonic development of this fish observed from the external features of the eggs obtained from induced spawning was described. The newly hatched larvae were reared under the same conditions as in hatching. The pituitary of the common carp, *Cyprinus carpio,* combined with a certain amount of Synahorin gave better result in inducing spawning of the Chinese carps than individual use of the common carp pituitary or Synahorin alone (Rottmann *et al*.,1991a) .

During the past few years the natural population of *Mastacembelus pancalus* has been rapidly declining due to various manmade and natural causes. There is no sufficient information on the early development of this fish. It is necessary to characterize its various stages of embryonic and larval development to understand the biological clock and cultural techniques of the species. Life starts with the unification of male and female gametes. As soon as the egg is fertilized by a sperm the zygote is formed and embryonic development starts and ends up at hatching. The larvae further undergo organogenesis and resembles their parents, thus end the larval stages (Karim and Hossain,1972).

Egg development in the ovary is maternally derived and is predetermined in the ovary but its genetics complex is determined at the very instant of fertilization. Rahman *et al*. (2009) studied the early embryonic and larval development of *Mastacembelus pancalus*. The parents stock was collected from different places of Mymensingh district. The eggs were obtained through

induction of spawning by use of hormones. At fertilization; the eggs were 0.50 mm in diameter. Samples were taken every 10 minutes interval till completion of morula and then every 1 hour interval up to hatching. After hatching, daily observations took place until the attainment of the fingerling stage. The eggs presented coloration varying from yellow to brownish-green. They were spherical, demersal and adhesive. The stages of embryonic development observed with cleavage, followed by blastula, morula, early gastrula, middle gastrula, late gastrula and until hatching of non-pigmented larvae which displayed total average length of 1.3 mm ± 0.22, 35 hours after fertilization. First cleavage was recorded within 1.05 hrs after fertilization and the embryonic rudiments of developing eggs appeared at 24.30 hrs at 27.0-31.0°C. The yolk sac was completely absorbed at 67 hrs during embryonic development on attainment of 5.50 mm total length. At the same time the digestive system became fully developed and the larvae searched for feeding.

S. fluviatilis is classified as vulnerable or endangered in the majority of the countries where it occurs. It is of urgent need to design and implement effective management and conservation measures to recover this endangered freshwater species. The full developmental sequence, from egg to adult was described as the information concerning the developmental biology of a species is of much need to allow captive breeding for the recovery of natural populations (Gil *et al.*, 2010).

2.10.3.Larval Rearing

The varied structural adaptations of the alimentary canal of fish larvae and the changes of these adaptations with development are characteristic of differing functional adaptations to diets and prey concentrations. There is some evidence that ingestion and digestion rates as well as assimilation efficiencies are adapted to maximize larval growth and that these adaptations differ among taxa (Houde and Schekter, 1980; 1983). Within taxa, digestion rates and assimilation efficiencies may change with prey availability and ration size (Werner and Blaxter, 1980; Boehlert and Yoklavich, 1984) as well as with development (Laurence, 1977, Buckley and Dillmann, 1982). Most fish larvae are visual,

raptorial planktivores (Hunter, 1981) regardless of whether their adult counterparts are indiscriminant filter feeders, pelagic carnivores, or benthic pickers. Larvae begin feeding on large phyto- and small zooplankters and follow by feeding on increasingly larger zooplankters (Hunter, 1981; Govoni *et al*., 1986).

The amount of food wastage can be reduced by ascertaining the amount of live food required, on a daily basis, to support robust growth and enhance survival (Barahona and Girin, 1977). The improved growth and survival observed in large containers may be due to a lower possibility of damage of the larvae from contact with the wall, more favourable water quality or possible development of a natural food chain as suggested by May *et al*. (1974). Increased feeding frequency usually improved feed utilisation and reduced the incidence of starvation in small fish in a group (Yager and Summerfelt, 1994; Duray *et al*., 1997). The success of larval rearing is greatly influenced by first feeding regimes and the nutritional quality of starter diets, with dietary lipids being recognized as one of the most important nutritional factors that affect larval growth and survival (Watanabe *et al*., 1983; Izquierdo *et al*., 2000).

Amino acids are also important catabolic substrates after the onset of first feeding and may account for 60% or higher of the energy dissipation. Since growth is primarily an increase in body muscle mass by protein synthesis and accretion and fish larvae have very high growth rates, they have a high dietary requirement for amino acids. Live foods such as phytoplanktons, rotifers, *Artemia* and various zooplanktons all contain a significant pool of FAA (Fyhn *et al*., 1993, 1995; Helland, 1995), although the mass-specific content of FAA varies between the various live foods (Ronnestad *et al*., 1999).

2.11.Transgenic technology

Transgenesis involves genomic alteration of an organism through insertion, modification or deletion of a gene with the objective of modifying characteristics of interest (Carter, 2004). In this manner, new stable and genetically determined characteristics can be incorporated into the genome of the

receptor organism and possibly transmitted to the next generations. In the last two decades, this technology has been successfully applied in fish due to the fact that these inferior vertebrates present reproductive and biological characteristics that allow easy manipulation of their genetic and physiological processes in the early stages of ontogenesis (Zhu and Shu, 2000). Studies of gene transfer have been carried out in more than 35 teleost species, most of which are important to aqua-culture (Zbikowska, 2003). However, genetically modified fish have also been developed as experimental models for biomedical research, especially in studies involving embryogenesis and organogenesis (Goldman *et al.,* 2001; Huang *et al.,* 2001*;* Takechi *et al.,* 2003) as well as in the study of human diseases (Ward and Lieschke, 2002), xenotransplantation (Pohajdak *et al.,* 2004) and recombinant protein production of important therapeutic agents (Anderson and Krummen, 2002).

Several techniques are currently available for transgenic fish production which have been developed to increase the efficiency of transgene integration or to produce a large number of transformed individuals simultaneously. Although these new methods of gene transfer are gaining importance due to the encouraging results reported (Tanaka and Kinoshita, 2001; Lu *et al.,* 2002; Grabher *et al*., 2003; Hostetler *et al.,* 2003; Kinoshita *et al.,* 2003; Kurita *et al.,* 2004), when microinjection is used to produce transgenic fish it almost invariably produces mosaic fish due to delayed trans gene integration, which occurs only after a few cycles of embryonic cell division. If the transgene is integrated in only one cell group or tissue but not into germ cells the transmission of the gene to descendants is difficult or impossible (Maclean, 1998). Gene transfer technology has produced a great impact in modern biology and biotechnology (Powers *et al*., 1998). Stuart *et al*. (1990) first showed that the DNA injected into the cytoplasm of fertilized zebrafish eggs could integrate into the fish genome and be inherited in the germ line. Culp *et al*. (1991) demonstrated that the frequency of germline transmission of a microinjected DNA could be as a high as 20% in zebrafish. This technology, however, still has as the major constrains the low efficiency generation of transgenics (Morales *et al*., 2001).

The gene coding for the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been widely used as a reporter gene because it does not require an exogenous substrate for its activity (Amsterdam *et al.,* 1995) and is stable and non-toxic in receptor organisms (Peters *et al.,* 1995). Green Fluorescent Proteins (GFP) are present in a wide variety of marine organisms, from corals to jellyfish. Its applications include use in *in vivo* imaging as well as in plant biology. Thus, the protein has become one of the most important tools used in contemporary biosciences. As GFP-like proteins have numerous applications under their spectrum, they can be aptly called as 'multi-purpose proteins' (Krishna and Ingole, 2009). In its journey from the tissues of the bioluminescent jellyfish *Aequorea victoria* to thousands of laboratories worldwide, GFP has found countless applications to research biology and bioengineering (Tsien, 1998) as a reporter tag to cell biology, the molecular engineering of GFP to improve its fluorescence properties, the applications of GFP as a reporter tag to bioengineering systems, and the introduction of additional functionalities into GFP via recombinant DNA technology. Further products of molecular engineering include enhanced GFP (EGFP), which fluoresces 35 times more brightly and emits red light (Zhang *et al.* 1996), and GFPuv, which fluoresces brightly under UV light but proves superior for GFP analyses in multicellular organisms, in particular insects (Cha *et al.*, 1997).

Although transgenics have been produced since the early 1980s, the production of transgenic fish was accomplished only a decade ago. Transgenesis can be accomplished using a number of techniques, such as microinjection that allows delivery of the transgene directly into the nucleus and electroporation that facilitates the formation of temporary pores on the surface of the target cells through which the transgene is introduced into the cytoplasm where it is then delivered to the nucleus by the cellular machinery (Pinkert *et al*., 1995). A variety of gene transfer techniques, such as calcium precipitation, microinjection, lipofection, retrovirus infection, electroporation, embryonic stem cells, sperm mediated gene transfer and particle gun bombardment, have been used to produce transgenics. Different gene transfer techniques present various advantages and

disadvantages depending on the species of animal to be used for gene transfer studies (Pinkert *et al*., 1995). For instance, methods such as microinjection and electroporation are currently used for gene transfer in egg-laying fish, whereas others, such as retroviral vector-mediated gene transfer are used in live-bearing fish species (Chen *et al*., 1993). Electroporation utilizes a series of short electric pulses to permeate the cell membrane and allow the transgenes to enter the cells. Electroporation has been shown to be the most effective means of gene transfer in fish since a large number of fertilized eggs can be treated in a short time by this method (Inoue *et al*., 1990; Chen *et al*., 1993a).

A transgene construct usually contains a promoter sequence, a binding site for RNA polymerase to initiate transcription of the transgene message, a signal sequence to address where the transgene product is going to be expressed and the transgene sequence. Currently, the preferred way of producing a transgenic fish is to introduce transgene into a newly fertilized egg. For this purpose, fertilized eggs can be collected immediately after fertilization and maintained in ice-cold Yamamato's saline to slow down embryonic development for a short period of time before gene transfer (Sambrook *et al*., 1989). Yamamoto's saline (Yamamoto, 1941) can be used as the electroporation medium.

After the gene transfer, treated embryos can be incubated under standard conditions established previously for each fish species until hatching. It has been reported that only a part of gene transfer trials are successful (Chen *et al*., 1993). Therefore, following the gene transfer, each individual needs to be analyzed for the presence of the transgene. For this purpose, following hatching, the treated individuals are reared to a stage at which a fin clip can be obtained without harming the individual to check for the presence of transgene. Genomic DNA from each surviving treated fish is prepared from a small piece of fin tissue fin clip. A variety of methods, such as dot blot and Southern blot hybridization, can be utilized to identify putative transgenic individuals using genomic DNA samples (Sambrook *et al*., 1989). F_o individuals are then crossed with nontransgenic individuals to investigate the transmission of the transgene and to

establish the transgenic fish lines. Progeny produced from the cross of a F_o transgenic and nontransgenic individuals are referred to as the F_1 generation. F_1 transgenic individuals are identified by the same method used to identify F_o transgenic individuals.

A number of previous studies (Chen *et al*., 1995; Inoue *et al*., 1990) demonstrated that some of the F_o generation transgenic fish are germline mosaics, meaning that these individuals contain the transgene in only a part of their germ cells; these therefore, produce transgenic offspring in limited numbers. Studies (Chen *et al*., 1993; Pinkert *et al*., 1995; Inoue *et al*., 1990) show that levels of transgene expression vary among transgenic individuals. This is because the numbers of integrated transgenes and integration sites vary among different individuals. Zhang *et al*. (1990) and Chen *et al*. (1991) reported that among the F_o and F_1 transgenic lines of common carp carrying rainbow trout growth hormone gene, the expression level of the transgene varied some tenfold. Stable integration of the transgene is required for continuous transmission to subsequent generations to establish a transgenic fish line.

There are important drawbacks to the use of these DNA elements in transgenic fish, including low expression efficiency (0-20%) (Culp *et al*., 1991; Higashijima *et al*., 1997) and the mosaic expression of transgene patterns (Stuart *et al*., 1990; Ju *et al*., 1999). Nevertheless, it is generally accepted that transgene integration mosaicism is inevitable (Liang *et al*., 2000). The search continues for a simple but effective means of improving the F_o expression of transgene and the stable transmission of phenotype in transgenic fish. Although Gibbs and Schmale (2000) demonstrated that transgenic GFP could be expressed throughout the life, the phenotypic difference occurred between the transgenic lines (Chou *et al*., 2001). Although some methodologies have presented promising results in increasing first generation (F_o) transgenic fish production, there is still the problem of rearing large numbers of F_o fish and the subsequent identification of those with the potential to generate stable germlines carrying the active transgene. This process is even more difficult for large fish such as carp, salmon,

tilapia or trout which need very complex culture facilities (Figueiredo *et al*., 2007).

In the past, microinjection of plasmid DNA into early embryos represented the state of the art in generating transgenic fish. However, this approach suffers from significant drawbacks like mosaic distribution of the injected transgene, late transgene integration at high copy numbers, low transgenesis frequency, making the generation of transgenic lines a laborious task (Soroldoni *et al*., 2009). Thus, it is necessary to develop simple gene transfer methods for use in aquaculture. However, it is important to consider that the success of transfection with foreign DNA seems to be a characteristic of the DNA, of the concentration, and the proportion of the linear and/or circular forms (Sciamanna *et al*., 2000; Shen *et al*., 2006; Hoelker *et al*., 2007). In this sense, it is important to establish new transfer techniques *en masse* for genes of interest and those that are important in studies of molecular ecology and biotechnology (Collares *et al*., 2010).

It is well known that the direct injection of plasmid DNA into muscle is simple, inexpensive, and safe non viral gene transfer technique *in vivo* (Draghia-Akli *et al*., 1999). Skeletal muscle can be transfected *in vivo* by direct plasmid DNA injection, where foreign genes can be expressed at significant levels for up to 19 months (Wolff *et al*., 1992; Tripathy *et al*., 1996; MacColl *et al*., 2000). These characteristics make plasmid-mediated gene delivery an excellent candidate for agricultural application (Hu *et al*., 2010).

Since 1985, a wide range of transgenic fish species have been produced (Chen and Power, 1990; Hackett, 1993) by microinjecting or electroporating homologous or heterologous transgenes into newly fertilized or unfertilized eggs. The prototype of a transgene is usually constructed in a plasmid to contain an appropriate promoter-enhancer element and a structural gene sequence. TK-l, or "Night Pearl", a derivate of green fluorescent medaka, is the first GM pet in the world (Dean, 2003; Holden, 2003). The only concern about marketing of GM pet fish is its ecological impact. Fortunately, more recently the technology of how to achieve 100% sterilization of Tk-l progeny is successfully developed. Thus, this

GM pet fish should be considered as environmentally and ecologically safe (Tsai, 2008).

2.11.1.Electroporation

The traditional method of microinjection of foreign DNA into fertilized embryos is tedious and time consuming. An electroporation apparatus can be used to generate transgenic fish by electroporation. The generation of transgenic fish by electroporation has several advantages over the traditional microinjection technique (Buono and Linser, 1991). Microinjection of the transgene vector into the pronucleus does not guarantee immediate integration. If integration occurs during the first cell cycle all cells would be expected to be transgenic. If integration doesn't occur until the second cell cycle, only 50% of cells would be transgenic, and for the third and fourth cell cycles the rate of transgenic cells would be 25 and 12.5% respectively, and so on. Integration before or after DNA replication at each cell cycle stage will also affect the efficiency of transgenesis (Chan *et al*., 1999).

DNA is a negatively charged molecule due to the presence of phosphate groups in its backbone. So when it is exposed to a current of electricity, DNA will migrate towards the positive anode. Electroporation is performed by exposing a cell culture mixed with transgenic DNA to a pulse of high voltage electricity. The DNA is pulled through the cell membranes by the electric current. Once inside the cell the DNA moves through the cytoplasm and becomes incorporated with the genetic material inside the cell (Taconic, 2003). This technique uses electric pulses to make small holes in the cell membrane through which DNA molecules can enter the cell. The procedure is rapid and simple, and it allows treatment of many embryos in a short period of time. Also, there is great variability in the amount of signal seen from one positive animal to the next. This may represent non-integration or low numbers of cells carrying copies of the transgene. It is therefore prudent to electroporate only one- and two-cell embryos to avoid generating chimeric or mosaic animals. Stable integration can only be demonstrated by the production of transgenic progeny from transgenic founder animals.

Although microinjection has been used successfully in the production of transgenic fish and is a commonly used technique, it is not adequate for the production of the large numbers of fish needed in screening for correct transgene expression. This method is not only time consuming and labor intensive but is also limited by the physiology of fish eggs. The nuclei of fish eggs are small and difficult to visualize, the outer membrane, or chorion, hardens soon after fertilization (Ginsburg, 1963), and in many species the eggs are opaque. Electroporation is an alternative that alleviates many of these problems and has the potential to make gene transfer more efficient. The general procedure was derived from cell culture studies in which an electrical permeabilization of the cell membrane was used to stimulate uptake of DNA (Neumann *et al.*, 1982; Shigekawa and Dower, 1988). Only embryos electroporated with pCMV-EGFP or CMV-PHY-GH1t-CMV-EGFP were grown to sexual maturity and used to test for germ-line transmission. Electroporation conditions were 25V, 100% modulation, 35kHz, 10 ms burst duration, 3 bursts, and a burst interval of 1.0 s. Of the 100 embryos electroporated with pCMV-EGFP (50 from each replicate), 70 survived to sexual maturity, and all were positive for the transgene (Hostetler *et al*., 2003).

Transient and stable GFP transgenics can be made in both medaka and zebrafish by injecting plasmid DNA into the embryo at early cleavage stages (Ozato *et al*., 1986). In zebrafish, injected plasmid DNA is converted into a high molecular weight form and amplified during the cleavage stage. It is subsequently degraded during gastrulation and retained in only some of the cells (Stuart *et al*., 1988). A great number of stable transgenic GFP lines have been created using tissue-specific enhancers in zebrafish (Megason *et al*., 2006). It is possible to electroporate unfertilized eggs or just fertilized eggs, but the latter is shown to be ideal, as it results in higher frequency of transgenics (Marian *et al*., 1997) and low percentage of deformities (Tsai *et al*., 1995). Low survival of the electroporated embryos (average 66%) compared to the control (88%) indicates the stress suffered by eggs subjected to electroporation (Sheela *et al*., 1998).

The expression was very strong in the site of injection in compare to electroporation group and the expression was very stable from the very beginning. Injection of plasmid with target DNA (in our case target gene was GFP gene) into the fish muscle was sufficient for expression of transgene into muscle cells that has also shown by some other workers (Xu *et al*., 1999; Tan and Chan, 1997; Lin *et al*., 2008).

Transgenic fish are appealing to some because attainment of desired traits is generally more effective, direct, and selective than traditional breeding, and could prove to be an economic benefit for improvement of production efficiency in aquaculture worldwide (Nam *et al*., 2002; Ramirez and Morrissey, 2003). In addition, fish species tend to be relatively tolerant to artificial manipulation of their genes during early development, making them ideal subjects for genetic modification (Foresti, 2000). However, use of transgenic organisms in aquaculture is a very controversial topic due to a number of environmental and human health concerns such as escapement and introduction of genetically modified organisms into the food chain. In response, some transgenic research has also been focused on inducing sterility to reduce the risk of transgenic organisms breeding with wild species. A method of chromosome manipulation, referred to as polyploidy, provides the option of creating sterile organisms, some of which also exhibit increased growth rates (Rasmussen and Morrissey, 2007). Methods of inducing sterility in transgenic fish are important for two reasons, firstly the escape of transgenic fish or shellfish from the sea cage environment is of real environmental concern, and has motivated researchers to develop strategies to render escapees sterile. Secondly, any company marketing a transgenic strain would benefit from selling only sterile individuals, thereby preventing others from growing and maintaining their own broodstock.

When a construct is inserted into an organism with the objective of improving a specific trait, that construct may affect more than one phenotypic character. These “pleiotropic effects” may be positive or negative (Dunham and Devlin, 1999). For instance, Chatakondi *et al.* (1995) found that common carp transgenic for the rainbow trout growth hormone had a positive influence on

survival from the fingerling size upwards, when subjected to a series of stressors and pathogens such as low oxygen, anchor worms and dropsy. Electroporation of sperm or gonads prior to fertilization is also possible and has been used successfully in the Chinook salmon *Oncorhynchus tshawytscha* (Sin *et al*., 1993). Electroporation is the favoured method in invertebrate transgenics also.

2.11.2.Identification of transgenic individuals (F_o)

The process of making transgenic animals is not efficient. Many resulting offspring do not take up the transgene, or they take it up but do not express it, so it is important to screen offspring for transgene integration and expression. Many methods for the detection of a transgene or its by products have been developed. Some screen for the transgene DNA itself, some for the mRNA produced from the transgene, and some screen for the transprotein encoded by the mRNA. Some transgenic techniques even allow screening before the embryo is developed, but most screening occurs when the newborn founder animal is about 3 weeks of age. And even if a founder proves positive, it is still important to continually check for transgene expression through the course of the animals' life.

Southern blotting is a technique that detects a specific DNA sequence in a complex mixture of DNA. This technique can detect the presence and copy number of a transgene inserted in the genome of an animal. The DNA most commonly tested is attained from tail, ear, or white blood cells. In the Southern process, the organisms' genome is cut using restriction enzymes into smaller fragments. These fragments are then separated by size in a process called gel electrophoresis. Using electricity to pull the negatively charged DNA through a gel, it can be separated based on size. The DNA in the gel is then denatured to form single stranded DNA (to allow it to anneal to a probe), and the DNA is transferred to a paper like membrane. The DNA on the membrane is washed with a solution containing a probe that contains a complementary DNA sequence to the transgene sequence of interest. The probe has either a radioactive or fluorescent label attached to it to allow visualization (Amsterdam *et al*., 1995).

Mosaicism can arise in a number of ways. 1. Position-dependent inactivation of gene expression in a fraction of cells that generate a particular tissue. This "on or off" expression state is then maintained through future cell divisions 2. High transgene copy number and tandem head-to-tail arrangements make the DNA more prone to rearrangement, and as a result different cell subsets may be generated and may result in silencing of expression in a subset of cells. 3. Integration of the transgene into the chromosomes at different stages of cell division. Recent advances in generating transgenic fish have improved the efficiency of germline transmission and enabled the generation of large numbers of transgenic animals. A suitable co-injection marker may help facilitate the preselection of transgenic embryos. For this purpose, a lens-specific marker appears to be a suitable candidate since the lens is a well-defined tissue that is easily accessible for examination of reporter gene expression. (Vopalensky *et al*., 2010).

The medaka β-actin/EGFP transgenic tilapia was produced by microinjecting embryos at the one-cell stage, as described by Kobayashi *et al*. (2007). Gamete collection and artificial fertilization procedures were followed as described by Biswas *et al*. (2005). The gene construct introduced was a plasmid containing the promoter, enhancer regions, and polyadenylation signal of the medaka β-actin gene, and the coding region of EGFP with a human codon usage, described previously by Hamada *et al*. (1998). Newly hatched *O. niloticus* were reared in the laboratory under the same conditions described above for parental fish. GFP-positive founders (F_0) were crossed with wild-type female for the production of the F_1 generation. The resultant F_1 transgenic fishes were identified by fluorescence observation. To determine the inheritance of the transferred DNA fragments from F_1 to F_2 generation, an F_1 GFP-positive male (screened by both fluorescence observation and PCR) derived from β-actin / EGFP-injected embryos was crossed with a wild-type female. The resultant F_2 progeny embryos were reared and their green fluorescent signals were observed at 140 days post-fertilization by direct observation of the caudal fin under a fluorescence

stereomicroscope. Fish were segregated into two groups according to their GFP fluorescence expression (Farlora *et al*., 2009).

The most successful commercial ornamental transgenic organism to date has been 'GloFish' (www.glofish.com), marketed by Yorktown Technologies. Since 2003, transgenic zebrafish (*Danio rerio*) have been sold to aquarium enthusiasts for approximately US $5.00 each. *D. rerio* were transformed with several different FPs – GFP, yellow fluorescent protein (YFP) and the red FP from the coral *Discosoma* sp. (DsRed) – under the control of a strong muscle-specific (mylz2) promoter (Gong *et al*., 2003). Illuminated with an ultraviolet aquarium lamp, GloFish appear brightly fluorescent red, and, in fact, the expression of the FP is great enough to color the fish red under room light. As marketing of ornamental fish has no food safety concern, ecological impact of the fluorescent transgenic fish become essentially the only concern. To address the concern, experiments were carried out to compare several parameters to determine their fitness relative to wild type fish. These parameters included juvenile viability, adult viability (life span), age of sexual maturity (growth rate), fecundity, fertility, and mating behavior, as recommended by Muir and Howard (2002). In all the experiments carried out, transgenic fish were less fit than the wild type fish. Thus, the transgenic fish should not pose a higher risk than the wild type fish for environmental impact.

With the successful commercialization of fluorescent transgenic fish or GloFish, the beauty of GloFish should not be just limited to ornamentation. It is expected that GloFish will serve as an excellent biotechnology model for public education to understand transgenic technology, GMOs (genetically modified organisms) and GM products. Indeed, GloFish is probably so far the best illustrated transgenic model for general public because the easily observed traits changed by genetic modification and the amusing appearance of these fish.

3. MATERIALS AND METHODS

3.1. Location

Plate 1 *Irrity River*

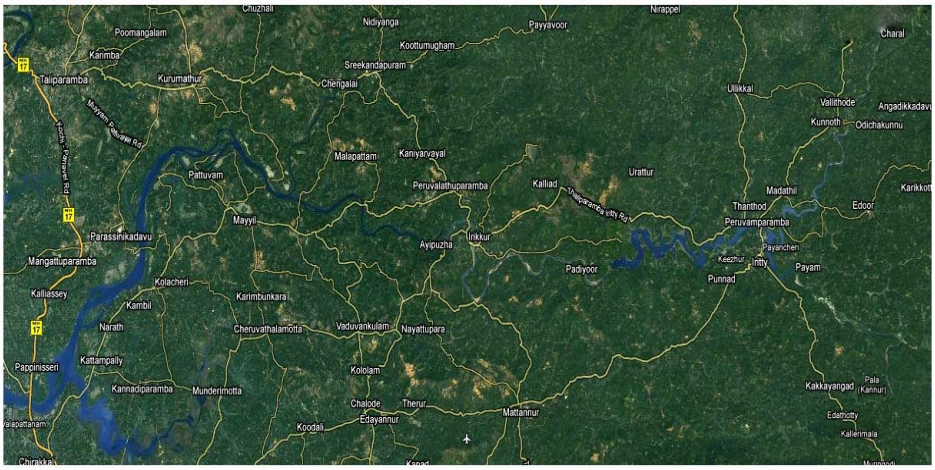


Plate 2 *Satellite image of Irrity River* 11°59'15"N 75°40'47"E

Plate 3 *Chaliyar River*

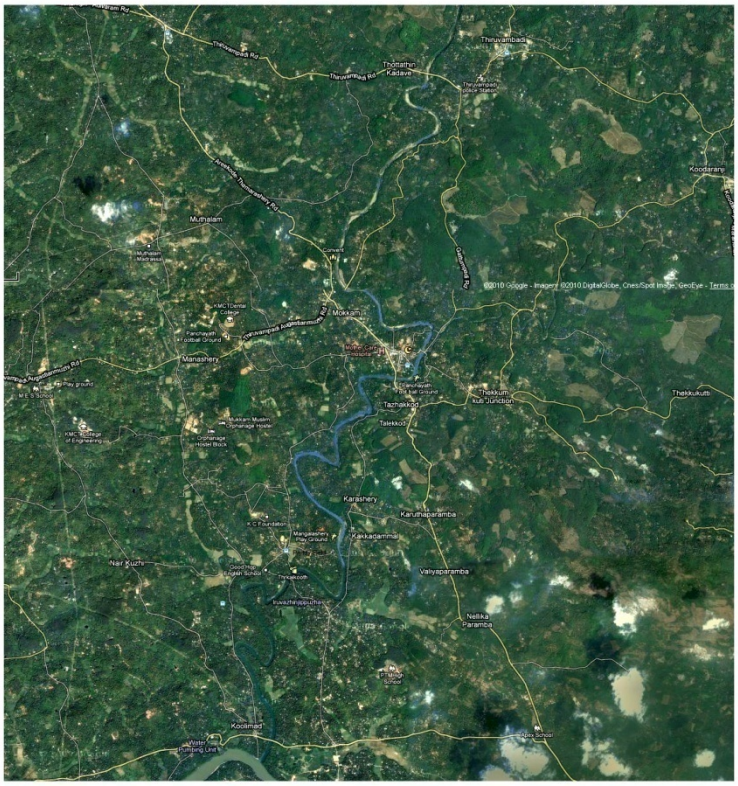


Plate 4 *Satellite image of Chaliyar River* 11°19'18"N 75°59'50"E

Plate 5 *Satellite image of Periyar River* 10°9'46"N 76°46'18"E

3.2. Truss Analysis

Freshly dead specimens of *Puntius denisonii* 30 numbers each were collected for truss analysis from fish collectors and transported to College of Fisheries, Panangad from different rivers of Kerala, namely the Irrity River, the Chaliyar River, and the Periyar River between November 2007 and January 2008 (Plate 1 to 5). The samples were placed individually into plastic bags and were kept deep-frozen (-20 °C) until truss studies were conducted in the laboratory. The three important steps in Truss Network analysis are;

1. Positioning: Fish were placed on acetate sheets, and body posture and fins were teased into a natural position. A block of expanded polystyrene (2 cm thick) was placed beneath the acetate sheet to facilitate pinning (Plate 7).

2. Pinning: Morphological features or landmarks that are distinctive and homologous from specimen to specimen were selected around the outline of the fish form. Each landmark was marked by piercing the acetate sheet with a dissecting needle. Measurements were made on one side of the each specimen throughout sampling. Additional data, such as eye diameter and standard length were also recorded and added in the truss data.

3. Digitizing: The acetate sheet, a camera, connected to a tripod, was set at the top of the table, and the image was captured to view interlandmark distances (Plate 6). The digital camera needs a very good lens that avoids aberration. This is a very important issue because measurements are taken from the pictures and these measurements have to be as much real as possible. Lights were adjusted so that shadows were minimized. Also reflection over the fish was avoided at the same time. Once this was achieved the system doesn't need to be changed anymore. The squares (1cm X 1cm) in the background was used to calibrate each picture. Squares had to appear in each picture, otherwise the picture will not be useful. Lines were well defined and exact. Entomological pins were used to mark the fish landmarks, some of them need to be driven into the base, therefore a soft base, and preferably a white polystyrene plate was used. The landmarks were easily recognized in the image analysis software. Of course, labels were waterproof and easily readable in the final picture. It is very important that the fish looks as natural as possible; therefore fish with broken parts or deformation (due to a bad treatment while frozen or whatever) were discarded. Before placing the fish on the white plate, total length and weight were recorded. The fish was placed on white base (polystyrene), the head in the left and the tail in the right, in order to take the measurements in the left part of the body. The fish was kept in a horizontal position, so that all landmark points were visible in the photograph.

The X-Y coordinate values for the positions of landmarks were digitized for each fish. All measurements were transferred to a spreadsheet file (Microsoft Excel), and X-Y coordinate data were transformed into linear distances by computer software (using the Pythagorean Theorem) for subsequent analysis.

As the samples of all populations of *P. denisonii* were collected from the different rivers of Kerala there was need to eliminate the size effect in the data set. Hence, this is an important stage in the data preparation for morphometric analyses because variation should be attributable to body shape differences, and

not related to the relative size of the fish. Several transformation methods are shown to be effective in removing such size-dependent variation. The transformations are:

I) RATIO: M_{adj}=M / SL, i.e. division by standard length.

II) LGRATIO: M_{adj}=logM / logSL = the log of ratio

III)ALLOM: M_{adj}=logM- β_1(logSL - $logSL_{mean}$)

Where, M is original truss measurement, M_{adj} is size adjusted truss measurement, SL is standard length of fish, SL_{mean} is overall mean of standard length and β_1 is coefficient of the overall linear regression of logM against logSL. Base-10 logarithms are used for all variables. The truss measurements were size adjusted using the first ratio.

Standard lengths were used in all cases, since it correlates strongly with other morphometric characters. Univariate statistics were used to compare the variation among samples for size-adjusted truss measurements. There was no distinct morphological differentiation between the sexes in different *Punitus denisonii* populations, otherwise females and males should be treated separately in multivariate analyses to remove the effect of sex from the result. The transformed data was submitted to principal components analysis (PCA) and canonical variance analysis (CVA) using a statistical package program (SPSS) and graphs were also generated using these programs.

Multivariate techniques simultaneously consider the variation in several characters and thereby assess the similarities between the samples. Correlations between original variables and the principal components (component loading) were used to interpret the importance of individual variables in the description of the variation of the data set. Principal components and canonical variance analysis were used to produce scatter diagram to visualise relationships among the individuals of different rivers. Each principal component contains the percentage of total variance of all variables. But in CVA, each function contains the percentage of the total between-groups variability. Therefore, these

observations were used to describe the pattern of phenotypic differentiation among samples of *P. denisonii*.

Plate 6 *Accessories for camera and polystyrene plate with graph sheet.*

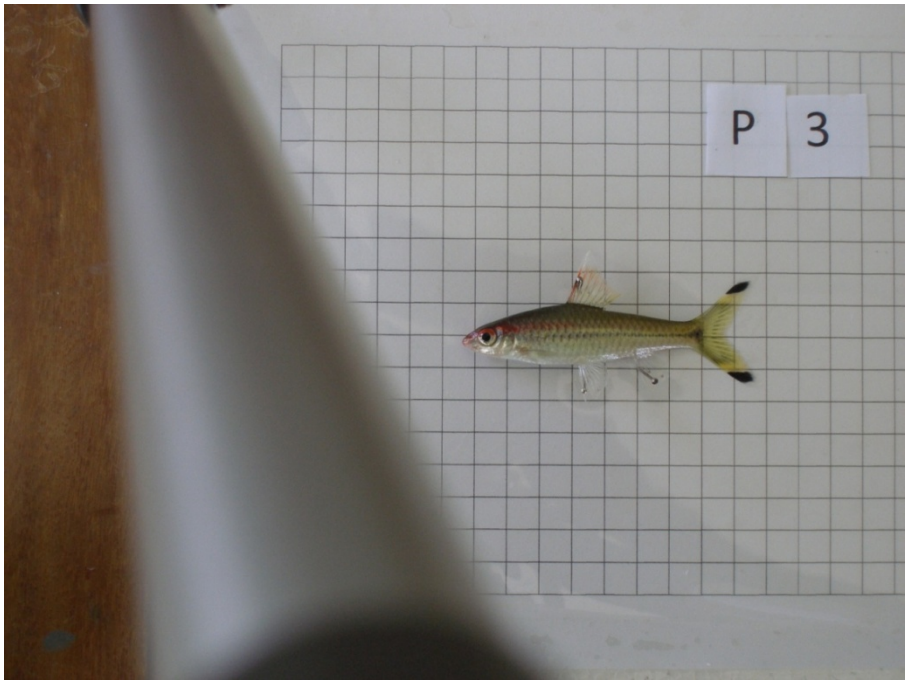


Plate 7 *Puntius denisonii positioned and pinned for taking photographs.*

3.3.Extraction of genomic DNA

Fish fins and scales are a reliable non-destructive source of DNA and these materials have been used to isolate DNA from fish species (Estoup *et al*., 1996; Nielsen *et al*., 1999; Adcock *et al*., 2000). DNA was isolated from freshly preserved caudal or anal fins of *P. denisonii* collected from Irrity, Chaliyar and Periyar river. Samples of freshly collected fins from 25 individuals of *P. denisonii* from each river were preserved in ethanol/EDTA. Preservation of nucleic acids depends primarily on the inhibition of tissue nucleases and denaturation, which can be achieved with EDTA and ethanol, respectively (Dessauer *et al*., 1996). The ethanol/EDTA storage solution also permits to maintain the softness of the tissue, facilitating its further dissociation in the digestion buffer. Approximately 100-300 mg of fins (1 to 2 cm^2) was initially stored in 95 % ethanol-100 M EDTA pH 8.0. Ethanol-fixed tissues were then dried on a filter paper, cut into small pieces, minced and placed in Lysis buffer (500µl). The Lysis buffer was prepared by, mixing 2M Tris, 0.5M EDTA and sterilised by autoclaving. Then 10% SDS was added after dissolving it by heating to 60˚C. Proteinase K @ 10µg/ml (5µl) was added and the tissue was incubated at 65˚C for 2 hours.

After tissue digestion, a phenol-chloroform isoamyl alcohol purification step was utilized, as reported by Taggart *et al*. (1992) and Sambrook and Russell (2001). The use of phenol-chloroform proved to be essential to obtaining pure DNA samples from fish fins. Crude extractions could result in a DNA contaminated with proteins that may not be stable for long-term storage. Hence, the DNA was isolated by adding equal amount (500µl) of phenol: chloroform: isoamyl alcohol (25:24:1) to the tubes. After inverting the tubes a few times, they were centrifuged for 5 min at 9,000g. The top aqueous layer was recovered to a new tube and 500µl of chloroform: isoamyl alcohol (24:1) was added. After mixing properly it was centrifuged at 9000g for 5 minutes. The supernatant was recovered and transferred into a new tube. Then, 0.1 volume of 6M NaCl and 3 volumes of absolute alcohol were added to precipitate the DNA, inverting the tubes several times. The DNA precipitate was recovered by centrifugation at

12000 g for 10 minutes. After discarding the supernatant, the DNA pellet was further washed briefly in 70 % ethanol, air dried and resuspended in 50µl of TE buffer (10 mM Tris pH 8.0; 1 mM EDTA pH 8.0). After completely dissolving in TE buffer, the DNA was stored at - 20°C. The DNA integrity was checked on 1.5% agarose gel stained with ethidium bromide. A spectrophotometer (Biophotometer plus, Eppendorf) was used to evaluate the total amount of obtained DNA and to analyse the purity of DNA. Samples were considered of adequate purity if A_{260}:A_{280} >1.5. DNA samples after quantification were adjusted to 100ng/µl and used as templates on PCR with universal primers for amplification of random polymorphic DNA.

3.4.Random Amplified Polymorphic DNA (RAPD) technique

RAPD procedures were first developed in 1990 (Welsh and McClelland, 1990; Williams *et al*., 1990) which is based on amplification of several random segments of a genome using single short arbitrary oligonucleotide primers that can detect polymorphism (reflect variation in nuclear DNA) in the absence of specific DNA nucleotide sequence information. Minute amounts of template DNA, one or more oligonucleotide primer (usually about 10 base pair in length), free nucleotides and polymerase with a suitable reaction buffer were the major requirements. Primers which are commercially available were screened. The complete genome of *P. denisonii* was screened, genetic polymorphisms in the respective primer sites of the genome was visualized and recorded. Because the primers are short and relatively low annealing temperatures (often 36–40^{0}C) are used, the likelihood of amplifying multiple products is great, with each product (presumably) representing a different locus.

Genetic variation and divergence within the taxa of interest were assessed by the presence or absence of each product. 5–20 bands were produced by a given primer. Such polymorphisms inherit in a Mendelian dominant fashion and are used as genetic markers in discriminating between closely related species, subspecies and populations in a wide range of organisms (Perez *et al*., 1998). Its

cost effectiveness provides an advantage in population genetic studies. The resulting pattern of bands was very sensitive to variations in reaction conditions, DNA quality, and the PCR temperature profile (Hoelzel and Green, 1998). Multilocus amplifications was separated electrophoretically on agarose gels and stained with ethidium bromide. In applying this method, it is assumed that populations are under the Hardy-Weinberg equilibrium, which polymorphic bands segregate in the Mendelian way, and that marker alleles from different loci do not comigrate to the same position in the gel (D'Amato and Corach, 1996). The information is not easily extended to related material, and, in general, for every different individual a new map has to be generated. Each primer directs amplification of several discrete loci in the genome so that allelism is not distinguishable in RAPD patterns. In other words, it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous.

The possibility of working with anonymous polymorphic DNA and the low expense makes it a simple and rapid methodology in the assessment of genetic differences among several groups of *P. denisonii*. Lynch and Milligan (1994) estimated that 2–10 times more individuals need to be sampled per locus for dominant markers compared to co-dominant markers. Krauss and Peakall (1998) suggested that this disadvantage may be overcome because of the large number of available polymorphisms, per gel-lane are possible. Popgene (Yeh and Boyle, 1997) a user-friendly Microsoft Window-based computer package was used for the analysis of genetic variation among and within populations using dominant markers and quantitative traits. This package provides the Windows graphical user interface that makes population genetics analysis more accessible for the casual computer user and more convenient for the experienced computer user.

A series of optimization experiments were conducted following the protocol of Williams *et al*., (1990), with various concentrations and purity of template DNA, dNTPs, $MgCl_2$ concentration, and Taq polymerase, to determine which conditions produced the strongest and most reproducible patterns. A total

number of 10 RAPD primers (OPA 1-10) were screened. Among them, 4 RAPD primers (OPA 1, 6, 7 and 10) produced clear and reproducible bands, so they were selected for amplification of DNA extracted from *Puntius denisonii* collected from Irrity, Chaliyar and Periyar. To test the reproducibility of the bands, 3 replicates were analysed for all selected primers in which contamination controls were added. The amplification reactions were performed in total volumes of 25 µL, containing 20 ng (5µl) of genomic template DNA, 2.5µl Taq buffer, 2µl of 10mM of dNTPs, 2.5µl of 100pmol primer, and 1µl of Taq DNA polymerase (3 units) (Genei). Amplification was performed in a Thermal Cycler (MJ Mini, Thermocycler, BIORAD) comprising of 3 min initial denaturation at 95˚C, 40 cycles of denaturation (1 min at 94˚C), annealing (1 min at 35–40˚C), and extension (2 min at 72˚C), and finally extension of 10 min at 72˚C using the fastest possible transitions between each temperature. The total volume of the PCR products after adding gel loading buffer were evaluated in 1.5% agarose gels and visualized by ethidium bromide staining. After electrophoresis, DNA bands profiling were observed under UV light, and the images were saved in a gel analyzer software (GelDOC, Quantity one).

Amplified fragments were scored as binary data, i.e. presence as 1 and absence as 0, for homologous bands. Only data generated from reproducible bands were used for statistical analysis. The number of polymorphic loci, percentage of polymorphic loci (%*P*), observed number of alleles (*na*), effective number of alleles (*ne*), Nei's gene diversity (*h*), and Shannon index (*H*') were estimated. Nei's (1987) coefficient of gene differentiation (*Gst*) and gene flow (*Nm*) were estimated using POPGENE program version 1.32 (Yeh *et al*., 1999). The electrophoretic bands obtained from the RAPD PCR were statistically analysed for Nei's Unbiased Measures of Genetic Identity and Genetic distance and dendrogram based on Nei's (1978) Genetic distance (using UPGMA -- Modified from NEIGHBOR procedure of PHYLIP Version 3.5) was constructed using the above software for estimating genetic diversity among *Puntius denisonii* individuals between and among populations.

3.5.Controlled Breeding of *P. denisonii*

Plate 8 *Live Puntius denisonii collected.*

3.5.1.Broodstock maintenance

Puntius denisonii wild stocks were collected during the period of May-August 2009 from Irrity and Chaliyar River. They were reared for induce breeding trials till November in outdoor cement tanks covered with shade net. The broodstock was fed with live as well as commercial pelleted feed. Captive maturation of broodstock were carried out in cement tanks (1000m^3), in glass tanks (400L) with recirculatory system and in glass tanks (200L) without recirculatory system. The water temperature in the stocking pond was around 28°C. Spawners were selected according to the following criteria: the belly of females had to be fully distended and bulging, soft and resilient to touch; males had to be little spermiating. The size ranged from 10 to 15g. Selected males and females were kept in separate 300-litre tanks in a hatchery with controlled temperature (25°C) and photoperiod (12 hrs light and 12 hrs dark).

3.5.2.Ovary type and fecundity studies

Ovary was dissected out from captive matured females showing fully distended belly and the fecundity was estimated. There is organized capture and trade in Irrity and Thriuvambadi. During collection, acclimation, sorting and packing a large no of specimens lose their life and is discarded by traders. During the period from November to February these freshly dead specimens were also collected and examined for fecundity studies. The number of ova in the ovary was totally counted and the ovadiameter from each lobes of the ovary was measured using micrometer. Both absolute fecundity and relative fecundity was recorded. Diameter of the types of eggs obtained and the size of the matured ovary was recorded (Hunter and Goldberg. 1980).

3.5.3.Breeding trials

Tank spawning is the simplest method for obtaining a hormone induced spawn. *P. denisonii* brood fish in different sex ratio were placed together in the spawning tank following injection(s). The female ovulates when she is physiologically ready. The males stimulate the female to release the eggs and fertilize the spawn. Common carp pituitary gland was collected. To gain access to the pituitary, the top of the skull was removed with a saw or knife. Fresh pituitary glands was used immediately or preserved by either freezing or acetone-drying.

Selected broodfish were divided into four groups - three experimental groups and control one - each group with a particular sex ratio (1:1 or 2:1, male:female). After five days of acclimation to 25°C, the fish were treated with respective hormonal injections of common carp pituitary extract, Ovaprim and WOVA-FH. Injections were given intramuscularly in the dorsal region of the body (Thalathiah *et al*., 1988). Before handling, fishes were sedated with clove oil (0.2 ml per l).

Induced or controlled breeding trials were conducted in Chinese hatchery, in cement cisterns, in plain glass aquarium, with aquatic plants, with rocky substratum, with sandy substratum, at different pH, hardness, water temperature, with or without waterflow, hypophysation, synthetic hormones-

Wova FH (a commercial product by Biostadt Pharma Ltd.) and Ovaprim (a commercial product prepared by the Syndel Laboratories, Canada, which is now marketed by Glaxo Laboratories Ltd), with different sex ratio and in the presence or absence of light. High-quality live food, for a limited conditioning period that last for only a few weeks, was the most crucial area of broodstock management, for obtaining success in breeding *P. denisonii*. Besides, finding and maintaining the right levels of pH, temperature, and dissolved solids ("hardness") was most important as in any other successful aquarium-fish breeding.

3.5.4.Early embryonic development stages and larval rearing

At fertilization; the eggs were 1.1± 0.01mm in diameter. Fertilized egg samples were taken every 10 minutes interval till completion of morula and then every 2 hour interval up to hatching. The eggs presented slight yellowish coloration, almost transparent. They were spherical, demersal and slightly adhesive. The different stages of embryonic development starting from cleavage upto hatching and time taken for development were documented. Photographs of developing eggs upto hatching were taken using Olympus digital camera (S 500) attached to a research microscope. The yolk sac was completely absorbed after 70h post hatching. At the same time the digestive system became fully developed and the larvae searched for feed.

After yolk sac absorption, the fry was fed initially with infusoria and then later with freshly hatched *Artemia* nauplii after 1 week. They were weaned to inert feed in another one week time. The black stripes were first seen within two weeks on the lateral sides of the body. Later, within two months reddish colouration over the eyes and on dorsal fins developed.

3.6.Development of transgenic varieties of *P. denisonii*

3.6.1.Artificial fertilization for freshly fertilized eggs

Fishes were maintained on an optimum light-dark cycle and bred in our laboratory according to standard conditions described as above. Otherwise, milt

of *P. denisonii* was collected with plastic syringes and kept at 4°C until further treatment. Females were checked each hour between 10 and 20 hours after injections. Eggs were stripped into a plastic vessel and were fertilized using "dry method" (Kucharczyk *et al*., 1997a). Only those sperms were taken for egg fertilization that showed the motility of more than 80% of spermatozoa. Two egg samples (250–300 eggs each) from each female were mixed with 0.5 ml of pooled milt sample. Embryos obtained from mass matings were also transferred separately to Hypo-osmolar electroporation buffer (Eppendorf). Embryos were pipetted into the electroporation cuvette, allowed to sink, with 20 - 50 embryos being placed in a single cuvette.

3.6.2.Plasmid DNA extraction

The *Escherichia coli* DH5α cells containing the pCMV-GFP were procured from Addgene Inc., USA. The plasmid was stored at -20ºC. Before conducting electroporation, plasmid DNA was extracted using the method of Sambrook *et al*. (1989). *E. coli* was inoculated in 5ml of sterile LB broth (Ampicillin 50 µg/ml) and incubated at 37ºC overnight. 1.5 ml of the cells were microcentrifuged and the pellet was resuspended in 100 µl GTE (Glucose- Tris-EDTA) solution for 5 minutes at room temperature. 200 µl of NaOH/SDS was added and kept for 5 minutes on ice after gentle mixing. 150 µl of potassium acetate was added and vortexed for 2sec. The tube was kept on ice for 5 minutes and then microcentrifuged at 4ºC for 3 minutes. The supernatant was transferred to fresh tube. 0.8ml of 95% ethanol was added and kept for 2 minutes at room temperature after mixing. Then it was microcentrifuged for 1 minute at room temperature and the supernatant was discarded. The pellet containing DNA was washed in 70% ethanol, and dried in vacuum. After resuspending DNA in 30 µl TE Buffer, it was stored at -20ºC till further use.

3.6.3. Electroporation of plasmid DNA

Circular GFP encoding plasmid DNA (150-200 ng/µl) was diluted to the desired concentration (10 ng/µl) in the electroporation buffer (Eppendorf). The electroporation device (Multiporator, Eppendorf) was used to generate electric

pulses. Optimizing the electroporation parameters is imperative as the application of high-voltage pulses can kill many cells. Parameters of voltage, pulse length, pulse intervals, and the number of pulses for electroporation were standardised. These parameters serve as a starting point and should be optimized for each electroporator and electrode set-up, as well as for each tissue and developmental stage being analyzed.

The electroporation apparatus, along with gene constructs, were used for the optimization of electroporation in the embryos. For each electroporation, 400µl of hypo osmolar electroporation buffer in a 2mm gap-width cuvette containing 20 - 50 embryos were used. Studies were conducted to determine the effect of each parameter on survival. After analysis of the data resulting from these combinations, a more focused set of parameters was tested. These parameters focused on optimizing voltage, burst duration, and burst interval, with three replicates for each. In order to study the effect of voltage, the voltage was varied from 10 to 100V in increments of 5V, while holding all other parameters constant at 3 bursts, and a burst interval of 1.0 s. After analysis of the above parameters, the conditions which consistently produced the largest number of live embryos were used for electroporation with the GFP construct.

3.6.4.Dot blot hybridization of electroporated *P. denisonii* fry

After electroporation, the embryos were gently removed from the cuvette and incubated in petridishes containing hatchery water at 28º C which was found as an optimal temperature for embryonic development. The incubation time to mass hatching was 29 hours. GFP integration was visualized in live hatchlings (*invitro*) using dot blot test as protein expression was not visible under UV light or blue light.

Genomic DNA was extracted from fin clips or entire electroporated fry after rearing them for a period of 2 months. DNA was isolated from transgenic and non-transgenic fish according to the methods of Jowett (1986). Tissue was digested in lysis buffer (100mM Tris, pH 8.0, 1% SDS, 100mM EDTA,

0.2mg/ml proteinase K) at 60°C for 1s h, and DNA purified using phenol–chloroform extraction and ethanol precipitation.

For conducting dot blot test, plasmid DNA (pCMV-EGFP) itself was used as control. Approximately 50 ng of Biotin -11- dUTP labeled pCMV-EGFP DNA was used as probe. Nucleic acid hybridization protocols and washes followed the methods of Sambrook *et al*. (1989). For labeling probe (Fermentas, Biotin DecaLabel DNA Labeling Kit), 10 µl of pCMV-GFP DNA (100ng -500ng) and decanucleotide 10 µl with 44 µl nuclease - Rnase free water was incubated in boiling water bath for 5-10 minutes. Then it was cooled on ice. 5 µl biotin labeling mix and 1 µl Klenow fragment exo- (5U) was added. After mixing it was incubated at 37°C upto 20 hours. The reaction was terminated by adding1 µl 0.5M EDTA, pH 8.0.

DNA isolated from the transgenic fish for dot blot test was heat denatured, blotted onto nitrocellulose membrane, and dried at 50°C for 1 hour, and covalently cross linked to the membrane by ultraviolet irradiation for 3 minutes. Prehybridization solution (Sambrook *et al*., 1989) 6X SSC pH 7.0 (900 mM sodium chloride/90 mM sodium citrate), 0.5% SDS was prepared. The membrane was placed in appropriately sized container and prehybridisation solution was added @ 0.2 ml/cm^2 and incubated for 42°C for 2-4 hours with shaking. Biotin labelled probe was denatured at 100°C for 5 min and chilling afterwards on ice. Add denatured probe to the pre hybridisation solution to obtain a final probe concentration of 25-100 ng/ml. The prehybridisation solution was discarded and approximately 60 µl/cm^2 hybridisation solution was added. Then the membrane was incubated overnight at 42°C with shaking. Membrane was incubated twice for the purpose of washing, with 2X SSC, 0.1% SDS for 10 min at room temperature. Later, the membrane was incubated twice with 0.1X SSC, 0.1% SDS for 20 min at 65°C. Excess liquid was removed by placing membrane on filter paper.

For detection (Fermentas, Biotin Chromogenic Detection Kit), the membrane was incubated in 30 ml 1X Blocking/Washing buffer (Dilute 1V of 10X Blocking/Washing buffer with 9V of water, Store refrigerated) for 5 minutes

at room temperature with moderate shaking. After blocking the membrane by incubating for 30 minutes at room temperature with moderate shaking using 30 ml blocking solution (1% blocking reagent in 1X Blocking buffer, shake at 50-60C for dissolution and stored at -20C), 20 ml of dilute Streptavidin – AP conjugate (Dilute concentrated Streptavidin – AP conjugate 5000 fold in blocking solution just prior to use), was added and again incubated for 30 minutes at room temperature with moderate shaking. The solution was discarded after incubation with 60 ml blocking buffer for 15 min and the same was repeated once with fresh. The membrane was incubated with 20ml of 1X detection buffer (Dilute 1V of 10X Detection buffer with 9V of water, Store refrigerated) for 10 min and then the buffer was discarded. Finally the membrane was incubated in 10ml of freshly prepared 1X BCIP/NBT (Dilute 1V of 50X BCIP/NBT with 49V of 1X Detection buffer) at room temp in the dark for 15-30 min till blue colour was developed indicating integration of pCMV-GFP in electroporated *P. denisonii*. The substrate solution was discarded and the membrane was air dried to document the results after washing in water for a few seconds.

4. RESULTS

4.1. Truss Network

4.1.1.Different length measurements taken for morphometric analysis

1- Standard length **(X)**

2- Snout to dorsal fin **(A)**

3- Snout to pelvic fin **(B)**

4- Snout to anal fin **(C)**

5- Eye diameter **(D)**

6- Dorsal fin to dorsal caudal peduncle **(E)**

7- Dorsal fin to ventral caudal peduncle **(F)**

8- Dorsal fin to anal fin **(G)**

9- Dorsal fin to pelvic fin **(H)**

10- Dorsal caudal peduncle to yellow spot **(I)**

11- Width of caudal peduncle **(J)**

12- Anal fin to dorsal caudal fin **(K)**

13- Pelvic fin to dorsal caudal fin **(L)**

14- Ventral caudal peduncle to yellow spot **(M)**

15- Ventral caudal peduncle to anal fin **(N)**

16- Anal fin to pelvic fin **(O)**

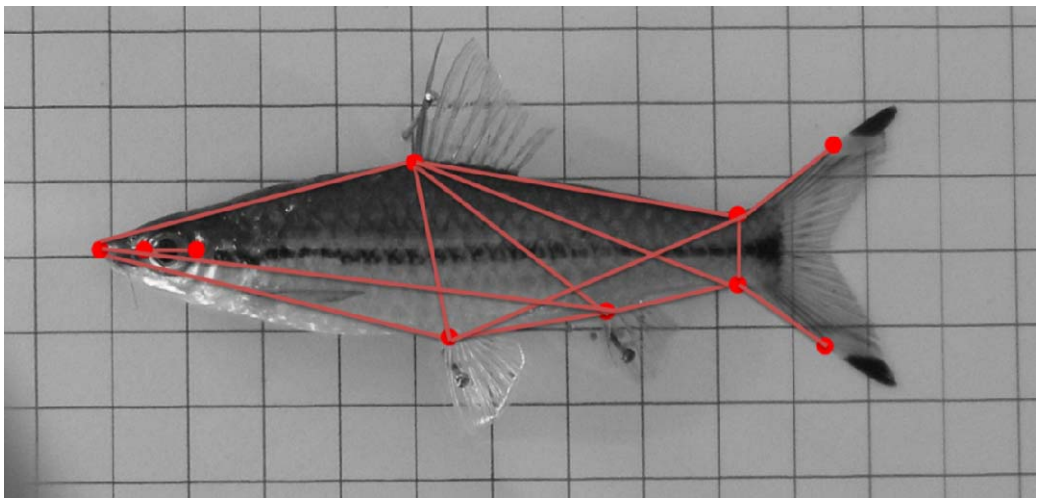


Plate 9 *Landmarks used in P. denisonii for taking truss measurements*

4.1.2.Measurements after size adjustment

Table 1 **Size adjusted truss measurements of Irrity population**

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	0.4881	0.5289	0.7320	0.0813	0.5108	0.5525	0.3443	0.2689	0.1702	0.1317	0.2975	0.5034	0.1622	0.2605	0.2163
2	0.4818	0.5339	0.7527	0.0706	0.5114	0.5423	0.3487	0.2754	0.1643	0.1268	0.2857	0.5214	0.1587	0.2405	0.2447
3	0.4813	0.5229	0.7322	0.0794	0.5019	0.5508	0.3513	0.2746	0.1702	0.1288	0.2856	0.4980	0.1597	0.2539	0.2244
4	0.5015	0.5430	0.7438	0.0797	0.5016	0.5242	0.3437	0.2809	0.1781	0.1251	0.2970	0.5062	0.1872	0.2401	0.2189
5	0.4830	0.5308	0.7513	0.0777	0.5124	0.5393	0.3625	0.2799	0.1797	0.1194	0.2798	0.5067	0.1867	0.2297	0.2385
6	0.4854	0.5756	0.7646	0.0758	0.4933	0.5148	0.3406	0.2598	0.1610	0.1247	0.2950	0.4913	0.1748	0.2357	0.2117
7	0.4836	0.5561	0.7591	0.0726	0.4973	0.5236	0.3417	0.2738	0.1625	0.1243	0.2918	0.5146	0.1663	0.2393	0.2341
8	0.4798	0.5490	0.7636	0.0721	0.5136	0.5262	0.3628	0.2715	0.1537	0.1165	0.2966	0.5124	0.1698	0.2314	0.2333
9	0.4898	0.5321	0.7350	0.0802	0.5056	0.5604	0.3487	0.2749	0.1732	0.1363	0.2894	0.4958	0.1548	0.2623	0.2169
10	0.4840	0.5352	0.7516	0.0748	0.4984	0.5402	0.3453	0.2697	0.1716	0.1223	0.2770	0.5063	0.1586	0.2441	0.2396
11	0.4841	0.5347	0.7278	0.0835	0.4943	0.5400	0.3438	0.2732	0.1717	0.1214	0.2873	0.4839	0.1703	0.2562	0.2077
12	0.4961	0.5437	0.7458	0.0741	0.4881	0.5304	0.3470	0.2817	0.1856	0.1182	0.2776	0.4886	0.1805	0.2425	0.2215
13	0.4836	0.5287	0.7535	0.0702	0.5030	0.5354	0.3636	0.2822	0.1894	0.1173	0.2705	0.5026	0.1946	0.2251	0.2444
14	0.4817	0.5710	0.7573	0.0733	0.4896	0.5146	0.3342	0.2552	0.1815	0.1240	0.2906	0.4862	0.1744	0.2373	0.2094
15	0.4773	0.5536	0.7551	0.0721	0.4845	0.5055	0.3400	0.2717	0.1831	0.1154	0.2727	0.4966	0.1831	0.2191	0.2343
16	0.4738	0.5506	0.7622	0.0769	0.5094	0.5259	0.3610	0.2714	0.1613	0.1210	0.2889	0.5064	0.1729	0.2254	0.2335
17	0.4799	0.5509	0.7561	0.0732	0.4966	0.5106	0.3400	0.2582	0.1668	0.1261	0.2907	0.5054	0.1768	0.2255	0.2288
18	0.4834	0.5498	0.7623	0.0777	0.4851	0.5037	0.3465	0.2698	0.1659	0.1173	0.2745	0.5002	0.1724	0.2145	0.2399
19	0.4607	0.5059	0.7480	0.0681	0.5382	0.5493	0.3750	0.2566	0.1824	0.1162	0.2826	0.5191	0.2001	0.2226	0.2523
20	0.4732	0.5201	0.7512	0.0660	0.5131	0.5565	0.3717	0.2703	0.1783	0.1222	0.2849	0.5094	0.1569	0.2463	0.2441

21	0.4778	0.5306	0.7596	0.0640	0.5244	0.5307	0.3773	0.2796	0.1747	0.1184	0.2936	0.5192	0.1819	0.2201	0.2439
22	0.4677	0.5123	0.7354	0.0664	0.5412	0.5637	0.3634	0.2639	0.1527	0.1168	0.3196	0.5366	0.1598	0.2693	0.2341
23	0.4736	0.5045	0.7465	0.0668	0.5381	0.5599	0.3744	0.2681	0.1435	0.1194	0.2962	0.5324	0.1383	0.2426	0.2525
24	0.4660	0.5231	0.7507	0.0625	0.5234	0.5485	0.3730	0.2694	0.1757	0.1111	0.2851	0.5097	0.1747	0.2368	0.2416
25	0.4720	0.5047	0.7385	0.0654	0.5403	0.5580	0.3727	0.2741	0.1653	0.1115	0.2903	0.5236	0.1676	0.2394	0.2450
26	0.4744	0.5150	0.7442	0.0732	0.5369	0.5593	0.3747	0.2610	0.1732	0.1048	0.2882	0.5071	0.1766	0.2441	0.2353
27	0.4777	0.5167	0.7536	0.0699	0.5374	0.5606	0.3779	0.2779	0.1541	0.1166	0.3003	0.5334	0.1541	0.2473	0.2498
28	0.4765	0.5103	0.7409	0.0711	0.5442	0.5743	0.3706	0.2724	0.1757	0.1068	0.2901	0.5212	0.1644	0.2567	0.2411
29	0.4782	0.5465	0.7621	0.0813	0.5083	0.5248	0.3629	0.2730	0.1620	0.1234	0.2936	0.5108	0.1749	0.2267	0.2353
30	0.4819	0.5532	0.7599	0.0748	0.4921	0.5032	0.3446	0.2632	0.1671	0.1197	0.2863	0.5015	0.1815	0.2191	0.2305

Table 2 **Size adjusted truss measurements of Chaliyar population**

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**
1	0.4596	0.5126	0.7307	0.0734	0.5053	0.5310	0.3526	0.2530	0.1901	0.1101	0.2809	0.4956	0.1603	0.2361	0.2308
2	0.4700	0.5155	0.7464	0.0712	0.5097	0.5353	0.3743	0.2683	0.1900	0.1059	0.2559	0.4828	0.1790	0.2109	0.2416
3	0.4613	0.4945	0.7357	0.0725	0.5240	0.5443	0.3686	0.2519	0.1746	0.1210	0.2802	0.5112	0.1695	0.2245	0.2491
4	0.4646	0.5099	0.7340	0.0645	0.5088	0.5430	0.3579	0.2753	0.1765	0.1079	0.2705	0.5050	0.1620	0.2370	0.2443
5	0.4717	0.5201	0.7414	0.0641	0.5170	0.5357	0.3563	0.2638	0.1760	0.1167	0.2756	0.5015	0.1753	0.2240	0.2362
6	0.4671	0.5212	0.7424	0.0695	0.5193	0.5420	0.3573	0.2615	0.1795	0.1144	0.2815	0.5060	0.1700	0.2343	0.2366
7	0.4767	0.5198	0.7422	0.0646	0.5047	0.5276	0.3613	0.2670	0.1794	0.1117	0.2715	0.4917	0.1718	0.2208	0.2346
8	0.4683	0.5250	0.7355	0.0715	0.5208	0.5419	0.3566	0.2712	0.1717	0.1211	0.2761	0.4952	0.1578	0.2262	0.2269
9	0.4906	0.5101	0.7504	0.0767	0.5070	0.5466	0.3619	0.2500	0.1825	0.1111	0.2823	0.5063	0.1686	0.2484	0.2461
10	0.4790	0.5092	0.7467	0.0725	0.5339	0.5498	0.3674	0.2573	0.1851	0.1117	0.2988	0.5261	0.1788	0.2442	0.2452
11	0.4742	0.5109	0.7259	0.0541	0.5159	0.5369	0.3596	0.2732	0.1823	0.1145	0.2773	0.4936	0.1734	0.2270	0.2263

12	0.4770	0.5147	0.7377	0.0702	0.5166	0.5412	0.3536	0.2462	0.1661	0.1209	0.2898	0.5025	0.1634	0.2401	0.2299
13	0.4696	0.5030	0.7499	0.0720	0.5399	0.5506	0.3599	0.2446	0.1666	0.1019	0.2875	0.5327	0.1872	0.2398	0.2578
14	0.4894	0.5087	0.7694	0.0769	0.4869	0.5285	0.3674	0.2400	0.1913	0.1081	0.2677	0.5021	0.1756	0.2308	0.2673
15	0.4611	0.5045	0.7459	0.0741	0.5537	0.5843	0.3643	0.2384	0.1525	0.1048	0.3143	0.5442	0.1369	0.2833	0.2492
16	0.4713	0.4992	0.7326	0.0704	0.5324	0.5667	0.3558	0.2431	0.1915	0.1065	0.3084	0.5287	0.1784	0.2779	0.2396
17	0.4597	0.5015	0.7467	0.0678	0.5158	0.5499	0.3692	0.2528	0.1751	0.1082	0.2848	0.5189	0.1603	0.2464	0.2571
18	0.4769	0.5111	0.7579	0.0676	0.5347	0.5706	0.3811	0.2635	0.1884	0.1142	0.2925	0.5264	0.1603	0.2532	0.2551
19	0.4852	0.5069	0.7342	0.0732	0.5499	0.5460	0.3496	0.2435	0.1826	0.0987	0.3002	0.5242	0.1734	0.2446	0.2324
20	0.4751	0.5221	0.7648	0.0665	0.5151	0.5456	0.3724	0.2567	0.1795	0.1249	0.3037	0.5285	0.1753	0.2470	0.2546
21	0.4740	0.5136	0.7687	0.0634	0.4918	0.5444	0.3679	0.2478	0.1879	0.1123	0.2681	0.5082	0.1507	0.2428	0.2687
22	0.4696	0.5073	0.7417	0.0714	0.5079	0.5375	0.3518	0.2392	0.1570	0.1007	0.2769	0.5027	0.1673	0.2432	0.2436
23	0.4760	0.5096	0.7602	0.0615	0.5072	0.5350	0.3638	0.2437	0.1800	0.1083	0.2716	0.5104	0.1691	0.2279	0.2607
24	0.4732	0.5331	0.7832	0.0731	0.5075	0.5361	0.3754	0.2404	0.1803	0.1143	0.2732	0.5045	0.1725	0.2218	0.2615
25	0.4589	0.5181	0.7541	0.0756	0.5373	0.5383	0.3536	0.2337	0.1681	0.1048	0.3017	0.5353	0.1745	0.2421	0.2508
26	0.4766	0.5131	0.7500	0.0758	0.5271	0.5660	0.3522	0.2335	0.1654	0.1089	0.2878	0.5163	0.1396	0.2645	0.2444
27	0.4646	0.5272	0.7494	0.0634	0.5207	0.5532	0.3488	0.2439	0.1957	0.1046	0.2834	0.5094	0.1681	0.2551	0.2385
28	0.4742	0.5080	0.7672	0.0738	0.5237	0.5606	0.3815	0.2470	0.1830	0.1236	0.2733	0.5145	0.1555	0.2291	0.2660
29	0.4809	0.5116	0.7611	0.0673	0.5175	0.5514	0.3680	0.2438	0.1793	0.1122	0.2626	0.5020	0.1437	0.2275	0.2565
30	0.4775	0.5107	0.7533	0.0772	0.5154	0.5500	0.3624	0.2517	0.1948	0.1140	0.2836	0.5174	0.1676	0.2452	0.2531

Table 3 **Size adjusted truss measurements of Periyar population**

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	0.4748	0.5060	0.7192	0.0739	0.5194	0.5601	0.3418	0.2495	0.1660	0.1246	0.3180	0.5223	0.1642	0.2839	0.2214
2	0.4641	0.4971	0.7183	0.0730	0.5112	0.5542	0.3528	0.2588	0.1372	0.1117	0.2768	0.4965	0.1703	0.2528	0.2315
3	0.4640	0.4964	0.7246	0.0767	0.5346	0.5513	0.3634	0.2553	0.1809	0.1144	0.2883	0.5100	0.1911	0.2369	0.2350
4	0.4617	0.5096	0.7175	0.0779	0.5275	0.5575	0.3565	0.2607	0.1631	0.1198	0.2989	0.5020	0.1737	0.2564	0.2165
5	0.4655	0.5003	0.7193	0.0676	0.5174	0.5495	0.3523	0.2612	0.1757	0.1134	0.2839	0.5031	0.1696	0.2479	0.2301
6	0.4635	0.4986	0.7276	0.0743	0.5272	0.5637	0.3574	0.2508	0.1825	0.1206	0.2768	0.5034	0.1691	0.2456	0.2375
7	0.4678	0.5101	0.7260	0.0701	0.5214	0.5698	0.3661	0.2849	0.1688	0.1247	0.2872	0.5075	0.1626	0.2582	0.2312
8	0.4558	0.4908	0.7156	0.0675	0.5188	0.5518	0.3419	0.2330	0.1741	0.1038	0.2804	0.5012	0.1727	0.2559	0.2325
9	0.4774	0.5050	0.7242	0.0735	0.5219	0.5555	0.3415	0.2405	0.1398	0.1196	0.2915	0.5053	0.1363	0.2588	0.2254
10	0.4640	0.5040	0.7190	0.0718	0.5404	0.5590	0.3572	0.2474	0.1710	0.1190	0.2937	0.5014	0.1729	0.2455	0.2196
11	0.4606	0.4999	0.7172	0.0757	0.5427	0.5627	0.3562	0.2474	0.1742	0.1152	0.2911	0.5036	0.1689	0.2481	0.2227
12	0.4772	0.5052	0.7190	0.0718	0.5254	0.5581	0.3410	0.2555	0.1580	0.1229	0.3223	0.5317	0.1678	0.2821	0.2238
13	0.4681	0.4983	0.7283	0.0713	0.5427	0.5687	0.3678	0.2617	0.1736	0.1215	0.2999	0.5230	0.1736	0.2532	0.2372
14	0.4712	0.5194	0.7264	0.0751	0.5370	0.5737	0.3584	0.2686	0.1642	0.1240	0.3080	0.5136	0.1700	0.2714	0.2172
15	0.4700	0.5105	0.7231	0.0672	0.5259	0.5433	0.3496	0.2624	0.1702	0.1171	0.2952	0.5100	0.1767	0.2441	0.2245
16	0.4675	0.5038	0.7342	0.0804	0.5379	0.5718	0.3613	0.2556	0.1766	0.1207	0.2831	0.5129	0.1699	0.2501	0.2397
17	0.4753	0.5131	0.7281	0.0686	0.5233	0.5633	0.3627	0.2819	0.1728	0.1227	0.2934	0.5111	0.1747	0.2570	0.2287
18	0.4600	0.4928	0.7213	0.0691	0.5128	0.5598	0.3449	0.2316	0.1782	0.1131	0.2776	0.4981	0.1646	0.2612	0.2352
19	0.4782	0.5065	0.7191	0.0793	0.5247	0.5518	0.3372	0.2449	0.1406	0.1174	0.2976	0.5083	0.1447	0.2605	0.2201
20	0.4692	0.5051	0.7151	0.0761	0.5471	0.5567	0.3488	0.2484	0.1713	0.1160	0.3048	0.5113	0.1753	0.2524	0.2154
21	0.4700	0.5059	0.7339	0.0753	0.5538	0.5635	0.3661	0.2500	0.1689	0.1166	0.2945	0.5159	0.1765	0.2399	0.2328
22	0.4703	0.5127	0.7298	0.0755	0.5458	0.5698	0.3595	0.2478	0.1800	0.1191	0.2947	0.5051	0.1738	0.2530	0.2218
23	0.4802	0.5108	0.7246	0.0747	0.5207	0.5607	0.3446	0.2513	0.1693	0.1178	0.3135	0.5186	0.1729	0.2825	0.2214

24	0.4655	0.5009	0.7314	0.0691	0.5373	0.5601	0.3724	0.2633	0.1852	0.1160	0.2896	0.5125	0.1886	0.2406	0.2378
25	0.4645	0.5102	0.7163	0.0683	0.5288	0.5556	0.3538	0.2637	0.1661	0.1162	0.2991	0.5038	0.1784	0.2566	0.2158
26	0.4730	0.5089	0.7314	0.0630	0.5362	0.5440	0.3553	0.2631	0.1586	0.1177	0.3014	0.5250	0.1728	0.2407	0.2342
27	0.4679	0.5033	0.7331	0.0727	0.5419	0.5730	0.3584	0.2564	0.1734	0.1193	0.2874	0.5189	0.1707	0.2538	0.2401
28	0.4734	0.5157	0.7320	0.0724	0.5320	0.5604	0.3651	0.2803	0.1684	0.1246	0.2958	0.5153	0.1761	0.2479	0.2299
29	0.4597	0.4943	0.7241	0.0729	0.5328	0.5631	0.3443	0.2348	0.1650	0.1163	0.2873	0.5160	0.1667	0.2568	0.2386
30	0.4799	0.5092	0.7192	0.0769	0.5148	0.5502	0.3328	0.2460	0.1526	0.1130	0.2848	0.4965	0.1450	0.2596	0.2190

Table 4 **Mean and standard deviation of size adjusted variables**

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**
Chaliyar															
Mean	0.4725	0.5124	0.7486	0.0699	0.5189	0.5463	0.3624	0.2515	0.1791	0.1113	0.2827	0.5115	0.1662	0.2399	0.2468
Std dev	0.0083	0.0084	0.0135	0.0054	0.0151	0.0129	0.0089	0.0118	0.0106	0.0067	0.0138	0.0144	0.0119	0.0161	0.0122
Irrity															
Mean	0.4799	0.5344	0.7499	0.0732	0.5112	0.5376	0.3568	0.2707	0.1698	0.1201	0.2886	0.5083	0.1712	0.2385	0.2335
Std dev	0.0083	0.0190	0.0104	0.0056	0.0187	0.0200	0.0140	0.0074	0.0107	0.0067	0.0096	0.0134	0.0132	0.0142	0.0126
Periyar															
Mean	0.4685	0.5049	0.7242	0.0728	0.5305	0.5598	0.3540	0.2550	0.1680	0.1179	0.2936	0.5098	0.1696	0.2549	0.2279
Std dev	0.0064	0.0068	0.0060	0.0040	0.0111	0.0081	0.0101	0.0130	0.0122	0.0045	0.0113	0.0088	0.0110	0.0119	0.0078

4.1.3.Principal component analysis (PCA)

Table 5 **PCA loadings**

	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5	Axis 6	Axis 7	Axis 8	Axis 9	Axis 10	Axis 11	Axis 12	Axis 13	Axis 14	Axis 15
s-d	0.145	-0.105	0.179	-0.049	0.134	-0.218	0.052	-0.827	0.225	-0.140	0.154	-0.247	0.162	0.003	0.029
s-pel	0.447	-0.244	0.401	0.024	-0.068	-0.078	-0.423	0.282	0.007	-0.152	-0.191	-0.044	0.491	-0.017	0.089
s-anal	0.319	0.370	0.406	-0.219	-0.030	-0.143	-0.215	0.006	-0.160	-0.106	0.026	-0.096	-0.649	0.015	-0.116
eye	0.009	-0.093	0.025	-0.042	-0.024	-0.063	-0.155	-0.358	-0.324	0.028	-0.218	0.824	-0.011	-0.001	-0.003
df-dc	-0.437	0.157	0.165	0.324	-0.229	0.238	-0.464	-0.156	0.267	-0.077	-0.153	-0.043	-0.137	-0.290	0.313
df-vc	-0.471	0.070	0.048	0.051	0.384	-0.231	-0.289	0.001	-0.240	-0.114	-0.245	-0.202	0.145	0.140	-0.522
df-af	-0.051	0.411	0.141	0.257	0.250	0.029	-0.098	0.048	-0.271	-0.019	0.617	0.129	0.255	0.224	0.285
df-pelf	0.161	-0.122	0.243	0.562	0.534	0.063	0.317	0.104	0.194	-0.134	-0.154	0.168	-0.222	-0.156	-0.052
dc-cfs	0.089	0.231	-0.248	0.194	-0.043	-0.719	-0.162	0.115	0.425	0.271	0.007	0.165	-0.004	0.003	0.001
wcp	0.042	-0.151	0.119	0.092	0.186	-0.025	-0.059	-0.099	-0.321	0.793	-0.163	-0.264	-0.073	-0.065	0.258
af-dc	-0.202	-0.239	0.430	0.081	-0.314	-0.089	0.104	0.066	0.021	0.242	0.456	0.118	0.036	-0.325	-0.450
pelf-dc	-0.210	0.182	0.471	-0.020	-0.227	-0.007	0.353	0.011	0.209	0.141	-0.298	0.072	0.102	0.593	0.069
vc-cfs	0.105	0.039	-0.117	0.538	-0.477	-0.241	0.222	-0.096	-0.474	-0.225	-0.137	-0.213	-0.001	-0.001	-0.002
vc-af	-0.361	-0.313	0.162	-0.257	0.104	-0.471	0.168	0.187	-0.142	-0.275	0.044	-0.005	-0.133	-0.136	0.500
af-pelf	0.015	0.553	0.106	-0.222	0.061	-0.030	0.312	-0.001	-0.075	0.025	-0.245	0.014	0.351	-0.585	0.012

Table 6 **Principal components and Percentage variation**

	Eigen value	**Percentage variation**
1	0.001083	40.614
2	0.000521	19.518
3	0.0003	11.237
4	0.000245	9.1859
5	0.000164	6.1576
6	0.000136	5.1167
7	7.36E-05	2.7613
8	4.11E-05	1.5413
9	3.62E-05	1.3589
10	2.94E-05	1.1016
11	2.41E-05	0.90378
12	1.32E-05	0.49548
13	1.15E-07	0.004329
14	7.77E-08	0.002913
15	3.76E-08	0.001409

Figure 1 **PCA loadings of Axis 1**

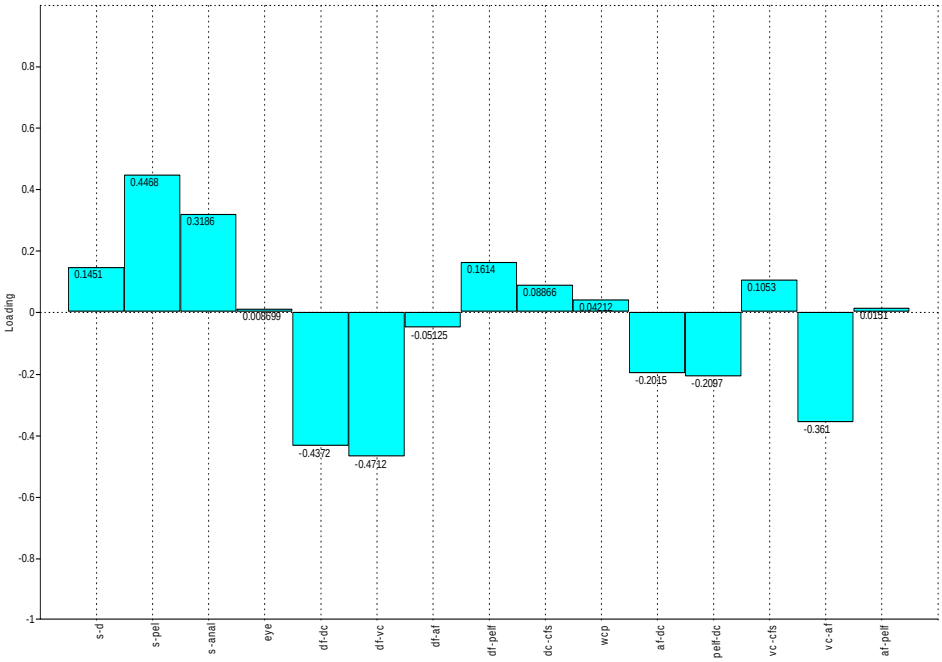


Figure 2 **PCA loadings of Axis 2**

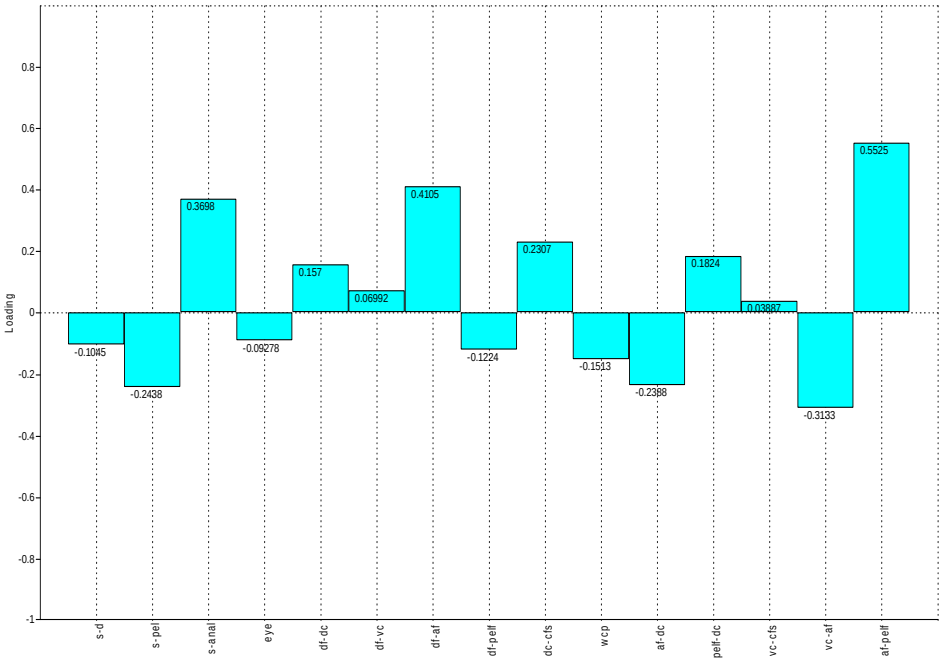


Figure 3 **PCA loadings of Axis 3**

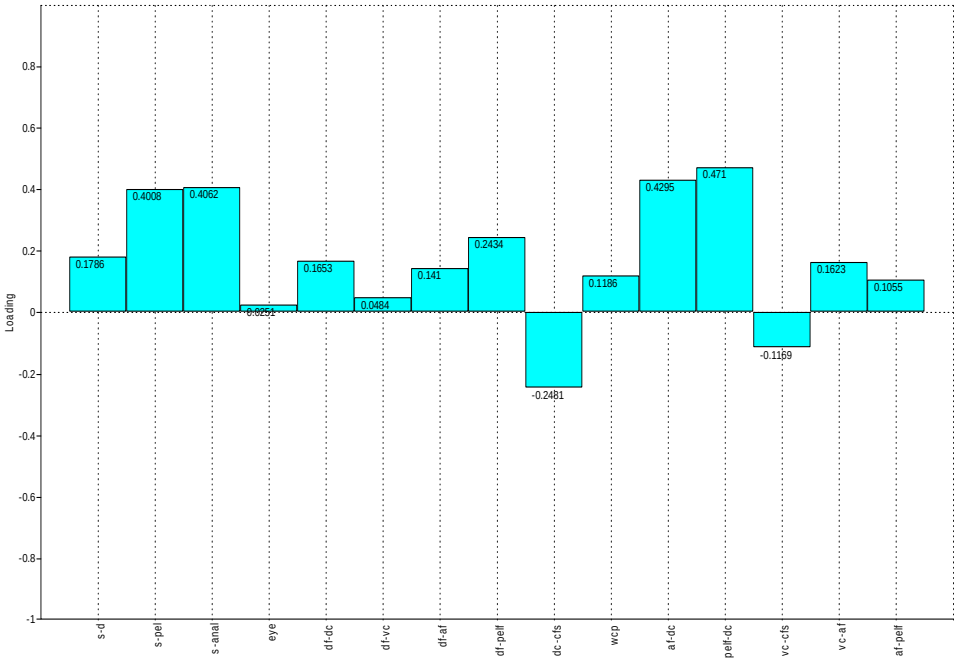


Figure 4 **Scatter diagram of Principal Component 1 and 2**

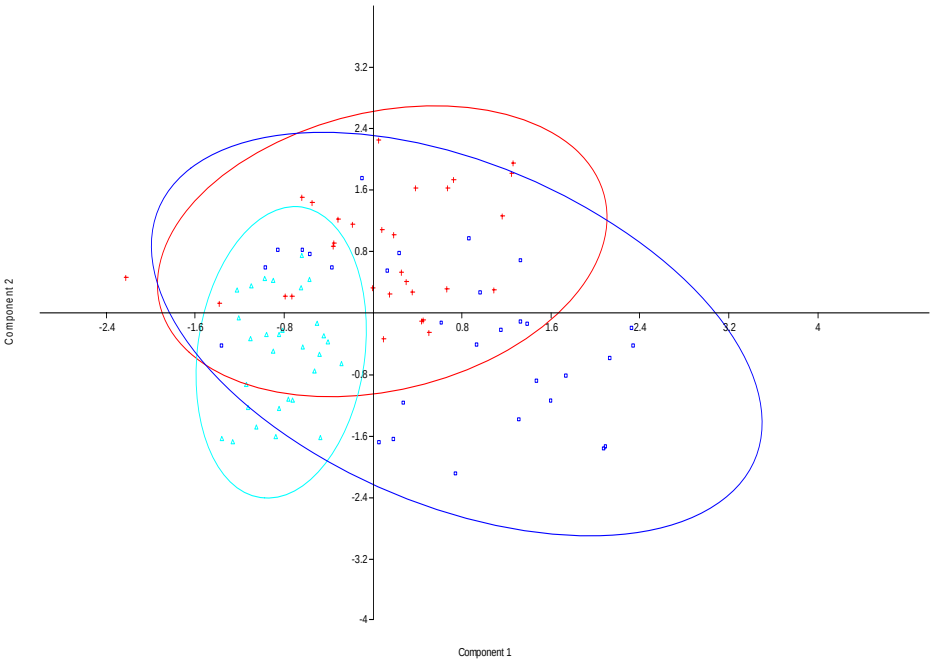
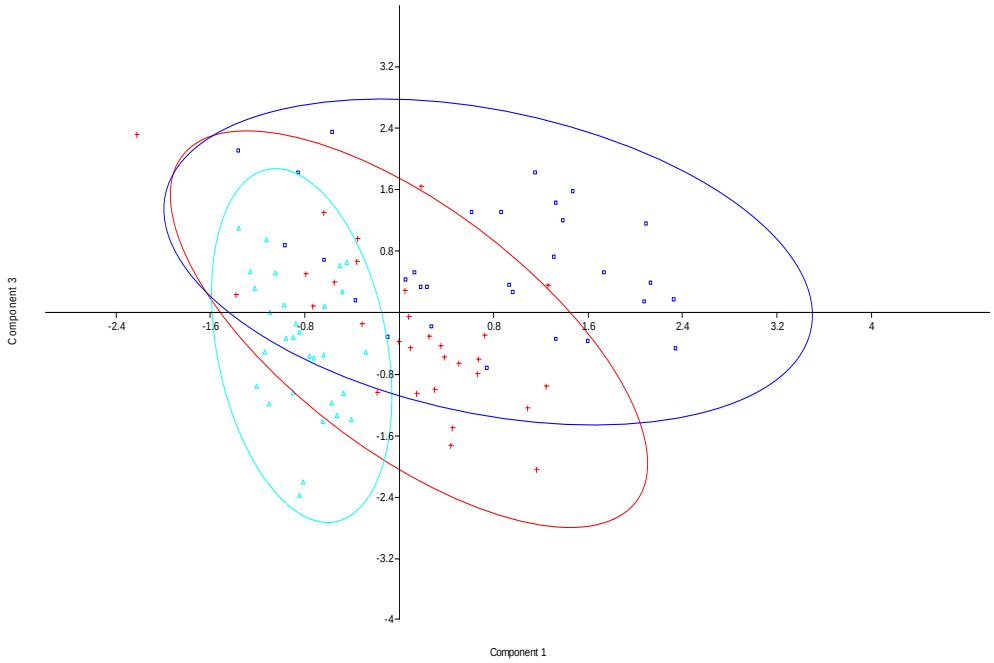


***Figure 5* Scatter diagram of Principal Component 1 and 3**



4.1.4.CVA analysis

***Figure 6* CVA scatter diagram of Axis 1 and 2**

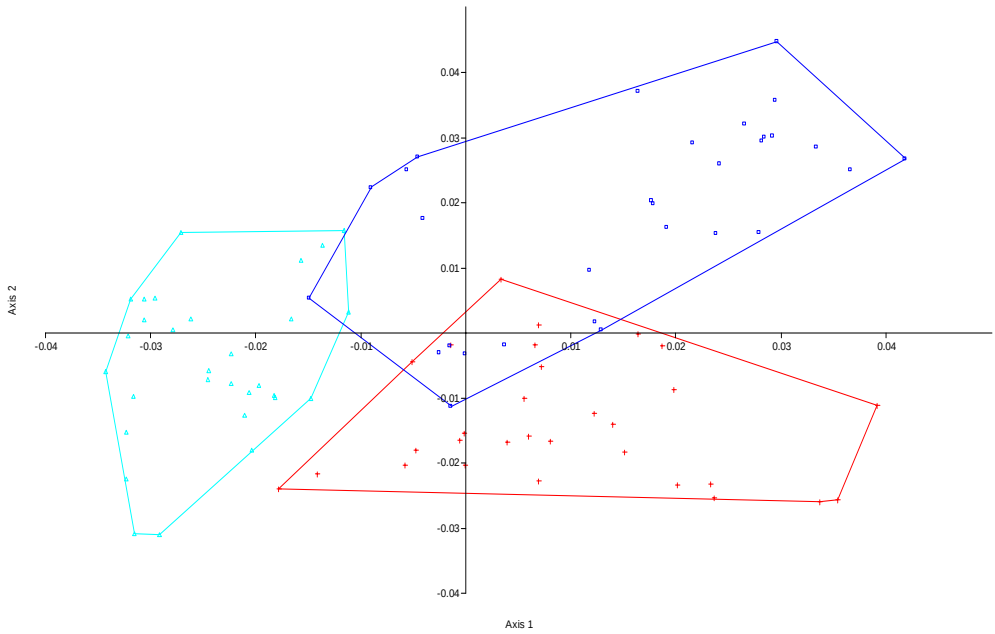
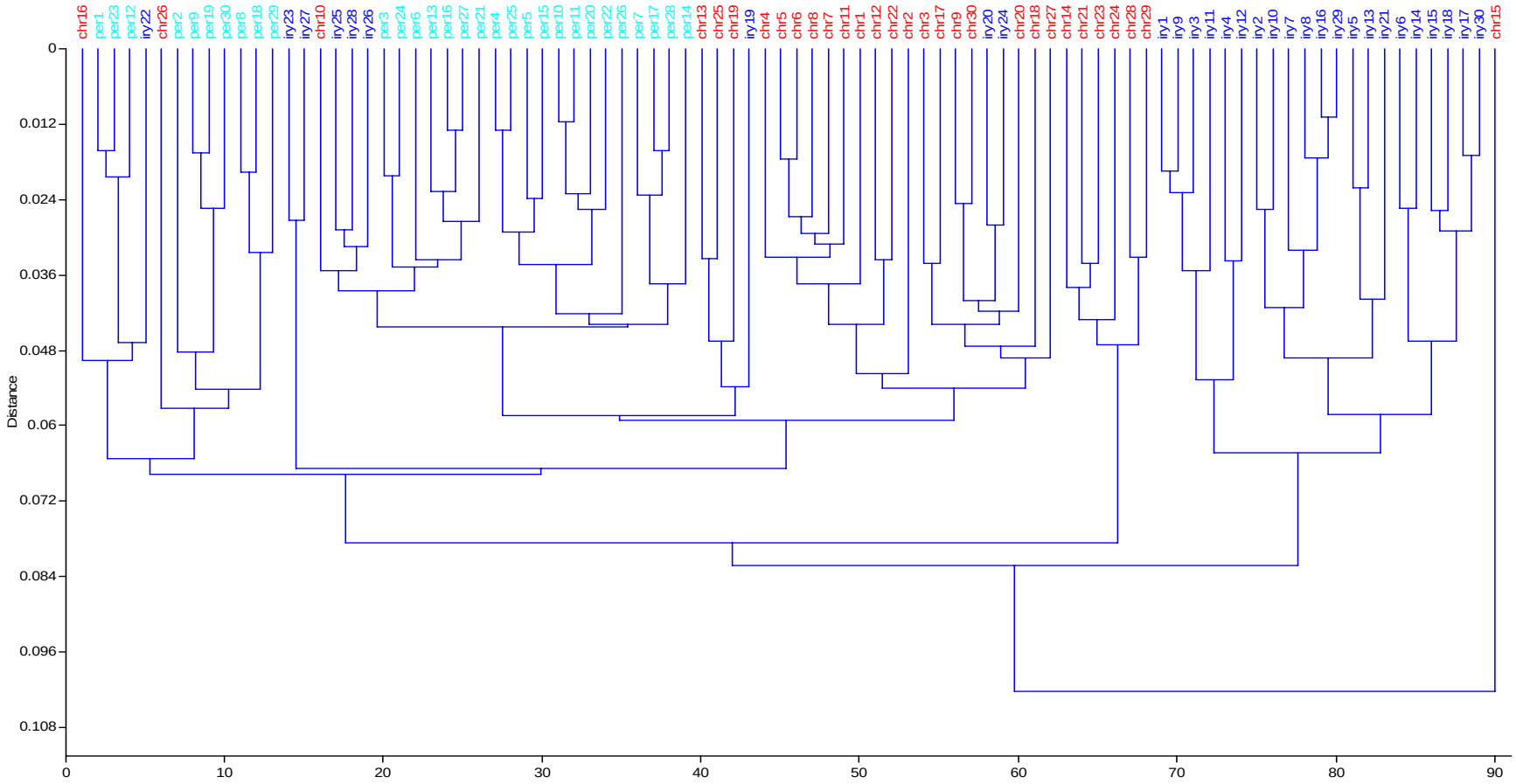


Table 7 **CVA loadings**

	Axis 1	**Axis 2**	**Axis 3**	**Axis 4**	**Axis 5**	**Axis 6**	**Axis 7**	**Axis 8**	**Axis 9**	**Axis 10**	**Axis 11**	**Axis 12**	**Axis 13**	**Axis 14**	**Axis 15**
s-d	0.154	0.157	-0.045	0.225	-0.489	-0.167	0.308	-0.289	0.279	0.450	0.005	-0.324	0.217	-0.143	0.018
s-pel	0.208	0.587	-0.484	0.075	0.331	-0.289	-0.113	-0.018	-0.174	-0.048	-0.127	0.023	0.052	-0.316	0.118
s-anal	0.599	-0.110	0.119	-0.161	-0.074	-0.131	0.055	0.036	0.117	-0.071	-0.275	-0.123	-0.656	-0.015	0.148
eye	0.013	0.144	0.100	0.071	0.064	-0.072	-0.448	0.492	0.668	0.215	0.088	0.088	0.039	0.005	0.053
df-dc	-0.236	-0.030	-0.077	0.158	-0.101	-0.201	-0.057	0.119	-0.055	-0.196	0.591	-0.372	-0.415	-0.315	-0.209
df-vc	-0.248	-0.069	-0.512	0.228	-0.350	0.137	-0.335	-0.144	0.014	-0.049	-0.121	-0.004	-0.248	0.337	0.391
df-af	0.185	0.064	-0.158	-0.491	-0.108	0.238	-0.415	-0.181	0.084	-0.176	-0.027	-0.462	0.223	0.060	-0.342
df-pelf	0.183	0.437	0.421	0.133	-0.202	0.463	-0.164	-0.189	-0.091	-0.194	0.281	0.138	-0.023	-0.160	0.304
dc-cfs	0.239	-0.376	0.020	0.535	0.182	-0.093	-0.196	-0.365	0.253	-0.390	-0.067	0.069	0.189	-0.146	-0.139
wcp	-0.350	0.297	0.268	0.203	-0.164	-0.040	0.094	0.218	0.046	-0.397	-0.582	-0.276	-0.006	-0.023	-0.127
af-dc	-0.110	-0.143	0.010	0.016	0.500	0.294	0.175	-0.001	0.146	0.047	0.004	-0.555	0.060	-0.085	0.508
pelf-dc	0.289	0.174	-0.324	0.236	0.042	0.360	0.442	0.287	0.163	-0.219	0.191	0.003	0.001	0.378	-0.252
vc-cfs	-0.272	0.227	-0.003	-0.349	0.113	-0.215	0.252	-0.440	0.501	-0.288	0.139	0.194	-0.134	0.147	0.076
vc-af	-0.193	0.110	-0.042	0.133	0.242	0.429	-0.048	-0.273	0.111	0.396	-0.212	0.099	-0.412	-0.190	-0.427
af-pelf	-0.056	-0.230	-0.300	-0.233	-0.271	0.280	0.188	0.198	0.191	-0.187	-0.116	0.253	0.084	-0.638	0.099

4.1.5.Cluster analysis

Figure 7 **Dendrogram showing distribution of *P.denisonii***



The X-Y coordinates were converted into distances. The size adjusted morphometric distances (Table 1, 2 and 3) of *P. denisonii* collected from different rivers were subjected to PCA and CVA. Principal components analysis was applied to the individual data. Morphological traits segregated quite clearly onto the first three PCs.

The first two principal components (PC's) accounted for 60.132 % of the total variance. PC1, accounting for 40.614 % of total variance (Table 6), was essentially a contrast between body variables B, E, F and N (Figure 1), while PC2 (19.518 % of total variability) essentially by significant contributions of variables C, G and O (Figure 2). In the scatter diagram of PCA loadings the Periyar stocks of *P. denisonii* were located on the negative sector of the second component, while the Irrity and Chaliyar populations were situated mostly on the positive sector of this component (Figure 4 and 5). The second component is merely defined by the length from snout to anal fin, length from dorsal fin to anal fin and distance between anal fin and pelvic fins.

The groups of *P. denisonii* were again separated distinctly by canonical variance analysis, and their validity was confirmed by the scatter plots of the CVA loadings (Figure 6, Table 7). The populations of the Irrity, Chaliyar and Periyar seemed to be clearly distinct from each other, with the Periyar population being grouped on the negative sector of the second CV component. Dendrogram of the sampling areas (Figure 7) was constructed by unweighted pair-group method with arithmetic means (Sneath and Sokal, 1973) to assess the degree of similarity between the samples. Some individuals of Chaliyar and Irrity populations are grouped along with the Periyar population in the cluster diagram.

4.2.Random Amplified Polymorphic DNA Studies

4.2.1.RAPD PCR gel

Plate 10 *Primer OPA1*

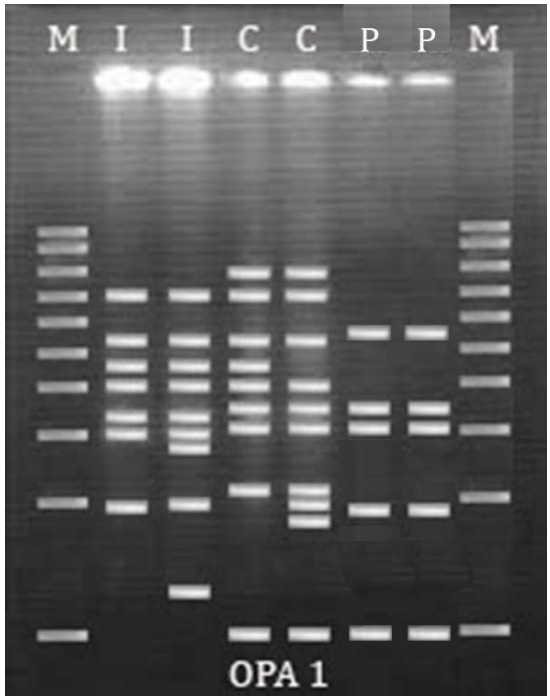


Plate 11 *Primer OPA6*

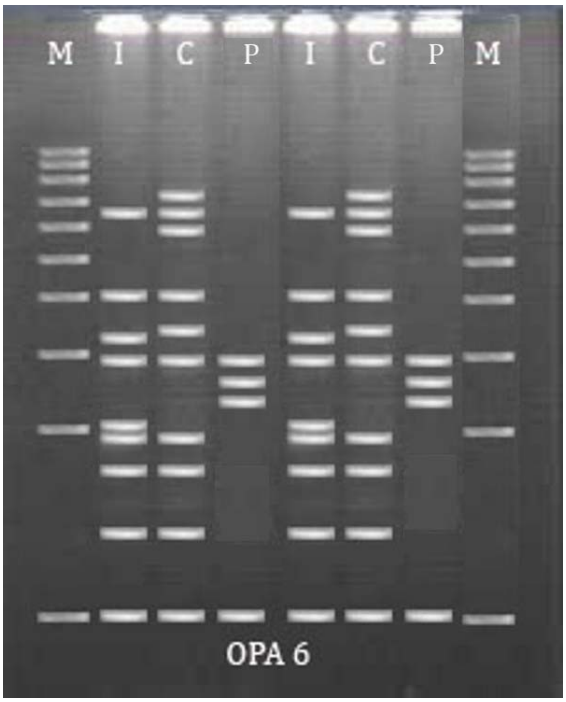


Plate 12 *Primer OPA7*

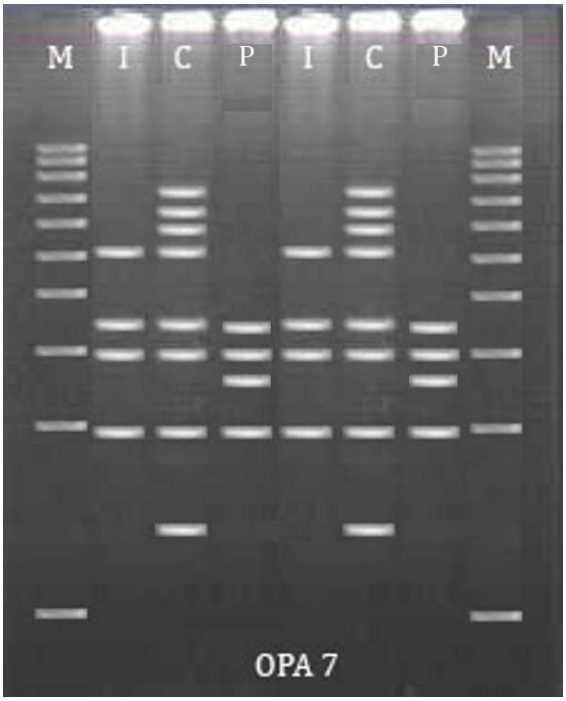
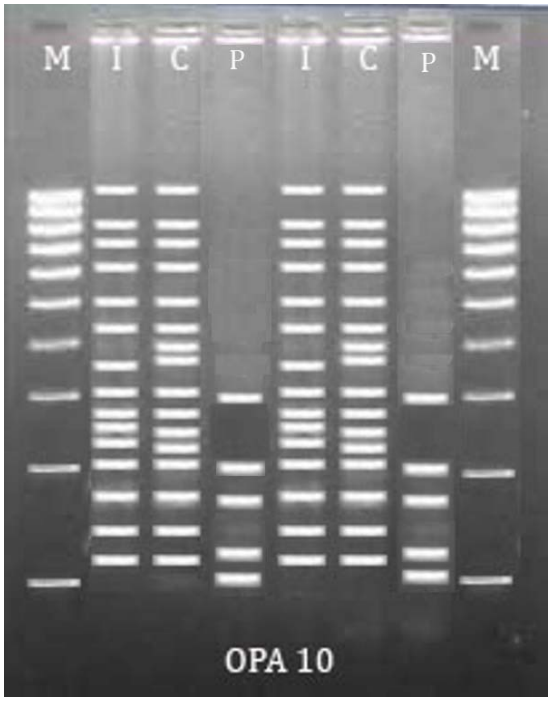


Plate 13 *Primer OPA10*



I-Irrity, C-Chaliyar, P-Periyar and M-Molecular marker (100-1000bp)

4.2.2.RAPD Analysis

Table 8 **RAPD analysis of Irrity population**

Locus	Sample Size	na*	ne*	h*	I*
opa1100	25	1	1	0	0
opa1125	25	1	1	0	0
opa1150	25	2	1.6756	0.4032	0.593
opa1175	25	1	1	0	0
opa1200	25	2	1.5743	0.3648	0.5511
opa1250	25	2	1.8546	0.4608	0.6534
opa1275	25	1	1	0	0
opa1300	25	2	1.8546	0.4608	0.6534
opa1350	25	2	1.4706	0.32	0.5004
opa1400	25	2	1.5743	0.3648	0.5511
opa1450	25	2	1.5743	0.3648	0.5511
opa1550	25	2	1.7705	0.4352	0.6269
opa1700	25	2	1.6756	0.4032	0.593
opa1900	25	1	1	0	0
opa6100	25	2	1.4706	0.32	0.5004
opa6125	25	2	1.6756	0.4032	0.593
opa6150	25	2	1.7705	0.4352	0.6269
opa6175	25	2	1.5743	0.3648	0.5511
opa6200	25	2	1.5743	0.3648	0.5511
opa6225	25	1	1	0	0
opa6250	25	1	1	0	0
opa6275	25	2	1.5743	0.3648	0.5511
opa6325	25	2	1.4706	0.32	0.5004
opa6350	25	1	1	0	0
opa6400	25	2	1.7705	0.4352	0.6269
opa6550	25	1	1	0	0
opa6650	25	2	1.4706	0.32	0.5004
opa6750	25	1	1	0	0
opa7175	25	1	1	0	0
opa7200	25	2	1.7705	0.4352	0.6269
opa7225	25	1	1	0	0
opa7250	25	1	1	0	0
opa7275	25	1	1	0	0
opa7300	25	2	1.4706	0.32	0.5004
opa7350	25	2	1.3676	0.2688	0.4397
opa7500	25	2	1.3676	0.2688	0.4397
opa10100	25	1	1	0	0

opa10125	25	2	1.6756	0.4032	0.593
opa10150	25	2	1.4706	0.32	0.5004
opa10175	25	2	1.6756	0.4032	0.593
opa10200	25	2	1.3676	0.2688	0.4397
opa10225	25	1	1	0	0
opa10250	25	2	1.5743	0.3648	0.5511
opa10275	25	2	1.6756	0.4032	0.593
opa10300	25	2	1.3676	0.2688	0.4397
opa10350	25	2	1.4706	0.32	0.5004
opa10375	25	1	1	0	0
opa10400	25	2	1.5743	0.3648	0.5511
opa10425	25	2	1.8546	0.4608	0.6534
opa10450	25	1	1	0	0
opa10500	25	2	1.6756	0.4032	0.593
opa10550	25	1	1	0	0
opa10600	25	2	1.9968	0.4992	0.6923
opa10700	25	2	1.8546	0.4608	0.6534
opa10750	25	1	1	0	0
opa10800	25	2	1.9231	0.48	0.673
opa101000	25	2	1.7705	0.4352	0.6269
Mean	25	1.6491	1.4084	0.2466	0.3673
St.dev		0.4815	0.3315	0.1899	0.2782

The number of polymorphic loci is : 37

The percentage of polymorphic loci is : 64.91 %

Table 9 **RAPD analysis of Chaliyar population**

Locus	**Sample Size**	**na***	**ne***	**h***	**I***
opa1100	25	2	1.6756	0.4032	0.593
opa1125	25	1	1	0	0
opa1150	25	1	1	0	0
opa1175	25	1	1	0	0
opa1200	25	2	1.6756	0.4032	0.593
opa1250	25	1	1	0	0
opa1275	25	1	1	0	0
opa1300	25	2	1.7705	0.4352	0.6269
opa1350	25	2	1.3676	0.2688	0.4397
opa1400	25	2	1.5743	0.3648	0.5511
opa1450	25	2	1.5743	0.3648	0.5511
opa1550	25	2	1.9231	0.48	0.673

opa1700	25	2	1.7705	0.4352	0.6269
opa1900	25	1	1	0	0
opa6100	25	2	1.8546	0.4608	0.6534
opa6125	25	2	1.5743	0.3648	0.5511
opa6150	25	2	1.8546	0.4608	0.6534
opa6175	25	1	1	0	0
opa6200	25	1	1	0	0
opa6225	25	1	1	0	0
opa6250	25	1	1	0	0
opa6275	25	2	1.6756	0.4032	0.593
opa6325	25	1	1	0	0
opa6350	25	2	1.4706	0.32	0.5004
opa6400	25	2	1.6756	0.4032	0.593
opa6550	25	2	1.5743	0.3648	0.5511
opa6650	25	2	1.7705	0.4352	0.6269
opa6750	25	2	1.7705	0.4352	0.6269
opa7175	25	2	1.7705	0.4352	0.6269
opa7200	25	1	1	0	0
opa7225	25	2	1.4706	0.32	0.5004
opa7250	25	1	1	0	0
opa7275	25	2	1.7705	0.4352	0.6269
opa7300	25	1	1	0	0
opa7350	25	1	1	0	0
opa7500	25	2	1.7705	0.4352	0.6269
opa10100	25	1	1	0	0
opa10125	25	2	1.5743	0.3648	0.5511
opa10150	25	2	1.5743	0.3648	0.5511
opa10175	25	2	1.8546	0.4608	0.6534
opa10200	25	1	1	0	0
opa10225	25	2	1.6756	0.4032	0.593
opa10250	25	2	1.6756	0.4032	0.593
opa10275	25	2	1.9231	0.48	0.673
opa10300	25	2	1.5743	0.3648	0.5511
opa10350	25	1	1	0	0
opa10375	25	2	1.7705	0.4352	0.6269
opa10400	25	2	1.8546	0.4608	0.6534
opa10425	25	1	1	0	0
opa10450	25	2	1.9716	0.4928	0.6859
opa10500	25	2	1.9231	0.48	0.673
opa10550	25	2	1.7705	0.4352	0.6269
opa10600	25	2	1.9231	0.48	0.673
opa10700	25	1	1	0	0

opa10750	25	2	1.4706	0.32	0.5004
opa10800	25	2	1.5743	0.3648	0.5511
opa101000	25	2	1.7705	0.4352	0.6269
Mean	25	1.6491	1.4599	0.2662	0.3889
St.dev		0.4815	0.3618	0.2021	0.2922

The number of polymorphic loci is : 37

The percentage of polymorphic loci is : 64.91 %

Table 10 **RAPD analysis of Periyar population**

Locus	**Sample Size**	**na***	**ne***	**h***	**I***
opa1100	25	2	1.8546	0.4608	0.6534
opa1125	25	1	1	0	0
opa1150	25	1	1	0	0
opa1175	25	1	1	0	0
opa1200	25	2	1.6756	0.4032	0.593
opa1250	25	1	1	0	0
opa1275	25	1	1	0	0
opa1300	25	2	1.7705	0.4352	0.6269
opa1350	25	2	1.8546	0.4608	0.6534
opa1400	25	1	1	0	0
opa1450	25	2	1.7705	0.4352	0.6269
opa1550	25	2	1.5743	0.3648	0.5511
opa1700	25	1	1	0	0
opa1900	25	1	1	0	0
opa6100	25	2	1.6756	0.4032	0.593
opa6125	25	1	1	0	0
opa6150	25	1	1	0	0
opa6175	25	1	1	0	0
opa6200	25	1	1	0	0
opa6225	25	2	1.9968	0.4992	0.6923
opa6250	25	2	1.9231	0.48	0.673
opa6275	25	2	1.8546	0.4608	0.6534
opa6325	25	1	1	0	0
opa6350	25	1	1	0	0
opa6400	25	1	1	0	0
opa6550	25	1	1	0	0
opa6650	25	1	1	0	0
opa6750	25	1	1	0	0
opa7175	25	1	1	0	0

opa7200	25	2	1.9716	0.4928	0.6859
opa7225	25	1	1	0	0
opa7250	25	1	1	0	0
opa7275	25	2	1.9231	0.48	0.673
opa7300	25	2	1.8546	0.4608	0.6534
opa7350	25	2	1.9231	0.48	0.673
opa7500	25	1	1	0	0
opa10100	25	2	1.9968	0.4992	0.6923
opa10125	25	2	1.8546	0.4608	0.6534
opa10150	25	1	1	0	0
opa10175	25	2	1.9968	0.4992	0.6923
opa10200	25	2	1.8546	0.4608	0.6534
opa10225	25	1	1	0	0
opa10250	25	1	1	0	0
opa10275	25	1	1	0	0
opa10300	25	2	1.9968	0.4992	0.6923
opa10350	25	1	1	0	0
opa10375	25	1	1	0	0
opa10400	25	1	1	0	0
opa10425	25	1	1	0	0
opa10450	25	1	1	0	0
opa10500	25	1	1	0	0
opa10550	25	1	1	0	0
opa10600	25	1	1	0	0
opa10700	25	1	1	0	0
opa10750	25	1	1	0	0
opa10800	25	1	1	0	0
opa101000	25	1	1	0	0
Mean	25	1.3333	1.2864	0.1533	0.2173
St.dev		0.4756	0.4143	0.2197	0.3108

The number of polymorphic loci is : 19

The percentage of polymorphic loci is : 33.33 %

Table 11 **Summary of Genic Variation Statistics for All Loci**

Locus	Sample Size	na*	ne*	h*	I*	Gst	Nm
opa1100	75	2	1.9827	0.4956	0.6888	0.4189	0.6935
opa1125	75	1	1	0	0	****	****
opa1150	75	2	1.5743	0.3648	0.5511	0.6316	0.2917
opa1175	75	1	1	0	0	****	****
opa1200	75	2	1.6423	0.3911	0.5799	0.0018	274.5
opa1250	75	2	1.5052	0.3356	0.5183	0.5424	0.4219
opa1275	75	1	1	0	0	****	****
opa1300	75	2	1.8	0.4444	0.6365	0.0016	312
opa1350	75	2	1.5743	0.3648	0.5511	0.0409	11.7143
opa1400	75	2	1.9996	0.4999	0.6931	0.5135	0.4737
opa1450	75	2	1.6423	0.3911	0.5799	0.0073	68.25
opa1550	75	2	1.7705	0.4352	0.6269	0.0196	25
opa1700	75	2	1.9912	0.4978	0.6909	0.4386	0.6401
opa1900	75	1	1	0	0	****	****
opa6100	75	2	1.6756	0.4032	0.593	0.0212	23.125
opa6125	75	2	1.9996	0.4999	0.6931	0.4879	0.5248
opa6150	75	2	1.9716	0.4928	0.6859	0.3939	0.7692
opa6175	75	2	1.6085	0.3783	0.566	0.6786	0.2368
opa6200	75	2	1.6085	0.3783	0.566	0.6786	0.2368
opa6225	75	2	1.4017	0.2866	0.4611	0.4194	0.6923
opa6250	75	2	1.4706	0.32	0.5004	0.5	0.5
opa6275	75	2	1.7082	0.4146	0.6051	0.012	41.1429
opa6325	75	2	1.6423	0.3911	0.5799	0.7273	0.1875
opa6350	75	2	1.6423	0.3911	0.5799	0.7273	0.1875
opa6400	75	2	1.9912	0.4978	0.6909	0.4386	0.6401
opa6550	75	2	1.6085	0.3783	0.566	0.6786	0.2368
opa6650	75	2	1.9996	0.4999	0.6931	0.4964	0.5072
opa6750	75	2	1.5398	0.3506	0.5352	0.5862	0.3529
opa7175	75	2	1.5398	0.3506	0.5352	0.5862	0.3529
opa7200	75	2	1.9417	0.485	0.678	0.3622	0.8806
opa7225	75	2	1.6423	0.3911	0.5799	0.7273	0.1875
opa7250	75	1	1	0	0	****	****
opa7275	75	2	1.9579	0.4892	0.6824	0.3765	0.8282
opa7300	75	2	1.9968	0.4992	0.6923	0.4786	0.5446
opa7350	75	2	1.9968	0.4992	0.6923	0.5	0.5
opa7500	75	2	1.9996	0.4999	0.6931	0.5306	0.4424
opa10100	75	2	1.4017	0.2866	0.4611	0.4194	0.6923

opa10125	75	2	1.7082	0.4146	0.6051	0.012	41.1429
opa10150	75	2	1.9968	0.4992	0.6923	0.5427	0.4213
opa10175	75	2	1.8794	0.4679	0.6607	0.0289	16.8158
opa10200	75	2	1.9996	0.4999	0.6931	0.5135	0.4737
opa10225	75	2	1.5743	0.3648	0.5511	0.6316	0.2917
opa10250	75	2	1.9996	0.4999	0.6931	0.4879	0.5248
opa10275	75	2	1.9716	0.4928	0.6859	0.4026	0.7419
opa10300	75	2	1.7399	0.4252	0.6164	0.112	3.9627
opa10350	75	2	1.6423	0.3911	0.5799	0.7273	0.1875
opa10375	75	2	1.5398	0.3506	0.5352	0.5862	0.3529
opa10400	75	2	1.9912	0.4978	0.6909	0.4471	0.6182
opa10425	75	2	1.5052	0.3356	0.5183	0.5424	0.4219
opa10450	75	2	1.436	0.3036	0.4814	0.459	0.5893
opa10500	75	2	1.9716	0.4928	0.6859	0.4026	0.7419
opa10550	75	2	1.5398	0.3506	0.5352	0.5862	0.3529
opa10600	75	2	1.8794	0.4679	0.6607	0.3024	1.1533
opa10700	75	2	1.5052	0.3356	0.5183	0.5424	0.4219
opa10750	75	2	1.6423	0.3911	0.5799	0.7273	0.1875
opa10800	75	2	1.9827	0.4956	0.6888	0.4319	0.6578
opa101000	75	2	1.9827	0.4956	0.6888	0.4146	0.7059
Mean	75	1.9123	1.6897	0.3855	0.558	0.4242	0.6788
St.dev		0.2854	0.2922	0.1374	0.1882		

The number of polymorphic loci is : 52

The percentage of polymorphic loci is : 91.23

* na = Observed number of alleles

* ne = Effective number of alleles [Kimura and Crow (1964)]

* h = Nei's (1973) gene diversity

* I = Shannon's Information index [Lewontin (1972)]

Gst = Nei’s (1987) coefficient of gene differentiation and

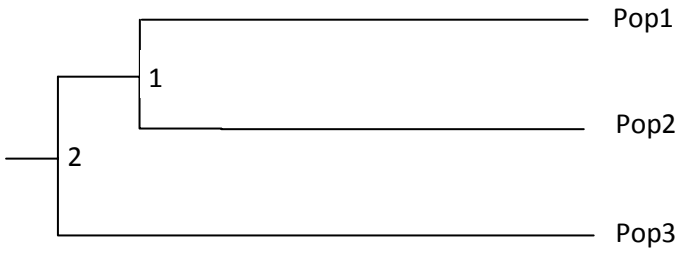
Nm = gene flow

Table 12 **Nei's Unbiased Measures of Genetic Identity and Genetic distance**

pop ID	Irrity	Chaliyar	Periyar
Irrity	****	0.6975	0.697
Chaliyar	0.3603	****	0.6753
Periyar	0.361	0.3926	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Figure 8 **Dendrogram Based on Nei's (1978) Genetic distance using UPGMA --Modified from NEIGHBOR procedure of PHYLIP Version 3.5**



The number and size of fragments amplified by primers OPA1 (Plate 10), OPA6 (Plate 11), OPA7 (Plate 12)and OPA10 (Plate 13) varied from 3-16 bands and 100-1000 bp, whereas the average number of polymorphic bands varied from 4-10 bands. The highest number of amplified fragments as well as polymorphic fragments was produced by primer OPA 10.

The percentage of polymorphic loci (Table 8, 9 and 10) of the Irrity, Chaliyar and Periyar populations are 64, 64 and 33 and Nei's gene diversity values are 0.24, 0.26 and 0.15 respectively. Upon testing 10 random decamer primers 4 primers giving reproducible results were selected and used for generating DNA fingerprints from a total of 75 individuals of *P. denisonii*, 25

each collected from these rivers. A total of 57 loci were scored, 91% of which were found to be polymorphic in nature. The average values of Nei's gene diversity and Shannon's Information Index were 0.38 and 0.55 respectively indicating a substantial level of genetic diversity (Table 11).

Dendrogram based on Nei's (1978) Genetic distance (using UPGMA -- Modified from NEIGHBOR procedure of PHYLIP Version 3.5) indicate two main branches and three well-differentiated groups (Figure 8) which indicates the presence of three genetically distinct regional populations of *P. denisonii.* The Periyar population which is denoted by population 3 is seperately placed as a separate or main branch. This result is again confirmed by Nei's genetic distances which were calculated between locations. The overall average genetic distance among all the three locations was 0.424. Nei's Unbiased Measures of Genetic Identity and Genetic distance (Table 12) show that the Chaliyar population is more genetically similar to Irrity population (0.6975) when compared to Periyar population (0.6753). Genetic similarity between the Irrity and Periyar populations are also less (0.697).

4.3.Controlled breeding of *P. denisonii*

4.3.1.Broodstock maintanence and selection of prospective brooders

250 numbers of *Puntius denisonii* were stocked in outdoor cement tanks of (1000m^3) capacity with daily water exchange and controlled temperature by covering with a shade net. They were fed with commercial pelleted feed (Crude Protein 40%, Fat 6%, Crude Fibre 3% and Moisture 11%) and live feed (earthworm, mosquito larvae) to enhance maturation. Monthly the broodstock was checked for spermeating males and ovulating females. The prospective spawners were selected (Table 13) by noticing the females with fully distended belly and males oozing milt on applying slight pressure on the abdomen. Both the sexes were found to be ripe and suitable for breeding operations during November to February. The males from all the rearing systems like cement tanks, glass aquariums with and without recirculatory systems were found spermeating but the best female spawners with matured ripe ovary were obtained from outdoor 1000m^3 cement tanks covered by shade net.

Table 13 **Sexual dimorphism in *P. denisonii***

Male	Female
1. Slender and streamlined body, more aggressive while handling.	1. Abdomen slightly bulged due to developed ovary during breeding season
2. Spermeating when gently pressed on the abdomen	2. Ripe ova released when gently pressed on the abdomen.

4.3.2. Ovary type and fecundity studies

Plate 14 *Mature female Puntius denisonii with ripe eggs*

The ovary from the ripe captive matured stock was dissected out (Plate 14) and the type of spawning was determined based on the diameter of eggs developing in the ovary. Both large matured eggs (0.6-0.8mm) ready for spawning, a batch of smaller intermediate eggs (0.2-0.4mm) and a large stock of small immatured eggs (< 0.1mm) were found uniformly present in the ovary. The ovary is classified as the group synchronous type in which all the eggs are not fully developed, but only part ie., the larger matured ones are released during a spawning act or the developing eggs are released completely in two batches during a breeding season (Figure 9).

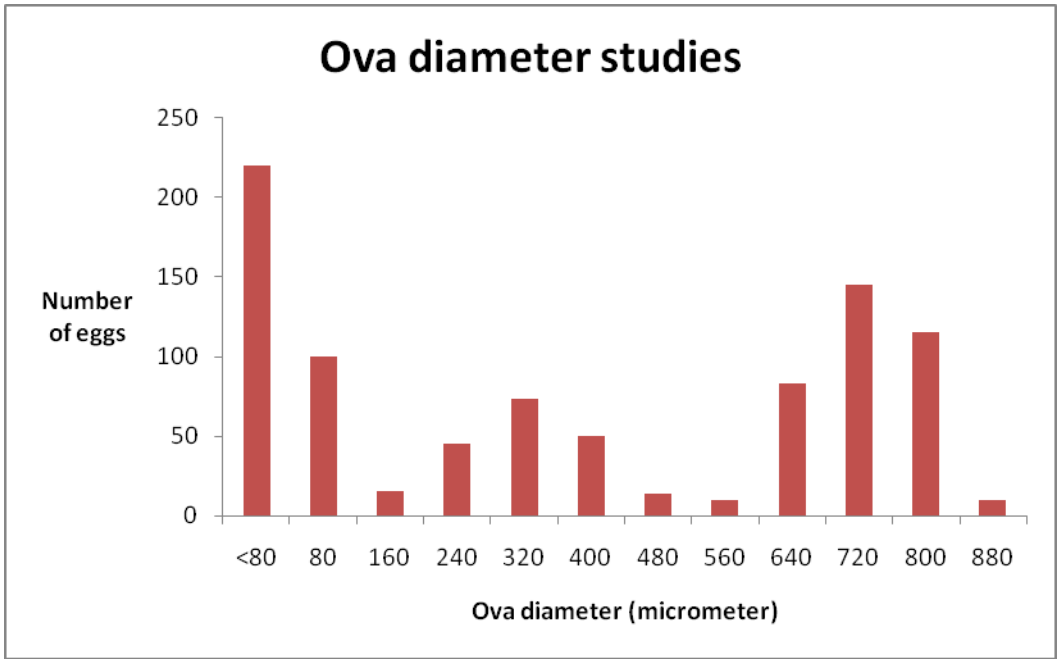
Plate 15 *A single lobe of the ripe ovary of Puntius denisonii*

Plate 16 *Mature male of Puntius denisonii with well developed testis*

Plate 17 *Ripe lobes of mature testis of Puntius denisonii*

The ovary can be classified as cystovary where ovary lumen has continuity with the oviduct. The ovary is bilobed, suspended by mesentries from the dorsal side of the peritoneal cavity. Mature fishes of 12- 15g size had ovaries weighing 0.8 to 1.1g (Plate 15) and the total number of eggs varied from 880 to 1220 numbers. The relative fecundity was calcuated as 58 to 82 eggs per gram of fish or 800 to 1110 eggs per gram of ovary in the present study. 38.1% of the ova was in immature condition while the intermediate maturing eggs were 21.8% and the matured ripe eggs were 40.1% of the total number of ova.

Figure 9 **Ovadiameter studies of ripe *P. denisonii***



4.3.3. Induced breeding trials

After selection of prospective brooders in the least stressful way, the brooders were sedated with clove oil, the fishes were grouped into three experimental and one control (sex ratio 1:1 and 2:1) and separately injected with common carp pituitary extract, commercial hormone preparation Wova FH and Ovaprim (Table 14). The fishes were showing typical spawning behaviour in

glass aquariums only when other parameters like temperature (25^0C), hardness (20ppm) and pH (6.5-7) were adjusted to resemble their natural habitat. It was not necessary that the fishes showing chasing behaviour should surely spawn. Substratum containing clean sand and polished rocks was also provided to simulate their natural environment. Water flow was found to increase success of spawning as both the sexes were found very active with intense colouration and rapid chasing movements. After environmental manipulation studies, best results in spawning were obtained with intramuscular injection of synthetic hormone 'Ovaprim' (standard dose 0.5ml/kg) using fine needle (insulin syringe) after sedation. The best sex ratio showing chasing behaviour and successful spawning in most of the breeding trials was 1:1 (4 numbers in 40L glass tank). Similar breeding trials conducted in Chinese hatchery met with limited success due to difficulty in maintaining optimal water quality as large volumes of water is required for its operation.

Table 14 **Breeding trials with different inducing agents in *P. denisonii***

Breeding trials	**Sex ratio**	**Spawning**	**Latency Period**	**Incubation time**
Common carp pituitary	1:1	No success	-	-
	2:1	No success	-	-
Common carp pituitary + Environmental simulation	1:1	Rarely	15 hours	32 hours
	2:1	Rarely	15 hours	32 hours
Wova FH	1:1	Rarely	13 hours	30 hours
	2:1	No success	-	-
Wova FH + Environmental simulation	1:1	Success	13 hours	30 hours
	2:1	Rarely	13 hours	30 hours
Ovaprim	1:1	Success	13 hours	30 hours
	2:1	Rarely	13 hours	30 hours
Ovaprim + Environmental simulation	1:1	Success	13 hours	30 hours
	2:1	Success	13 hours	30 hours

Chasing began almost immediately after males and females were kept together in the breeding tank and glass aquarium, which was however, no surety that the fish would breed. Just before the beginning of active chasing the fishes would change colour to vivid brightness of intense red hue, and the male would quiver in front of the prospective partner with movements making use of the dorsal and caudal fins. Successful spawning took place within 13 hours of the injection dispersing eggs among the pebble substratum (Table 15). While the males were still chasing females, there was visible indication of spawning in the tank, in the form of a line of froth adhered to the tank along the water edges.

Table 15 **Spawning behaviour in *P. denisonii***

Male	Female
At the start of courtship behaviour, there is an increase in colour of the reddish streak, and quivering of the dorsal, pectoral and anal fins. Starts chasing the female and continue for a long period.	Female responds well to the courtship behaviour of the male and starts spawning at short intervals often coming towards the surface of the water.

4.3.4.Early embryonic developmental stages of *P. denisonii*

Fertilized eggs of *P. denisonii* were demersal, spherical, slightly adhesive, opaque and light yellowish. They were collected from the spawning tank by siphoning into a glass beaker with a water cushion. The diameter of the unfertilized eggs (Plate 18 b) obtained was 0.86 ± 0.04 mm. The fertilized eggs (Plate 18 a, 1.1± 0.01mm) were not transparent unlike the case in many other cyprinids. Yolk was seen with distinct blastodisc, and did not show the presence of an oil globule. The details of development of the fertilized egg until hatching are given in Table 16 and photographs in Plate 18.

Plate 18 *Embryonic developmental stages of P. denisonii*

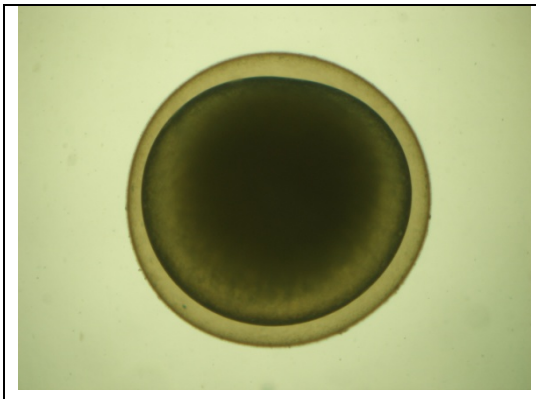
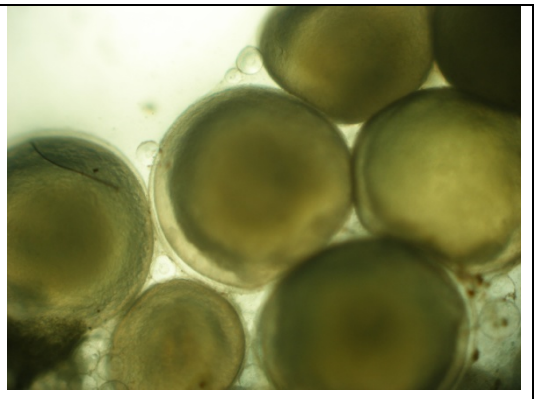
	

Plate 18 a *Fertilised egg*	***Plate 18 b*** *Unfertilised eggs*

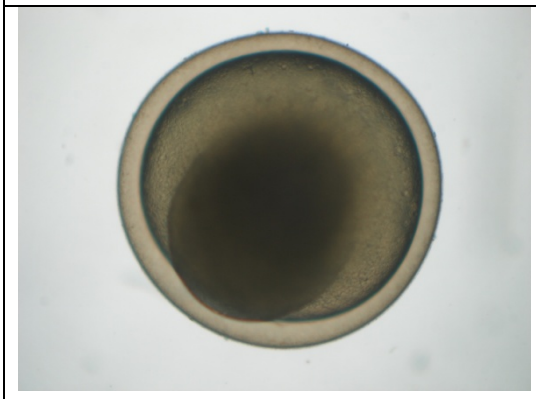
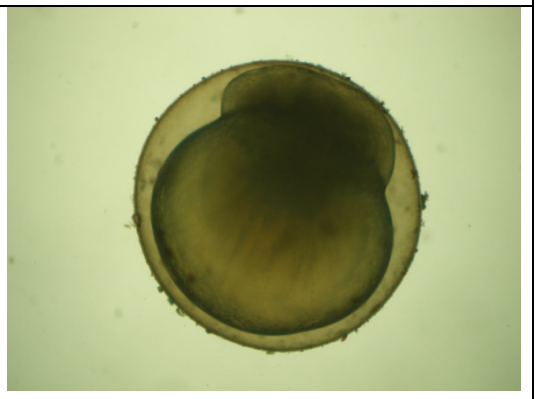

	

Plate 18 c *Cleavage*	***Plate 18 d*** *Blastula*

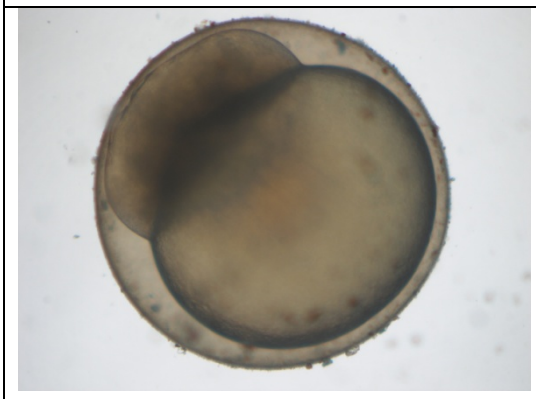
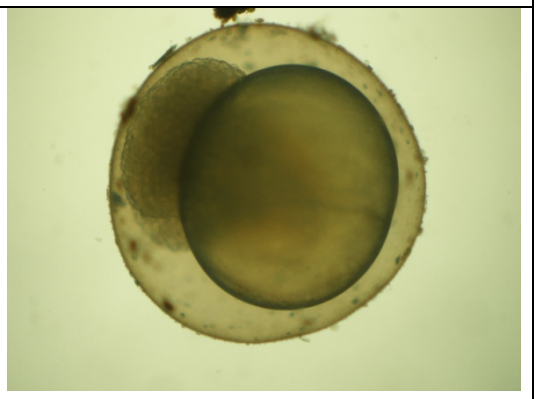

	

Plate 18e *Dividing cells*	***Plate 18 f*** *Morula*

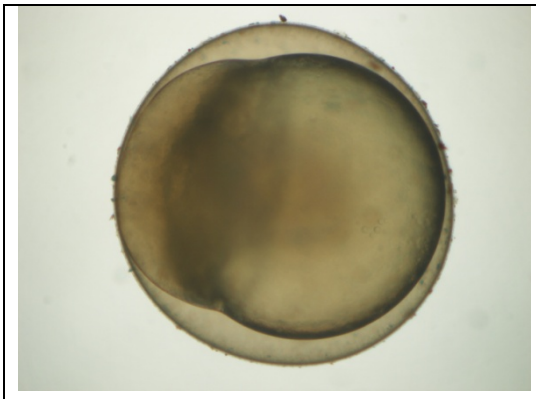
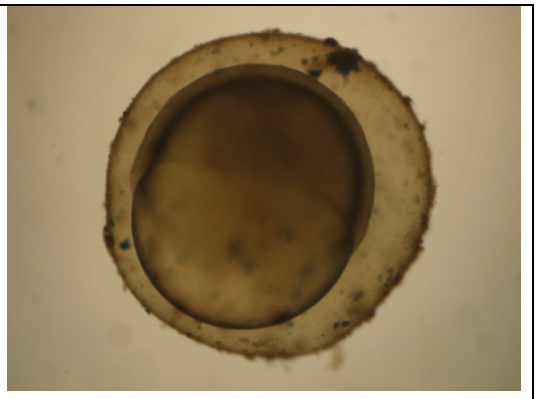
	

Plate 18 g *Late Blastula*	***Plate 18 h*** *Early Gastrula*

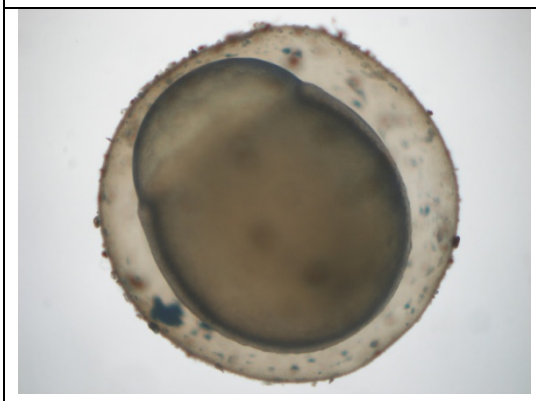
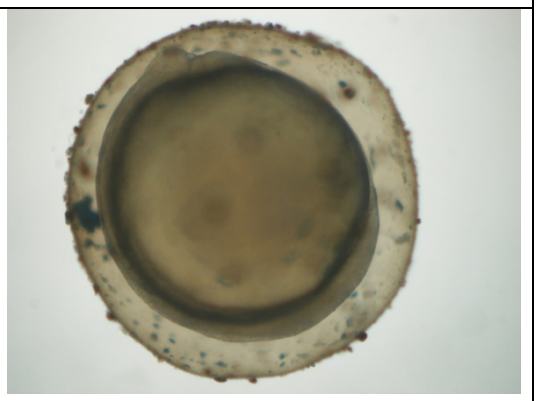

	

Plate 18 i *Involution*	***Plate 18 j*** *Gastrula*

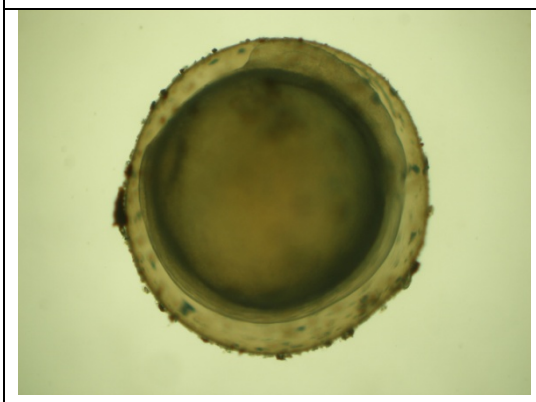
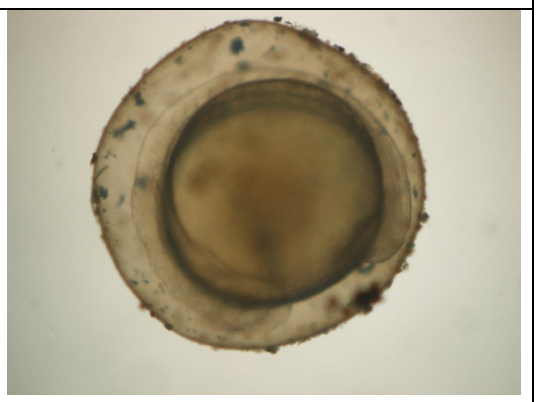

	

Plate 18 k *Differentiation of cells*	***Plate 18 l*** *Myomeres visible*

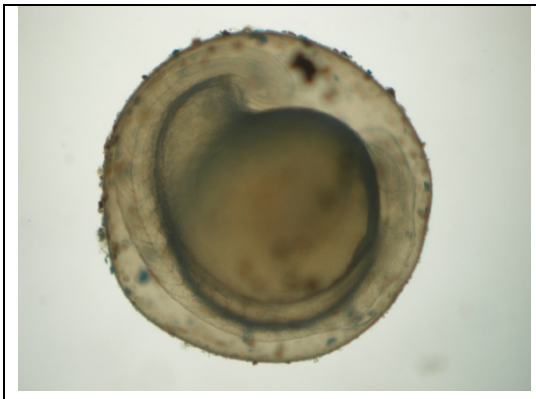
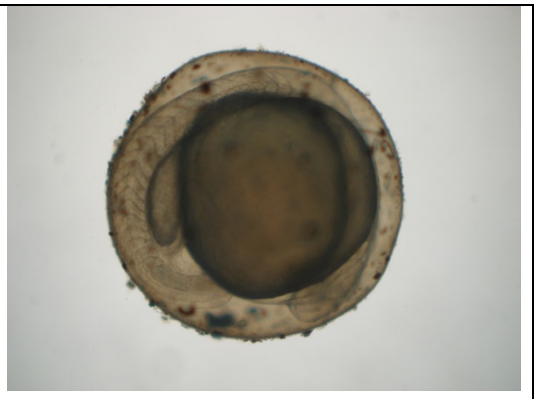
	

Plate 18 m *Head and caudal somite visible*	***Plate 18 n*** *Tail extending and covering the entire yolk*

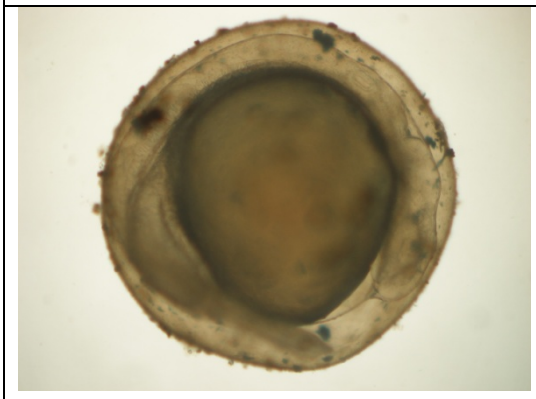
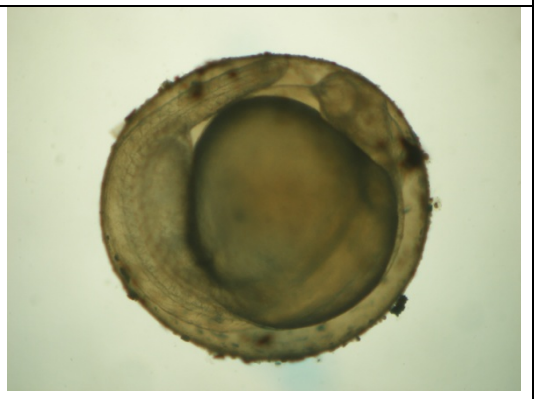

	

Plate 18 o *Tail free from the yolk sac*	***Plate 18 p*** *Optic lobes develop*

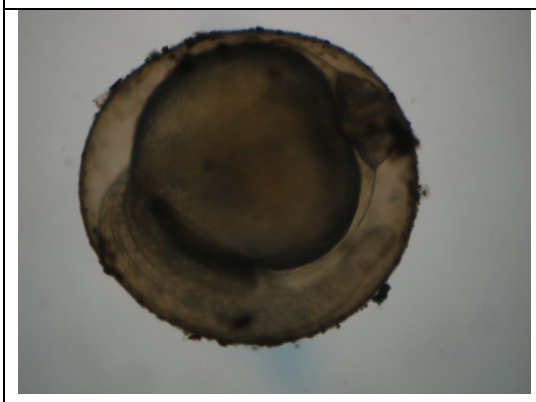
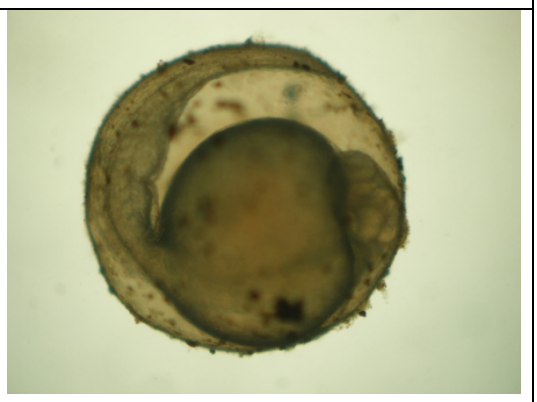

	

Plate 18 q *Twitching movement starts*	***Plate 18 r*** *More body part freed from the yolk sac*

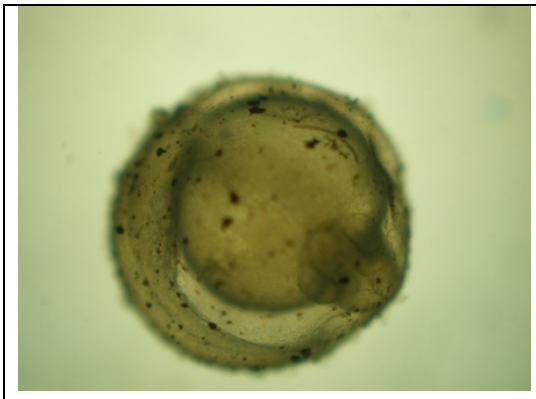
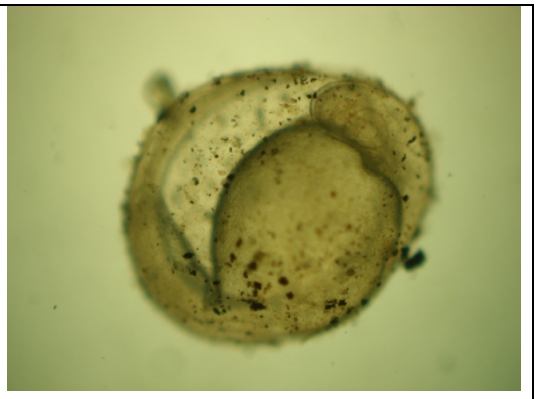
	

Plate 18 s *Embryo occupying entire space*	***Plate 18 t*** *The chorion becomes flexible*

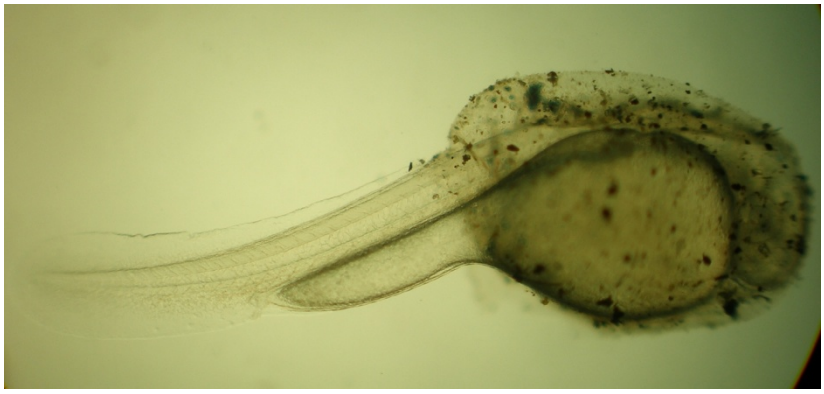


Plate 18 u *Yolk-sac embryo with part of chorion remaining on head region.*

Table 16 **Description of developmental stages of *P. denisonii* with time**

Time after spawning	Stage of egg	Description
0 min	Fertilized egg	Mean diameter 0.8± 0.01mm (Plate 18 a).
15 min	First cleavage	Division of blastodisc into two blastomeres.
1.5 – 2 h	Morula	Cleavage and a number of blastomeres are formed (Morula) (Plate 18 f).
5 - 6 h	Blastula	Blastoderm increased in size by spreading over the yolk, and reaching the equator of the yolk sac (Plate 18 g).
8 h	Early gastrula	Start of epiboly, blastoderm flattening and beginning to spread downward over the yolk. When it had progressed past the equator, leaving

		a small surface area exposed, the blastopore, at the vegetative pole.
10 h	Gastrula	Embryonic streak and Germ shield visible commenced development as a thickening of the blastoderm along a line that started at the blastopore margin, and proceeded about halfway around the perimeter of the blastoderm opposite the blastopore. The two ends of the embryonic streak were destined to form the head and tail (Plate 18 i).
14 – 16 h	Organogenesis	Neural plate and groove visible; myomeres started appearing; the embryo becomes segmented into somites that initially were more visible along the mid-region (Plate 18 l). First caudal somites visible. The pericardial cavity was obvious below the head; the tail margin became distinct but remained flush with the surface of the yolk sac
21 h		Tail developed continuously, growing outward and rising above the surface of the yolk sac when viewed from side.
22 h		As the head developed the optic lobes became visible; optic vesicles apparent and the yolk sac begin separation (Plate 18 p).
23 h		The tail completely separated from the yolk sac; first melanophores appear on the sac (Plate 18 q). First movement of embryo begins with 17-19 contractions per minute. Twitching movement becomes very frequent at later stages. The tail extends free from the yolk sac. Embryo is two-thirds around yolk sac at first and later occupying almost whole of the perivitelline space. Flexing of the body and tail occurs (Plate 18 t).
29.35 h	Hatching	The yolk sac embryo emerged out with a quick jerking movement, tail out first (Plate 18 u).

The division of the blastodisc into two blastomeres (first cleavage) occurred within 15 min of fertilization. The segmentation was typically meroblastic. As successive cleavages progressed the blastomeres decreased in

size and the morula stage was reached between 1.5 – 2 h after fertilization (Plate 18 f). The embryo attained blastula stage (Plate 18 g) within 5 – 6 h of fertilization, which followed the start of epiboly, the blastoderm flattening and beginning to spread downwards over the yolk, and progressing towards one end leaving the blastopore exposed at the vegetative pole. Within 10 h of fertilization, the embryonic streak and the germ shield were visible, beginning to develop as a thickening of the blastoderm along the line that started at the blastopore margin (Plate 18 i). The two ends of the embryonic streak developed further and the first myomeres became visible within 14 h of fertilization (Plate 18 l), clearly demarcating the head and tail region. There were initially 13-14 myomeres, more towards the anterior region at this stage.

The antero-posterior axis became distinguishable with more somites added (20 – 23) and the tail involuting the yolk and the head becoming more prominent with optic vesicles (Plate 18 p). The Kupffer's vesicle could be seen at this time. The tail fold appeared to develop faster than the head fold with the appearance of embryonic fin folds. Within 22 h the tail became free, and melanophores started appearing on the sac (Plate 18 q). At this stage the first movement of the embryo was observed with 15-18 contractions per minute. The head fold also became prominent with two optic cups becoming visible and the embryo occupying a larger area within the egg. The outer chorion membrane was softened and was seen flexible according to the twitching movement of the embryo (Plate 18 t). After 29 h the embryo hatched out breaking the outer chorion and the hatchling was showing flexing of its tail and jerking movements lying on the lateral sides (Plate 18 u). The yolk sac embryo tried to move short distances in the same posture, but remained most of the time stationary near the air stone supplying mild aeration.

4.3.5.Larval rearing

After 70 hours post hatching, yolk sac absorption was completed, and the larvae were fed initially with infusoria for a week. Later they were fed with freshly hatched *Artemia* nauplii for one month. Artificial feed was given from the third week onwards and they were weaned to artificial feed in another one week

time. The black stripes were first seen within two weeks on the lateral sides of the body. Later, within two months reddish colouration over the eyes and on the dorsal fins developed.

4.4.Development of transgenic varieties of *P. denisonii*

4.4.1. Extraction of plasmid DNA

The plasmid pCMV-GFP procured from Addgene, US, in *E. coli* was cultured in sterile conditions and plasmid DNA was isolated following the protocol of Sambrook *et al*. (1989). The concentration of plasmid DNA was quantified as 150-200 ng/ml using spectrophotometer (Biophotometer plus, Eppendorf). This was stored under -20^{0}C until electroporation and biotin labeling for dot blot hybridization.

4.4.2. Artificial fertilization and electroporation of freshly fertilised eggs

Broodstock was maintained in optimal conditions as in the case of Controlled breeding experiments in the sex ratio 1:1. After 13 hours post injection of Ovaprim, the freshly fertilized eggs in the cleavage stage were collected following artificial fertilization or dry stripping. The best conditions for electroporation of fertilized eggs were 20V with 3 bursts at a time interval of 1sec (Table 17). Other stages of eggs like gastrula and morula were also electroporated under similar conditions. After electroporation the survivors were grown for a period of 8 weeks.

Table 17 **Electroporation conditions and efficiencies**

Molecule	Stage	Optimal voltage protocol	Survival rate	Electroporation rate for survivors	n
pCMV-GFP	13 hpf Gastrula	20 V; 1s; 3X	75	40	246
pCMV-GFP	1hpf Morula	20V; 1s; 3X	70	70	200
pCMV-GFP	10 minutes pf One or two celled stage	20V; 1s; 3X	65	79	220

4.4.3.Dot blot hybridisation test

Expression of green fluorescent protein was not visible (*in vivo*) under UV or blue light. Plasmid DNA containing the GFP gene was biotin labelled (Fermentas). The DNA of the *P. denisonii* electroporated with GFP gene was spotted on the nitrocellulose membrane (Sambrook *et al*., 1989) for hybridisation test. Blue colour was developed finally when the membrane was incubated in freshly prepared 1X BCIP/NBT at room temp in the dark for 15-30 min indicating integration of GFP gene in *P. denisonii*. The substrate solution was discarded and the membrane was air dried to document the results (Plate 19) after washing in water for a few seconds.

Plate 19 *Dot blot hybridization of the transgenic juveniles using biotin labeled probe.*

5. DISCUSSION

5.1.Truss Analysis

Stock can be considered as a production or management unit in which all members are characterized by similarities induced by the environment, which are not heritable, and which may include members of several different subpopulations. A good knowledge of the population structure is essential for management of fisheries - exploitation or preservation or both. At the same time it is hazardous to generalize the demographic, geographic and genetic structural characteristics of the species.

Eventhough it is important to reliably identify management units of the same species, a single technique is not sufficient to understand the genetic variation between these units (Edmonds *et al*., 1989; Campana *et al*., 1995). Combining the results obtained with several techniques, gives considerable insight to the possible stock structure of a species (Elliott *et al*., 1995). Hence, both Truss network analysis and RAPD analysis was employed to study the population structure of *P. denisonii* seen in the Kerala rivers endemic to the Western ghats. This species is one of the highly sought after ornamental indiscriminately captured from its natural habitat for domestic and international trade, eventhough declared as vulnerable by the IUCN recently.

Genetic markers are generally oversensitive to a low level of gene flow. Therefore, molecular markers may not be sufficient to detect existing genetic variation among populations, and also only a small proportion of DNA is analysed by molecular markers. Phenotypic variation has advantages in the stock identification especially when the time is insufficient for significant genetic differentiation to accumulate among *P. denisonii* populations of Irrity and Chaliyar.

Different types of body measurements have been traditionally used to characterize stocks which are concentrated along the body axis with only sampling from depth and breadth, and most measurements in the head. A regionally unbiased network of morphometric measurements over the two-

dimensional outline of a fish (Plate 9) gives more information about local body differences than a conventional set of measurements (Strauss and Bookstein, 1982). In the present study, it was necessary to remove the size effect from the data (Table 1,2 and 3) as variation should always be attributable to body shape differences, and not related to the relative size of the fish.

Environmental factors as well as the geographical region occupied by a species tend to influence phenotypic variation between stocks. In the present study, *Puntius denisonii* collected from three rivers of Kerala exhibited morphometric differences which were identified by truss analysis when subjected to Principal Component and Canonical Variance analysis. The fish samples collected from the Periyar, Chaliyar and Irrity River were found to segregate from each other in the PCA (Figure 4 and 5) and CVA scatter (Figure 6) diagram.

According to Haig (1998), the most important assessment of the viability of populations is to determine the relative amounts of genetic diversity within and among the populations. Genetic techniques of handling, conservation and breeding of *P. denisonii*, should depend on knowing the amount of variation within each reproductive or management unit as conservationists assume that a fall in genetic diversity in a population could adversely affect its short-term viability (Leberg, 1990). As all individuals of *P. denisonii* collected were distinctly separated into three populations, the conservation strategies should endeavour to preserve its diversity in that area. In future, if restocked with individuals bred in captivity which did not originate from the local population, alleles important for survival in that habitat may be diluted or lost and/or less well-adapted alleles fixed, possibly leading to local extinction (Leuzzi *et al*., 2004).

Diversity among salmon populations has been documented within and between rivers, within Europe. Similarly, the spatial isolation of river systems and a sometimes patchy, discontinuous distribution of spawning and rearing habitat within river systems, provides the opportunity for *P. denisonii* to segregate into distinct reproductive groups and provides the basis for the species to subdivide into local populations or stocks. It has to be noted that the intensity

of color of the red bands in the case of *P. denisonii* differ with river, showing the highest intensity in fishes in the Irrity river, and the lowest in Periyar.

Most multivariate studies of allometry have used principal component analysis (PCA) (Klingenberg, 1996). PCA combines and summarizes the variation associated with each of a number of measured variables into a smaller number of principal components (PCs) which are a linear combination of the variables that describe the shape variations in the pooled sample. Each principal component contains the percentage of total variance of all variables. Snout to pelvic fin, dorsal fin to dorsal caudal, dorsal fin to ventral caudal and ventral caudal to anal fin (Figure 1) are the most important mophometric measurements containing 40.614 % of total variance ie PC1 (Table 6). While snout to anal fin, dorsal fin to anal fin and anal fin to pelvic fin (Figure 2) are the important measurements with 19.518 % of total variability (PC2).

Principal components and canonical variance analysis was used to produce graphs to visualise relationships among the individuals of different *P. denisonii* populations by plotting population centroids of 95% confidence ellipses of first two PCs and CVA scatter diagram. In both PCA (Figure 4 and 5) and CVA (Figure 6) scatter diagrams, the Periyar population segregated as a separate group on the negative sector of the second component. While both Irrity and Chaliyar populations are grouped in the positive sector with slight overlapping. The fish collected from northern Kerala rivers are showing more morphological similarity when compared to the Periyar population (located in Central Kerala) which is seen as a separate group. The pattern of phenotypic discreteness suggests a direct relationship between the extent of phenotypic divergence and geographic separation.

To assess the degree of similarity between the samples, dendrogram (Figure 7) was constructed by unweighted pair-group method with arithmetic means. The presence of some individuals of Irrity and Chaliyar populations grouping along with the population of Periyar shows that certain locations or stretches of these rivers are almost identical or the individuals can be marked as separate stocks as they do not resemble the general structure of the individuals

segregating into separate groups or populations. This shows that the morphology of *P. denisonii* in the same river but different localities could differ. Further studies on stock structure of these populations with rivers are recommended.

5.2.Random amplified polymorphic DNA

RAPD analysis was conducted in samples collected from Irrity, Chaliyar and Periyar river. RAPD-PCR allows for a more introspective interpretation of diversity within a population. Understanding relative levels of variation within- and among-populations of *P. denisonii* can help to plan for long-term genetic diversity and for clarifying demographic and ecological issues early in species recovery. This helps in identification of populations in need of new individuals, populations that could safely donate individuals to more vulnerable populations, or populations in need of further demographic or environmental consideration (Fritzner *et al*., 2001).

In the present study, from Table 8, 9 and 10, the percentage of polymorphism in the fish samples collected from Irrity and Chaliyar was found high (64%) when compared to the Periyar population (33%), which reflects the fact that the species has still not experienced genetic deterioration in the rivers of Northern Kerala suggesting a greater potential for use in breeding programs. However, the vulnerable state of this species may create genetic deterioration in the future if proper steps for conservation and stock enhancement are not taken. Moreover, primary genetic data in the present study are useful not only to characterize each population, but also to highlight future tasks for a better hatchery management and for the biodiversity conservation of *P. denisonii*. The overall percentage of polymorphic loci was 91%, indicating a high level of polymorphism supporting the suitability of RAPD markers for effectively discriminating different *P. denisonii* populations.

Observed number of alleles (na), Effective number of alleles (ne) following Kimura and Crow (1964), Nei's (1973) gene diversity (h) and Shannon's Information index (I) following Lewontin (1972) of each population (Table 8,9 and 10) indicates that the genetic diversity is maximum in the Irrity

and Chaliyar when compared to the Periyar population. Dendrogram based on Nei's (1978) Genetic distance (using UPGMA --Modified from NEIGHBOR procedure of PHYLIP Version 3.5) indicates the presence of three genetically distinct regional populations of *P. denisonii* (Figure 8). The Periyar population which is denoted by population 3 is seperately placed as a separate or main branch which confirms the geographical effect on genetic diversity revealed by RAPD analysis.

Based on results of Nei's Unbiased Measures of Genetic Identity and Genetic distance, the Irrity and Chaliyar populations show higher genetic similarity (0.6975) when compared to the Periyar population (Table 12). The genetic distance is the least between Irrity and Chaliyar populations (0.3603) while that between the populations of Chaliyar and Periyar (0.3926) and that between Irrity and Periyar (0.361) are higher. This may be related to regional, environmental and spatial characteristics of these rivers. Additionally, differences in the band frequencies were observed with collection site. The findings suggest the occurrence of a structured population and the importance for the conservation of the genetic variability of distinct *P. denisonii* populations. Further studies to delineate stocks within populations are recommended.

5.3. Controlled breeding of *P. denisonii*

The demand for fish is steadily increasing in the recent years mainly for food, recreation, and ornamental aquariums. The fry of *Puntius denisonii* was only collected from the river systems of Kerala for trade where they breed naturally. But natural fish populations have declined during the last several decades because of environmental degradation and over-fishing. As a result the breeding grounds are losing their suitability to be used by the species, posing a threat of extinction to the existence of *P. denisonii*. Controlled spawning of *Puntius denisonii*, an ornamental indigenous to the Western Ghats, and artificial rearing of its fry are the best means to meet the large demand rising in domestic and international trade. However, a number of fish species including *Puntius denisonii* that have or potentially have great economic significance as ornamentals do not reproduce spontaneously in captivity.

Reproductive processes are by no means fully impaired in captivity. The progressive development of the gonads remains, in general, uninhibited up to the final stages of gamete maturation, and it is only at the point of gamete release that the sequence is arrested. Hormone-induced spawning is the only reliable method to induce reproduction in these fishes. As the wild stocks of *P. denisonii* has been classified as vulnerable by the IUCN, the present study for development of techniques of controlled propagation of this fish is very significant.

Providing optimal conditions for broodstock can eliminate or at least reduce the need for intervention. Fishes need certain environmental and social cues before they will mature and spawn. Captive conditions differ from those in the natural environment and are not optimal for the final stages of sexual development and the production of high quality gametes in *P. denisonii*. Hence, environmental optimization with hormone intervention was studied to bring captive fish to spawning readiness and spawning in confinement. Another issue is the selection of a suitable diet, which is usually different from that of fish in the wild. More effort was put into improving broodstock diet with live feeds during conditioning. Subjecting the fish to inappropriate rearing conditions and diets can result in disturbed gametogenesis (Bogdanova, 2004) and less effective fish reproduction (Miroslaw *et al*., 2010).

In *P. denisonii* hormonal regulation for vitellogenic event and final maturation occur during November to February of the year unlike the popular belief, and conservative reports that it coincides with the southwest monsoon season. These fish in nature select a spawning ground with intermittent sandy bottom and rocky patches for spawning. In confinement, the best female spawners were collected from large outdoor cement tanks ($1000m^3$) covered with shade nets to control temperature. This may be due to the increased exposure of broodstock to natural productivity in the outdoor system.

Physical injury and physiological stress of capturing, handling, transporting, injecting, and holding brood fish had a greater detrimental effect on spawning success in the present study than almost any other factor. Fish must be handled carefully and optimum water conditions must be maintained to minimize

stress so that the spawners may adapt themselves to the confined environment and be in a normal physiological condition to receive the hormone substances. Female brood fish ready for spawning are in a particularly delicate condition and should be selected quickly to avoid stress and mortality. Before sampling the testes or ovaries, the fish was made quiet with anaesthetic clove oil (Matin *et al*., 2009). It is best to keep the fish in the water during selection. Secondary female sex characteristics such as plumpness of the abdomen and redness of the vent are extremely subjective in *P. denisonii* and can be misleading.

The ovary is a typical teleost bilobed cystovary where ovary lumen has continuity with the oviduct. The ovadiameter studies in *P. denisonii* shows a group synchronous type of ovary containing a huge stock of immature eggs, a ripe stock of mature eggs and a intermediate stock of maturing eggs (Figure 9). It is a multiple spawner releasing eggs more than one time in a breeding season. An interesting unique feature is the uniform distribution of ripe ova inside the ovary during breeding season. Temperature, water quality (e.g., dissolved oxygen, pH, and hardness) photoperiod, spawning substrate (e.g., gravel, polished rocks) and nutrition are interrelated factors which determine success during induced breeding. Glass aquariums in which temperature, pH and hardness were adjusted to natural habitat gave the best results.

Common carp pituitary gland extract and commercial sGnRHa preparations were used for induced breeding of *P. denisonii*. Good results of induced ovulation in *P. denisonii* were obtained after hormonal stimulation by synthetic analogue of gonadotropin-releasing hormone (GnRH), with dopamine antagonists. These hormones last longer in the fish's system; only one dose was needed to induce spawning. The GnRH molecule itself is not highly species specific. Ovaprim is not only cheaper but also extremely dependable in *P. denisonii*, hence highly recommended for hatchery users.

For developmental studies, eggs were stripped into a plastic vessel and were fertilized using "dry method" (Kucharczyk *et al*., 1997a), the best fertilization technique preferred over wet stripping for *P. denisonii*, because it takes advantage of gamete physiology. Even for fish species which can undergo

natural spawning in captivity, induced ovulation followed by artificial fertilization is generally preferred in order to achieve a greater control over fry production. Furthermore, controlled reproduction of *P. denisonii* also makes it possible to develop method for selective breeding or transgenic production of superior strains and for furnishing parasite- and disease-free fish seeds.

5.4.Early embryonic development and Larval rearing

The eggs were transparent with light yellowish coloration. They were spherical, demersal and slightly adhesive. Samples of fertilized eggs were taken every 2 hours starting from cleavage up to hatching which took 30 hours. Embryo showed a discoidal meroblastic cleavage, where the large yolk materials restricts cell division to a small area at the animal pole close to the micropyle as in other teleosts (Hall *et al*., 2004). The yolk sac was completely absorbed at 70 hrs during embryonic development on attainment of 3mm total length. At the same time the digestive system became fully developed and the larvae searched for feeding. The full developmental sequence of *P. denisonii*, from egg to yolksac embryo (Table 16, Plate 18) is recorded as the information concerning its developmental biology is of much need to enhance captive breeding of this species. In addition, these studies can be useful in directing the husbandry efforts of the fish farmer to the specific state and requirements of each developmental stage and to optimize growth and survival.

The amount of live food required was ascertained, on a daily basis, to support robust growth, enhance survival and to reduce food wastage (Barahona-Fernandez and Girin, 1977). Increased feeding frequency usually improved feed utilisation and reduced the incidence of starvation in small fish in a group (Yager and Summerfelt, 1994; Duray *et al*., 1997). The success of larval rearing was greatly influenced by first feeding regimes and the nutritional quality of starter diets that affect larval growth and survival (Watanabe *et al*., 1983; Izquierdo *et al*., 2000). Live foods such as infusoria and *Artemia* contains a significant pool of FAA (Fyhn *et al*., 1993, 1995; Helland, 1995). Since growth is primarily an increase in body muscle mass by protein synthesis and accretion and fish larvae

have very high growth rates, they have a high dietary requirement for amino acids.

The need of artificial production of *P. denisonii* has grown tremendously in the recent years as there is scarcity of fish seed experienced in many of the previously located natural habitats. With the rapid industrialization of the State, factory effluents and urbanization effects pollute the waters and adversely affect the riverine fisheries. Besides, construction of dams across many rivers in Kerala have restricted to a large extent the spawning migration of fishes and brought about considerable changes in the environment and natural spawning grounds of many fishes, thus depleting the fishery in these waters. Captive bred quality fish seed of *P. denisonii* for domestic as well as international trade could be met to a large extent by application of the controlled breeding and larval rearing techniques described.

5.7. Development of transgenic varieties of *P. denisonii*

The increasing world demand for ornamental fish has opened the market for new varieties with novel colors which can be supplied through the use of transgenics. The transgenic technology was applied to ornamental fish industry since 1995, with the tremendous local interest in this sector. The availability of genes encoding fluorescent proteins such as GFP, RFP, BFP, YFP and CFP, has enabled the production of green, red, blue, yellow or cyan fish in an almost endless variety of combinations. In the present study, an attempt has been made to develop transgenic varieties of *P. denisonii* incorporating the green fluorescent protein gene, which could possibly glow, if expressed, in the presence of blue light.

Uniform GFP expression throughout the whole body by using β-actin promoter is reported in medaka (Chou *et al.,* 2001) and zebrafish (Higashijima *et al.,* 1997; Hsiao *et al.,* 2001). The gene coding for the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been widely used as a reporter gene because it does not require an exogenous substrate for its activity (Amsterdam *et al.,* 1995) and is stable and non-toxic in receptor organisms

(Peters *et al.,* 1995). Fortunately, more recently as the technology to achieve 100% sterilization of the progeny has been successfully developed, GM pet fish should be considered as environmentally and ecologically safe (Tsai, 2008).

Aquaculture species have been particularly amenable to the production of transgenics. Fish and shellfish tend to be highly fecund, producing a large quantity of gametes. *P. denisonii* was harvested for eggs and sperm, and fertilisation *in-vitro* is straightforward. Eggs are relatively large, and fertilised eggs develop outside the body, so no further manipulation, such as reimplantation is necessary. Methods such as microinjection and electroporation are currently used for gene transfer in egg-laying fish (Chen *et al*., 1993). Skeletal muscle can be transfected in vivo by direct plasmid DNA injection, where foreign genes can be expressed at significant levels for up to 19 months (Wolff *et al*., 1992; Tripathy *et al*., 1996; MacColl *et al*., 2000). In the present study, the transgene was purchased as plasmid, pCMV-GFP with an appropriate promoter-enhancer element and a structural gene sequence contained in *E. coli* DH5α cells (Addgene Inc., USA).

Microinjection is not only time consuming and labor intensive but is also limited by the physiology of fish eggs. Hence electroporation of fertilized eggs of *Puntius denisonii* was the alternative making gene transfer more efficient. Removal of the chorion was not applied because it is a tedious procedure and introduces additional stress on newly fertilized eggs of *P. denisonii*. A large number of fertilized eggs can be treated in a short time by this method (Inoue *et al*., 1990; Chen *et al*., 1995). Electroporation of fertilized eggs has been considered as a means of mass gene transfer for model fish medaka (Inoue *et al.,* 1990) and many commercially important species such as catfish *Ictalurus punctatus,* common carp *Cyprinus carpio* (Powers *et al.,* 1992), black porgy *Acanthopagrus schlegeli* (Tsai and Tseng, 1994), abalone *Haliotis rufescens* (Powers *et al.,* 1995) and tiger shrimp *Penaeus monodon* (Tseng *et al.,* 2000). Electroporation, therefore, was considered as an efficient and versatile massive gene transfer technology to develop transgenic varieties of *P. denisonii* which has short sperm motility time.

The shortcoming is that the protocol of optimal condition is inconsistent, stage- and species-dependent, resulting in the poor successful rate and reproducibility. The ideal voltage, pulse length and number of pulses are difficult to determine and changes for each setup (Bansal *et al*., 2009). As with any electroporation protocol, long duration and high voltage pulses killed cells (Cerda *et al*., 2006; Sale and Hamilton, 1967, 1968). Also great variability in the amount of signal seen from one positive animal to the next has been reported. Gastrula, Morula, one- and two-cell embryos of *P. denisonii* was electroporated to study the effect of time after fertilization on successful GFP integration (Table 17). The electroporation rate of survivors was highest in one and two celled embryos.

In zebrafish, injected plasmid DNA is converted into a high molecular weight form and amplified during the cleavage stage. It is subsequently degraded during gastrulation and retained in only some of the cells (Stuart *et al*., 1988). In the present study, eventhough plasmid CMV - GFP was transferred to the fertilized eggs of *P. denisonii*, it might have degraded, allowing restricted integration of GFP gene to the genome of survivors. Previous studies (Chen *et al*., 1993; Pinkert *et al*., 1995; Inoue *et al*., 1990) showed that levels of transgene expression vary among transgenic individuals. There are important drawbacks to the use of these DNA elements in transgenic fish, including low expression efficiency (0-20%) (Culp *et al*., 1991; Higashijima *et al*., 1997) and the mosaic expression of transgene patterns (Stuart *et al*., 1990; Ju *et al*., 1999). Hence, the process of making transgenic animals is not efficient. Stable integration of the transgene is required for continuous transmission to subsequent generations to establish a transgenic fish line. Many resulting offspring do not take up the transgene, or they take it up but do not express it, so it is important to screen offspring for transgene integration and expression. In the present study, Dot blot hybridization of genomic DNA with biotinylated probes was conducted to detect transgene integration (Plate 19).

Following hatching, the founder individuals was reared for 8 weeks, at which a fin clip can be obtained without harming the individual to check for the presence of transgene. In the present study, eventhough the expression of GFP

was not evident under blue or UV light, transgenic *P. denisonii* containing the GFP gene was successfully produced by electroporation and was confirmed by conducting dot blot test. It is expected that stable lines of fluorescent transgenic *P. denisonii* would emit light in night time to drive pet aquarium to a new horizon allowing its beauty to be enjoyed both day and night in the near future.

6. SUMMARY

The population structure of *Puntius denisonii*, the indigenous ornamental fish of Kerala endemic to the Western Ghats in three different rivers- namely Irrity, Chaliyar, and Periyar was studied. Truss network (morphometric) and Random Amplified Polymorphic DNA (genetic) analysis were conducted to study the population structure of *P. denisonii*.

Already declared vulnerable by the IUCN, the genetic diversity of the species was estimated based on the number of polymorphic loci and the percentage of polymorphism obtained from the RAPD electrophoretic pattern using popgene software. The effective number of alleles, genetic similarity and genetic distance were calculated. Dendrogram was drawn based on Nei's genetic distance to study the distance and similarities between the different populations. The Northern Kerala rivers show more genetic diversity than Periyar river. The Irrity and Chaliyar populations are genetically similar when compared to the Periyar population.

The truss measurements after size adjustment were subjected to multivariate statistical analysis namely, Principal Component Analysis and Canonical Variant Analysis. The PCA components were analysed to study the percentage of variation between individuals and the measurements with maximum PCA loadings were noted. The truss measurements were also subjected to CVA and the loadings were plotted to obtain scatter diagram. Both the scatter diagrams obtained by plotting the PCA and CVA loadings separated the Irrity and Chaliyar population to the positive sector of the component while the Periyar population was segregated to the negative sector of the second component. The Irrity and Chaliyar populations are morphometrically similar when compared to the Periyar population. The data was then submitted to Cluster analysis and the resulting dendrogram segregated the individuals into different populations. The occurrence of a few individuals of Irrity and Chaliyar river among the Periyar individuals in the dendrogram indicates the presence of subpopulations or stocks.

Controlled breeding of *P. denisonii* was successfully completed under hatchery conditions with the administration of Common carp pituitary hormone, Wova-FH and Ovaprim. Best results were obtained when injected with Ovaprim, accompanied by environmental simulation maintaining a sex ratio of 1:1. The important parameters that played a major role in successful spawning were stress free handling and injection of the broodstock, and supply of water with low hardness, pH and temperature to the spawning tank. In addition to the above a water flow and a substratum consisting of sand and polished rocks at the bottom of the spawning tank enhance spawning success.

P. denisonii has a cystovary, with group synchronous spawning, ripe during the cooler months of the year, from November to February. Ova diameter studies indicated the presence of a mature stock, an intermediate stock and a huge stock of immature eggs. It is a multiple spawner which releases the ripe stock of eggs first during the initial months of the breeding season while the remaining intermediate stock of ova is released towards the end of the breeding season. The different stages of embryonic development with time, starting from cleavage to hatching were recorded. The larvae were successfully reared to juveniles under laboratory conditions. Both live feeds like infusoria, artemia and inert feeds were given.

Inorder to develop transgenic *P. denisonii* with green fluorescent protein gene, electroporation technique was standardized. After artificial fertilization, the cleavage, morula and gastrula stage of embryos were electroporated with pCMV-GFP. The surviving transgenic embryos were reared in hatchery conditions. As *invivo* expression of the gene was not visible, dot blot hybridization technique was employed to check the presence of the GFP gene in the electroporated transgenic juveniles. After preparing biotinylated probe, blue colour was developed on the nitrocellulose membrane after hybridisation and incubation, indicating the presence of GFP gene in transgenic *P. denisonii*.

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GENETIC CHARACTERIZATION, CONTROLLED BREEDING AND DEVELOPMENT OF TRANSGENIC VARIETIES OF *PUNTIUS DENISONII* (DAY, 1865).

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ABSTRACT OF THE THESIS

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ABSTRACT

Puntius denisonii, a beautiful ornamental fish indigenous to the Western Ghats, which has been indiscriminately exploited from the different rivers of Kerala has been recently declared to be vulnerable by the IUCN. The population structure and genetic diversity of *P. denisonii* has not yet been studied and documented. Many previous attempts to breed this fish in captivity have yielded negative results. The increasing demand for this fish to decorate aquariums worldwide could be satisfied only by developing controlled breeding techniques and larval rearing of its fry.

In the present study, the present population structure of *P. denisonii* has been studied combining both phenotypic and genotypic techniques. Fishes were collected from Irrity, Chaliyar and Periyar rivers of Kerala. Truss network analysis was conducted and the size adjusted morphometric variables were subjected to Principal Component Analysis and Canonical Variance Analysis. Scatter diagram and Dendrogram was plotted using PCA and CVA loadings. The Irrity and the Chaliyar populations were grouped on the positive sector of the PC and CV component showing morphological similarities between the two populations while the Periyar population was placed in the negative sector of the component separated far from the other two. The PC scores were used to find out the variables showing maximum variation between fishes collected from different rivers.

RAPD PCR was conducted after isolating DNA from the fins of different populations of *P. denisonii*. Universal random primers were screened and the primers that produced reproducible bands were selected. Popgene analysis of the binary data yielded the genetic structure of different populations of *P. denisonii*. Number and percentage of polymorphic loci, Nei's (1973) gene diversity, Shannon's Information index Lewontin (1972), Nei's Unbiased Measures of Genetic Identity and Genetic distance and Dendrogram Based Nei's (1978) Genetic distance using UPGMA --Modified from NEIGHBOR procedure of PHYLIP Version 3.5 were studied. The results obtained supports the truss analysis in that the Irrity and Chaliyar populations in Northern Kerala are

genetically more similar while that of Periyar population in Central Kerala are distinct.

P. denisonii was successfully induced bred under controlled conditions with synthetic hormone preparations Ovaprim and WOVA-FH. Stress during transport and handling was minimized and live feed was supplemented to enhance maturation of the broodstock. The whole developmental sequence starting from fertilized eggs to hatching was photographed and documented. It took 29-30 hours for the eggs to hatch at 28^0C. Rearing of fry was successfully accomplished under laboratory conditions.

In an attempt to develop transgenic varieties of *P. denisonii*, pCMV-GFP was electroporated into newly fertilized eggs, maintained in hypoosmolar electroporation buffer. The electroporation parameters that yielded best results were 20V, 3 bursts at 1 second interval. Fin clips were taken from the transgenic individuals reared for a period of 6 weeks. Dot blot test was positive showing integration of the GFP gene in *P. denisonii*, eventhough expression was not detected under blue or UV light.

The genetic and phenotypic data of *P. denisonii* populations in the present study will aid as a base line for formulating conservation procedures to protect the genetic diversity of wild ones. Stock identification studies are recommended for more concise information on each population. Moreover, the larval rearing and controlled breeding techniques along with the genetic diversity studies will help to design captive breeding programs and enhance the production of hatchery bred ones to meet increasing demand. Further research is recommended for generating transgenic lines with uniform GFP expression.