"Population Genetic Structure of Silver pomfret (*Pampus argenteus*) along Indian coast"

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Under the Faculty of Marine Sciences of the Cochin University of Science and Technology Cochin – 682 022, Kerala, India

by

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Declaration

I do hereby declare that the thesis entitled **"Population Genetic Structure of Silver pomfret (*Pampus argenteus*) along Indian coast"** is the authentic and bonafide record of the research work carried out by me under the guidance of Dr. A. Gopalakrishnan, Director, Central Marine Fisheries Research Institute (CMFRI), Cochin in partial fulfillment for the award of Ph.D. degree under the Faculty of Marine Sciences of Cochin University of Science and Technology, Cochin and no part thereof has been previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

Cochin (Mohitha C)

24th December 2016

Dated: 22nd December 2016

CERTIFICATE

This is to certify that this thesis entitled, **"Population Genetic Structure of Silver pomfret (*Pampus argenteus*) along Indian coast"** is an authentic record of original and bonafide research work carried out by **Ms. Mohitha C (Reg. No. 4180)** at PMFGR Centre, National Bureau of Fish Genetic Resources (NBFGR), Central Marine Fisheries Research Institute, under my supervision and guidance in partial fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Faculty of Marine Sciences, **Cochin University of Science and Technology, Cochin,** Kerala. It is also certified that no part of the work presented in this thesis has been submitted earlier for the award of any degree, diploma or any other similar title. All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the doctoral committee of the candidate have been incorporated in the thesis.

(A. Gopalakrishnan)

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Preface

Silver pomfret (*Pampus argenteus*), is a commercially important pelagic fish in Middle East, Indian and other south East Asian countries, and more over a potential fin fish species for mariculture. The overfishing and overexploitation of this valuable species can reduce their chance of successful reproduction and this may result in changes in species genetic variability and population structure. Despite its importance as a valuable marine resource, little is known about its genetic diversity and population structure of this species in Indian waters. Therefore, there was an urgent need to study the baseline information on genetic stock structuring of this species across its range of natural distribution along Indian waters. Identifying the population structure of commercially important native resources would be the base for genetic upgradation, fisheries management and conservation programs and is significant for every nation.

The present study entitled "Population Genetic Structure of Silver pomfret (*Pampus argenteus*) along Indian coast" aims to determine the fine-scale population structure in *P. argenteus* collected from five geographical locations along Indian coast using mitochondrial ATPase 6/8 gene and polymorphic microsatellite markers. This study investigates the level of genetic diversity and population genetic structure of the species along the Indian coast and a possibility of population size contraction, since there are indications about the catch decline in the past two decades.

The thesis is presented in six chapters excluding summary and references. Latest techniques have been adapted in the study for evaluating the stock structure to arrive at conclusions. This work has been carried out under the guidance of Dr. A. Gopalakrishnan, Director, CMFRI, Cochin.

(Mohitha C)

Contents

List of Abbreviations

μg	:	Micro grams
μl	:	Micro litre
μM	:	Micro molar
AFLP	:	Amplified Fragment Length Polymorphism
AMOVA	:	analysis of molecular variance
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pairs
BPB	:	Bromophenol blue
CMFRI	:	Central Marine Fisheries Research Institute
COI	:	Cytochrome c Oxidase sub-unit I
DNA	:	Deoxyribo Nucleic Acid
dNTPs	:	Deoxynucleoside tri phosphates
EDTA	:	Ethylene Diamine Tetra Acetic acid
EST	:	Expressed Sequence Tags
FAO	:	Food and Agriculture Organization of the United Nations
F_{IS}	:	Inbreeding coefficient
F_{IT}	:	Fixation Index
F_{ST}	:	Co-efficient of genetic differentiation
He	:	Expected Heterozygosity
Ho	:	Observed Heterozygosity
HWE	:	Hardy-Weinberg Equilibrium
IAM	:	Infinite Alleles Model
IBD	:	Isolation by Distance
MDS	:	Multidimensional scaling
MFRs	:	Microsatellite Flanking Regions
mg	:	milligram
ml	:	millilitre
mM	:	millimolar
mm	:	Millimeter
MPEDA	:	Marine Products Export Development Authority
mtDNA	:	Mitochondrial DNA
MUs	:	Management Units
NBFGR	:	National Bureau of Fish Genetic Resources
NCBI	:	National Centre for Biotechnology Information
ng	:	nanogram
NGS	:	Next Generation Sequencing
NJ	:	Neighbour Joining
Nm	:	Rate of gene flow
nm	:	nanometer
OD	:	optical density

PAGE	:	Polyacrylamide gel electrophoresis
PCoA	:	Principal Coordinates Analysis
PCR	:	Polymerase Chain Reaction
PIC	:	Polymorphic Information Content
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
RGCB	:	Rajiv Gandhi Centre for Biotechnology
RNA	:	Ribo Nucleic Acid
rpm	:	Revolutions per minute
R_{ST}	:	Allele size based covariance
SD	:	standard deviation
SDS	:	Sodium Dodecyl Sulphate
SMM	:	Stepwise Mutation Model
SSRs	:	Simple Sequence Repeats
STRs	:	Short Tandem Repeats
TBE	:	Tris Borate EDTA
TPM	:	Two Phase Model
UPGMA	:	Unweighted Pair Group Method with Arithmetic Mean
UV	:	Ultra Violet
VNTRs	:	Variable number of Tandem Repeats

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Chapter I

INTRODUCTION

1.1 Overview

Marine fishery resources are very important component in providing food safety, income and accelerate the socio economic development of the country. Global marine fishery production is estimated as 79.7 million tonnes in 2012 (FAO, 2016). The marine fish landing of the country was estimated as 3.4 million tonnes (CMFRI, 2015) and contributes significantly in global fisheries being the second largest producer in the world. The proportion of assessed marine fish stocks declined from 90 percent in 1974 to 71.2 percent in 2011; where 28.8 percent of fish stocks were estimated as fished at a biologically unsustainable level and overexploited (FAO, 2016). So there is an urgent need for fishery management which is necessary in the scenario of overexploitation of the aquatic resources causing drastic decline in Indian coastal waters. Fisheries management consists of making accurate measures to obtain an optimum yield for fish populations by understanding the biological principles and characteristics of the species (Allendorf *et al*., 1986). Lack of information on the exact number of interbreeding populations of an exploited species may cause problems in management policies. For the effective fisheries management it is necessary to delineate groups genetically within a species, which are the genetic breeding unit referred as 'stock' (Thorpe *et al*., 2000). The group of fishes exploited in a particular area or by a specific method is known as stock in the aspect of fishery management (Carvalho and Hauser, 1995). The stock concept for harvested species is fundamental to the management of wild fisheries and represents natural management units because a connection between productivity and harvest rates can be established (Ovenden *et al*., 2015). The population structure of a species is important for developing an optimal policy for its efficient management. Many organizations take a categorical species approach to management which fails to identify the variation within a species. Such methods may verify unsuccessful, since in sexual organisms it is the interbreeding population and not the species that is clearly the evolutionary unit of importance

(Coyle, 1998). Knowledge about the population structure of a species is essential if managers are to take advantage of potentially useful ecological adaptation in future management and breeding programmes. There are different thresholds for the genetic and demographic connectivity between the stocks and this is because genetic connectivity is based on the number of migrants between the stocks but the demographic connectivity depends on the contribution to population growth versus local recruitments (Lowe and Allendorff, 2010).

The efficient management of fishery resources can be achieved by employing basic genetic principles combined with molecular genetics monitoring to minimize any harmful genetic change (Allendorf *et al*., 2008). The genetically distinct and isolated populations of a species in a particular geographical locality are referred to as stocks. These stocks should be managed carefully as separate units and the failure will lead to the loss of genetic diversity and finally their depletion (Hutchings, 2000; Begg *et al*., 1998). Technological advances in molecular biology and biochemistry have led to the progress of a variety of genetic markers that can be used to address questions of relevance to the management and conservation of fish species. Genetic markers have been applied particularly in three fisheries areas (stock structure analysis, aquaculture and taxonomy/systematics) (Ward and Grewe, 1994). The detection of genetic variation among individuals is a requirement in all applications of genetic markers.

Fish populations are normally made up of a number of diverse stocks; all are genetically isolated from the others through behavioral or distributional differences, which is known as mixed stock fishery (Ovenden *et al*., 2015). Intense fishing in certain habitats may cause the elimination of distinct, locally adapted stocks, resulting in loss of diversity and the adaptive potential of the species. It is important to adopt stock specific management for conservation and sustainable utilization of genetic resources. Species impacted by fishing should be managed to minimize the loss of genetic diversity. Protection of stock viability is greatly enhanced by the conservation of their gene pools, preserving genetic variability and hence, the possibility of future adaptation (Milligan *et al*., 1994). In the recent

times, capture fisheries is under enormous pressure because of overfishing, climate change, and other impacts. Aquaculture is seen as a substitute, which may have to increase in output to meet the widening gap in global rising demand and decreasing supply for aquatic food products (Muir, 2013).

Silver pomfret (*Pampus argenteus*) (Euphrasen, 1788) belonging to the family Stromateidae is a potential fin fish species for mariculture in Asian countries. *P. argenteus* is widely distributed throughout the Indo-West Pacific: from the Persian Gulf to Indonesia, Japan, West and Southwest of Korea and Eastern parts of China (Zhao *et al*., 2011). *P. argenteus* contributes an important fishery in the states of Tamil Nadu, Karnataka and Goa with the total landing of 30191 tonnes (CMFRI, 2015). Pomfrets are schooling, pelagic, medium sized fishes inhabiting shallow waters. They are highly relished table fish and demand high value in domestic and export market. The species attains a maximum size of about 60 cm (Fischer and Bianchi, 1984). The fishery resource of pomfrets in Indian waters showed a steady decline in catch since 1990s, mainly because of the undersized capture of the fish specimens using trawl nets. Along the Indian coast, silver pomfret showed a rapid decline from 34,072 tonnes (2010–11) to 27,515 tonnes (2011–12), in a short span of one year (CMFRI, 2013). Central Marine Fisheries Research Institute (CMFRI) recommended minimum legal size (300 grams) for export and its being implemented by MPEDA. Despite the importance of this species as a valuable marine resource, little is known about its genetic diversity and population structure.

Silver pomfrets (*Pampus argenteus*) being a commercially important species and as the fishery is in a dwindling stage along Indian coast, a better understanding of fish population is important for its effective fisheries management. Management of fishery resources needs detecting the sustainable levels of exploitation given stock characteristics and environmental conditions. Thus, fisheries conservation requires stock delineation, and mainly this is accomplished through genetic data analysis. Stock dynamics is based on both local adaptation and meta-population dynamics involving dispersal. Genetic markers

particularly nuclear microsatellites and mitochondrial markers can be used to address questions relevance to the stock structure analysis and the demographic patterns (Ward and Grewe, 1994). The above molecular markers used throughout this study have been chosen with basis on previous works of population genetics in marine species (Musammilu *et al*., 2014).

In the current scenario, to enhance productivity, it is highly essential to standardize the breeding and hatchery technology of pomfrets. The culture technology for silver pomfret was initially developed during 1998 by the Mariculture and Fisheries Department (MFD) of Kuwait Institute for Scientific Research (KISR) and succeeded in larval rearing of this species with the eggs collected from the wild (Almatar *et al*., 2000). In Asia, some other countries are also showing interest in developing the culture technology for this species because of the depletion of wild stock and high export demand (James and Almatar, 2008). For the successful development of the hatchery production, a prior knowledge of the genetic stock structure of the species is essential. Developing the baseline information on genetic stock structure of this species will be helpful in effective formulation of conservation management strategies as well as developing brood stocks with superior traits for mariculture programmes. Hence, there is an urgent need to study the genetic diversity and spatial population structure of silver pomfret across its range of distribution.

For genetic stock structure analysis of fishes, many researchers all over the world developed different methods to differentiate and characterize the fish stocks and assess the genetic variation. Stocks have generally been identified through continuous observations that disclose a interruption in some aspects of their life history such as large intra-annual differences in recruitment, timing of spawning activities, or differences in growth rates; or by differences in morphology (Waldman, 1999). Previously conventional morphometric measurements and truss network analysis were used for stock identification in fishes. But these methods have been graded as inefficient and biased. Nowadays genetic markers are used for the identification and quantification of the level of population structure within a

species (Abdul Muneer, 2014). These markers may involve assessment of variation directly at DNA level or through phenotypic expression that can be protein or morphological variants. The genetic markers detect intra-specific differences which reveal stock composition and genetic relatedness within a species. An important benefit of the genetic approach is that it measures long-term average levels of population connectivity. Depending upon the question to be answered, suitable markers need to be identified for the respective species (Ovenden *et al*., 2015).

Allozyme electrophoresis was one of the earliest techniques used for studying the genetic variation at codominant Mendelian inherited loci. Allozyme electrophoresis is used for the identification of genetic variations in enzyme levels (Okumus and Çiftci, 2003). RFLP (Restriction Fragment Length Polymorphism) analysis is another method for population studies in many fish species. In RFLP analysis, the DNA sample is digesting by using a specific restriction endonuclease and the resulting restriction fragments are separated by gel electrophoresis (Shaw *et al*., 1999). High genetic drift and low gene flow, requirement of very small tissues and detection of high levels of genetic variations are some of the advantages of RFLP (Hansen *et al*., 2000). The discovery of PCR (Polymerase Chain Reaction) enabled the population genetic analysis easier. Randomly Amplified Polymorphic DNA (RAPD) is one of the frequently used methods in fisheries studies. In this method, distinct regions of the genome are amplified using arbitrary primer sequences (usually deca-primers) (Ferguson *et al*., 1995). Amplified fragment length polymorphism (AFLP) dominant, PCR based technique. It is the combination of RFLP and RAPD markers. This method has a major advantage that a large number of polymorphisms can be identified by using a single poly acrylamide gel. It is inexpensive and requires very short time.

The mitochondrial DNA (mt DNA) based population studies were emerged in 1970s (Avise *et al*., 1979). Mitochondrial variations are more sensitive compared to nuclear DNA because of its smaller size. mt DNA is smaller, double-stranded, made up of nearly 16000 nucleotides and is inherited maternally. mt DNA is physically detached from the rest of the cell's DNA and it is comparatively

easy to isolate. Any tissue or blood can be used for isolation of mt DNA. Fast evolving regions like Control region, ATPase 6/8 genes etc can be used for assessing population structure and levels of connectivity in fish species (Ovenden and Street, 2003; Kathirvelpandian *et al*., 2014). ATPase gene of mtDNA is comparatively fast evolving (1.3% per million years) and can be used for stock structure analysis in fish species (Ovenden and Street, 2003).

Recently, many studies are based upon the genetic variation in the number of repetitive elements at a particular locus. These sequences are classified based on the sizes into satellites, minisatellites, and microsatellites. Microsatellites are short tandemly arrayed di, tri, or tetra nucleotide repeat sequences with repeat size of 1–6 bp repeated several times flanked by regions of non-repetitive unique DNA sequences (Tautz, 1989). These neutral markers are also known as SSR (simple sequence repeats). Microsatellites exhibit higher levels of polymorphism and abundance in genomic DNA compared to other markers such as allozymes and proteins (Schlotterer, 2000). The most important advantages of microsatellite markers are codominant transmission, hypervariability, high polymorphism, easy sample preparation and high mutation rate (Abdul Muneer, 2014). One of the factors restricting the broader use of microsatellites is the fact that the early identification of the marker is costly and labour-consuming, and requires cloning and sequencing. To overcome these disadvantages, researchers often adapt information about microsatellite markers originally developed for one species (resource) for use in other closely related species (the target). This strategy is called cross-species microsatellite amplification and was widely applied, frequently with success (Moore *et al*., 1991).

mt DNA sequencing has been widely applied on phylogenetic, phylogeography, population structure and evolutionary studies on diverse species including teleost fish, hence this technique was chosen for this work. A small effective population size owing to maternal inheritance and the haploid nature give the idea that the population divergence will be faster for mt DNA sequence compared to nuclear gene and mt DNA markers are more sensitive to genetic drift.

The mt DNA analysis provided information about the historical fluctuations in population demography (Hurwood *et al*., 2005). Fast evolving mt DNA has been used to determine if localized populations are genetically differentiated from each other and this method is widely employed in aquaculture production (Liu and Cordes, 2004). Mitochondrial DNA is only a small part of the whole genome but showing hypervariability and can be used for population studies (Fuchs *et al*., 2008). ATPase 6/8 gene has been reported to be useful for detecting intraspecific variation in several species across orders like Atheriniformes (McGlashan and Hughes, 2000) Characiformes (Sivasundar *et al*.,2001), Clupeiformes (Hughes and Hillyer, 2006), Tetrodontiformes (Reza *et al*., 2008), Orectolobiformes (Corrigan and Beheregaray, 2009) Salmoniformes (Hughes and Hillyer, 2006), Siluriformes (Betancur *et al*., 2007), Cypriniformes (Nguyen *et al*., 2006) and Perciformes (Chow and Ushiama, 1995; Kathirvelpandian *et al*., 2014; Vineesh *et al*., 2016; Linu *et al*., 2016).

However, recent studies using microsatellite loci for population analysis have started to reveal genetic structuring in the species which previously thought to be homogeneous over geographic ranges using mtDNA marker. The combination of mitochondrial and nuclear genes have established for understanding the genetic structure of populations as they differ in nature, variability and sensitivity. Microsatellites evolve faster than mt DNA and their higher diversities allow inferring present connectivity patterns than mt DNA, which carries a longer persisting signature of post events (Seloe and Toonen, 2006). Even if the uniparental inheritance of mtDNA tends to highlight genetic differences among population compared to nuclear genes, it does not confine the whole genetic history which is fundamental in the case of defining population structure as required for fisheries management. Codominant inheritance and fast and easy assay approach of microsatellites makes it as good molecular marker for population studies (Weber and May, 1989). These markers are widely used in stock structure analysis of number of fish species because of their very high rate of polymorphism (Muneer *et al*., 2009; Kathirvelpandian *et al*., 2014; Musammilu *et al*., 2014).

The target species *P. argenteus* is commercially important aquaculture species in several countries and, as the hatchery techniques are being standardized, globally for restocking programs for revival and sustainable utilization of its fishery. This research work is mainly meant for identifying the polymorphic molecular markers which can be used in population genetic analysis of *P. argenteus* and for revealing the genetic stock structure of *P. argenteus* along Indian coast using molecular markers. The population structure developed in this study can be used in conservation and management measure for the fishery of this particular species. The data generated from the study will also be useful to the planners and fishery resource managers for stock specific ranching programmes. Our study will help to generate information on the genetic stock identification of silver pomfrets, providing an example of holistic approach to stock identification and conservation.

1.2 Species description

Pampus argenteus (Figure 1.1), is marine, bentho pelagic migratory fish which can live in a depth range of 5-110 meters.

1.2.1 Taxonomic status

The current taxonomic position of *P. argenteus* (Source: Euphrasen, 1788):

Phylum	:	Chordata
Subphylum	:	Vertebrata
Class	:	Actinopterygii
Order	:	Perciformes
Suborder	:	Stromateoidei
Family	:	Stromateidae
Genus	:	*Pampus*
Species	:	*Pampus argenteus*

1.2.2 Distinguishing taxonomic Characters

Body of silver pomfret is very deep and compressed, caudal peduncle is short and compressed, with no keels or scutes. Head is found to be deep and broad, snout short and blunt; eyes are small, centrally located and surrounded by adipose tissue which extends forward around the large nostrils. The mouth of the species is sub-terminal, small and curved downward, the maxilla scarcely reaching to below eye and the angle of gape located before eye; premaxilla is not protractile; maxilla is immobile and covered with skin and united to cheek; teeth are minute, uniserial and flattened, with very small cusps. The gill covers are seen as very thin, gill membranes broadly united to the isthmus in all Indian Ocean species, gill opening is a straight slit. Single dorsal and anal fins preceded by none or 5 to 10 flat, blade-like spines, pointed on both ends and resembling the ends of free interneurals; pectoral fins are long and pelvic fins are absent; caudal fin is usually forked. Scales are small, cycloid (smooth) and very easily shed; head is naked, with prominent canals visible under the thin skin. Color is gray above grading to silvery white towards the belly, with small black dots all over the body. Fins of silver pomfrets are faintly yellow and the vertical fins are with dark edges.

1.2.3 Common names

White pomfret (English), Aavoli (Malayalam), Paplet Saranga (Marati), Vichuda (Gujarati), Gheya (Orissa) and Vavval (Tamil).

Figure 1.1 ***Pampus argenteus*** **(Euphrasen, 1788)**

1.3 Objectives of the study

In the present study, investigation of genetic stock structure and dispersal patterns of the candidate species *P. argenteus* distributed along the Indian coast will be performed with the following objectives.

1. Stock identification of *P. argenteus* in Indian waters using mitochondrial ATPase 6/8 genes.
2. To identify potential polymorphic microsatellite loci to be used for stock analysis of *P. argenteus* through cross species amplification and from resource species.
3. To study genetic stock structure of *P. argenteus* along the Indian coasts using polymorphic microsatellites.

Chapter II

REVIEW OF LITERATURE

2.1 The concept of stock

The organisms are continually undergoing evolutionary processes and showing variations from the morphological level to DNA sequence level, causing variations in the organisms. No two organisms (except identical twins or other multiple identical births) can be likely to have the similar genotype for all the genes (Hartl and Clark, 1997). As phenotypic characters of an organism are determined by genes, so the changes that occurred in genes generate individuals, which are unlike either at the molecular level or at the organismal level. These individuals may form separate groups within the species and these intraspecific groups were referred to as 'stocks' and the management of commercially important marine organisms is based upon the concept of stock. The stock concept is having two fundamental principles: that fishes are divided into local populations, and there will be genetic differences between local populations which are adaptive (MacLean and Evans, 1981).

Stock concept in fisheries has been defined in many ways, but the most commonly used are genetic and operational definitions. According to the genetic definition, a stock is a reproductively isolated unit that is genetically dissimilar from other stocks (Carvalho and Hauser, 1995). This definition is having high degree of reliability because only a small number of migrants are adequate to avoid the progress of genetic differentiation between monospecific groups. The operational definition was suggested by Begg and Waldman, 1999 and specified that a stock is a semi distinctive group of fishes with clear cut features (such as growth, mortality, reproduction, different response to fishery exploitation) of concern to the organizers. Yet, this does not essentially reveal the true sub structuring of the species. To overcome this problem, various biological stock definitions have been put forward. A good definition has been put forward by Ihssen *et al*., 1981: a stock is an intraspecific group of randomly mating individuals

with temporal and spatial integrity. Later, Shaklee *et al*., 1990 defined a stock as "a panmictic population of related individuals within a single species that is genetically distinct from other such populations". The postulation on stock is that within a stock there will be genetic homogeneity and between stocks genetic divergence may occur. The lack of knowledge on the total number of inbreeding populations of an exploited species will not help in the management strategies to attain long lasting conservation goals. So the information on stock structure and the basic genetic profile of a particular species is required for the effective management and conservation.

2.2 Population Genetics

A population is a group of freely interbreeding individuals in a particular habitat at a specific time (Slatkin, 1993). Population genetics is mainly aimed at the genetic composition of populations and to record the forces that change their genetic composition and thus helps to understand the process of evolution. Population genetics has contributed a large extent to our knowledge that how fish species are genetically structured into reproductively isolated populations throughout their distribution and this knowledge is having prime importance in fisheries management. Many fish populations have been declined by changes in the local environments and these populations are in danger of extinction and some native populations are already extinct (Reynolds *et al*., 2005). The identification of local adaptations in natural populations has recently been highlighted as valuable of special attention (Mortiz *et al*., 2002). Population genetics offers new promises for conservation in many ways. Firstly, for most species the number of neutral genetic markers will be available and this will help to understand about the effects of demographic processes, effective population size, identification of wild and farmed individuals etc. (Morin *et al*., 2004). Another important application the detection of the genetic basis of local adaptation and this idea will greatly help to manage the genetic diversity in natural populations.

The lack of information about the genetic structure of the populations may result in the differential harvest of the populations that will eventually have a

severe and long-term effect. To avoid this, there is always a need for examination surrounding the genetic variations at the intra and inter-population levels as well as at the intra and inter- specific levels of the fish species (Allendorf and Utter, 1979). For the cautious exploitation and management of fishery, the assessment of the genetic make-up and variability of fish stocks is very important. This can be achieved by characterizing the genetic structure of the populations being harvested. The genetic diversity data generated has various applications in research on evolution, conservation and genetic improvement programmes.

The genetic variation within and between populations can be measured by gene frequencies and the factors such as migration, mutation, selection and genetic drift. Mutations are defined as the changes that happen within the DNA and that can fabricate new allelic forms and contribute to population demarcation. The reason may be a change at the single nucleotide level (transitions, transversions) or they can involve deletion, or the addition of one or more nucleotides or processes such as transposition, unequal crossing-over, slippage, gene conversion and duplication. Mutations may be silent or non-synonymous depending upon the amino acid. If the allelic frequencies of the populations are changing by chance, that process is called genetic drift. This can cause reproductively isolated populations to randomly deviate based on genotype frequencies. The consequence of genetic drift on populations depends on their effective population size, the most important component of genetic drift, and its variation in time. In small populations genetic drift can cause strong population divergence and severe changes in genotype frequencies from generation to generation.

Genetically, effective migration (gene flow) happens when individuals move about from a population to another and introduce new alleles changing allelic frequencies within the recipient population. The process of migration is acting as a stabilizer of genetic deviation since it increases effective population sizes and reduces genetic drift (Slatkin, 1985). If there is no gene flow between subpopulations the genetic differentiation evolves timely (Chakraborty and Leimar, 1987). Gene flow rates of 10% or less may defend treatment as separate stocks.

That means constraint on gene flow may guide to genetic subdivision. But marine fishes usually showing lower levels of population differentiation compared to fresh water species because of the fewer barriers to migration and gene flow. Selection is the process in which individuals with required phenotypes for some specific traits are recognized and are utilized as future brood stocks to produce progenies that are also superior to that characteristic (Allendorf and Leary, 1986).

There are three basic models of population structure, which will result in the genetic differentiation. Firstly the whole population may consist of single unit (free exchange) known as the "panmictic model". Second model is "island model" or "stepping stone" which contains a series of small subpopulations isolated from other subpopulations. Third one is the "isolation by distance model" which contains individuals exchanging genes only with others in the geographically closer areas (May and Kruger, 1990; Baverstock and Mortiz, 1996). So before understanding the population structuring of a specific species we should consider which of these models best explains the population structure. The basic assumption for population genetic analysis is the Hardy-Weinberg principle and this law states that "in a large random mating population with no selection, mutation or migration, the allele frequencies and the genotypic frequencies are constant from generation to generation (Hardy, 1908; Weinberg, 1908).

2.3 Genetic markers in conservation and Fisheries management

Measurement of genetic diversity in wild fish populations or aquaculture stocks is necessary for interpretation and proper conservation of these populations or stocks. Genetic diversity can be assessed by classical systematic analysis of phenotypic traits. Ecological parameters, tagging, parasite distribution, physiological and behavioural traits, morphometrics and meristics, calcified structures, cytogenetic, immunogenetics and blood pigments can be used to analyze stock structure in fish populations (Ihssen *et al*., 1981).

Multivariate morphometric investigations are based on different types of body measurements and are used to examine the discreteness and interdependence

of stocks within a species (Turan, 1999). These morphometric methods are criticized because there are several biases, unevenness and weaknesses inherent in conventional characters. As an alternative, a new system of morphometric measurements called the Truss Network System had been used for stock identification. Truss network measurements are a sequence of quantification calculated between landmarks that form a regular pattern of connected quadrilaterals or cells across the body form. Sen *et al*., (2011) analyzed the stock structure of *Decapterus russelli* from east and west coast of India using truss morphometry. Sajina *et al*., (2011) reported the stock structure analysis of *Megalaspis* along the Indian coast based on truss network analysis. Jayasankar *et al*., (2004) was adopted a comprehensive approach to analyze possible population differences in Indian mackerel (*Rastrelliger kanagurta*) from selected centers in the East and West coasts of India using truss analysis combined with genotypic methods. Gopikrishna *et al*., (2006) carried out truss network analysis using data of the Asian seabass (*Lates calcarifer*) sampled from five different locations along the Indian coast for examining the stock differences. But in fisheries science and management these methods have found only narrow applications and these methods can't be used for wild populations (Hallerman, 2003). The concentration of applied genetics on fisheries is a far more extensive field of genetics which involves the study of structure, function and dynamics of genes and can give greater resolution. The development of molecular markers has led to the progress of genetic analysis of populations in the last two decades.

Generally, molecular markers are used in fisheries for detecting whether the samples from culture facilities or natural populations are genetically distinct or not. The basic objective of using molecular markers is to find out the dissimilarities among the recognized stocks in either nuclear alleleic types or mt DNA haplotypes (Danzmann and Ihssen, 1995). Basically two types of genetic markers are categorized- protein and DNA (molecular). The protein markers are fast and inexpensive but the presence of null alleles, silent substitutions and synonymous substitutions are some of the disadvantages (Tanya and Kumar, 2010).

A molecular (DNA) marker is a DNA sequence used to "mark" or track a particular location (locus) on a particular chromosome, i.e. marker gene. It is possible to observe and exploit genetic variation in the entire genome. Molecular markers are of two types; mitochondrial DNA and nuclear markers. Nuclear DNA markers such as Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Variable Number of Tandem Repeats loci (VNTRs: minisatellites, microsatellites) and Single Nucleotide Polymorphisms (SNPs) are biparently inherited. Mitochondrial DNA markers are maternally inherited; display high rates of mutation and are non-recombining such that, they have one quarter the genetic effective population size (Ne) of nuclear markers (Ferguson and Danzmann, 1998).

Amplified fragment length polymorphism (AFLP) is a technique suitable for finger printing simple and complex genomes from different species (Vos *et al*., 1995; Felip *et al*., 2000). In AFLP, genomic DNA is digested by restriction endonucleases and amplified by PCR using primers that contain common sequences of the adapters and one to three random nucleotides as selective sequences (Lin and Kuo, 1995). From l990's, many studies have been published making use of random parts of a genome. This approach involves PCR amplification of unknown DNA fragments commonly referred as Random Amplified Polymorphic DNA (RAPD) (Williams *et al*., 1993) to amplify stretches of DNA identified by random primers. A single 10 bp primer and low annealing temperature are combined to produce specific amplification patterns from individual genomes. Priming sites are randomly distributed throughout a genome and polymorphisms in such sites result in differing amplification products, detected by the presence and absence of fragments. These polymorphisms are usually inherited in a Mendelian fashion and can be used as genetic markers (Appleyard and Mather, 2002). RAPD fingerprinting has been used in many studies for the analysis of phylogenetic and genetic relationship among organisms (Manimekalai and Nagarajan, 2006; Muneer *et al*., 2009; Gopalakrishnan *et al*., 2009; Divya *et al*., 2010; Muneer *et al*., 2011; Bhat *et al*., 2012). More powerful markers like

mtDNA and microsatellite markers can be used to assess the population structure of fish species.

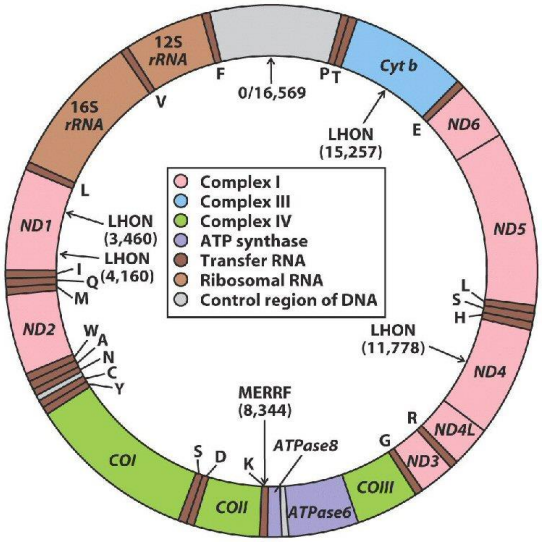
2.3.1 Mitochondrial DNA markers

Mitochondrial DNA (mtDNA) analysis is widely used in population demographic studies and phylogenetic surveys of organisms. From the studies of vertebrate species, it is clear that sequence divergence accumulates more rapidly in mitochondrial than in nuclear DNA (Brown, 1985). The mt DNA is maternally inherited with no recombination, representative of 25% of the effective population size of nuclear markers, which makes it more sensitive to detect reductions in genetic variation (Ferguson and Danzmann, 1998). The mtDNA molecule exists in a high copy number in the mitochondria of cells and has a circular structure. The vertebrate mitochondrial genome is made up of about 16 to 20 kb in different organisms, coding for 37 genes responsible for 2 ribosomal RNAs, 22 transfer RNAs, and 13 proteins essential in respiration (Ferris and Berg, 1987; Hartl and Clark, 1997). It also has a non-coding region (+ 1000bp) accountable for replication, known as the "control region" or "d-loop", that evolves 4 - 5 times faster than the entire mtDNA molecule which itself evolves 5 to 10 times faster than nuclear DNA (Brown *et al*., 1979), mainly due to the reason that mitochondria do not have repair enzymes for errors in the replication and for the damages of the DNA (Clayton, 1982). One consequence of maternal inheritance is that the effective population size for mtDNA is smaller than that of nuclear DNA, so that mtDNA variation is a more sensitive indicator of population phenomena such as bottlenecks and hybridizations. Sex-specific differences in gene flow could also be revealed by contrasting nuclear with mitochondrial DNA. Due to its non-Mendelian mode of inheritance, the mitochondrial DNA molecule is considered a single locus in genetic investigations (Avise, 2012).

The technical advantages like requirement of only minute amounts of fresh, frozen or alcohol-preserved tissue make mtDNA a very practical genetic tool and mtDNA sequencing has become the molecular marker of choice when studying cospecific populations (Gold *et al.*, 1993). There are some disadvantages that must

not be forgotten. For example, maternal mode of inheritance does not give information about males in populations, which may result in different dispersal behaviour to females. No inferences about the neutrality and equilibrium of populations, as well as other aspects based on allelic frequencies can be addressed due to the haploid nature of this marker (Hansen, 2003).

Figure 2.1 Schematic representation of Vertebrate mitochondrial genome



Analyses of mtDNA markers have been used extensively to investigate stock structure in a variety of vertebrates including fishes (Avise and Ellis, 1986; Gold *et al*., 1993; Chow and Inoue, 1993), birds (Merilä *et al*., 1997; Zink *et al*., 2000) and reptiles (Avise *et al*., 1998; Podnar *et al*., 2005). The mitochondrial DNA control region, which includes the D-loop in vertebrates, also known as AT rich region in invertebrates, does not code for a functional gene and thus it is under fewer structural and functional constraints, owing to a high average substitution rate (Saccone *et al*., 1987). It is generally the fastest evolving region in the mitochondrial DNA of vertebrates and invertebrates and therefore more sensitive than protein loci as a marker to score intraspecific variations of many organisms (Avise, 2000). Partial sequences of mitochondrial DNA genes especially 16S rRNA and Cytochrome C Oxidase I has proved suitable than other gene sequences to resolve the phylogenetic relationships within the family in several group of eukaryotes including fishes owing to a large number of informative sites (Barucca

et al., 2004). Conservative protein coding genes like Cytochrome b (Cyt b) tend to display intraspecific variation mainly in 3rd position of codon which can be used to identify stocks.

2.3.1.1 ATPase 6/8 genes in genetic stock structure analysis

ATPase 6 and 8 are the two genes with overlapping sequences in between, codes for ATPase enzyme that provides energy for the cell to use through the synthesis of adenosine triphosphate (ATP). ATPase gene of mtDNA is comparatively faster evolving (1.3% per million years) and is extremely useful in assessing population structure, levels of connectivity and influence of historical processes in fish species (McGlashan and Hughes, 2000; Ovenden and Street, 2003). These genes of mtDNA are generally variable in vertebrates (Zardoya and Meyer, 1996). A study by Yan *et al*., 2009 recommended that ATPase 6/8 gene is a valuable genetic marker to track genealogies and variations in progenies of hybrids. Besides, it has also been used in studying genetic analysis based population structure investigation. ATPase 8 and ATPase 6 genes have been potentially used for analyzing both phylogeny as well as phylogeography and stock characterisation of several fish species, (Chow and Ushiama, 1995; Dammannagoda *et al*., 2008; Vergara *et al*., 2009; Vineesh *et al*., 2016 and Linu *et al*., 2016).

A number of attempts have been made to study the population structure using ATPase 6/8 gene in fishes. Mc Glashan and Hughes (2000) reported significant levels of genetic subdivision among populations from 8 sites of the Australian freshwater fish, *Craterocephalus stercusmuscarum* using seven polymorphic allozyme loci and sequence information on the ATPase gene of mitochondrial DNA. The phylogenetic relationships and evolutionary patterns in the cyprinid genus *Barbus* was studied using the complete sequences of mitochondrial ATPase and cytochrome *b* genes (Machordom and Doadrio, 2001). In mangrove jack, *Lutjanus argentimaculatus*, mtDNA control region and ATPase 6/8 genes were utilized to study the stock structure analysis along the Queensland coast and the north-western coast of Western Australia (Ovenden and Street, 2003). Hughes and Hillyer (2006) used allozymes and ATPase gene of mtDNA to study

the genetic differentiation between drainages of two fish species, *Nematolosa erebi* and *Retropinna semoni* from inland rivers in Queensland, Australia. Habib *et al*., (2012) evaluated the potential of complete ATPase 6/8 gene to determine the phylogeography of *Channa marulius* from unlinked river basins; Mahanadi, Teesta and Yamuna. Kathirvelpandian *et al*., (2014) revealed the genetic divergence in *C. dussumieri* populations of northeast and northwest coasts of India. Luharia *et al*., (2014) studied the genealogy and phylogeography of Cyprinid fish *Labeo rohita* from Indian rivers using this gene.

2.3.2 Microsatellites

Microsatellites have emerged as one of the most accepted choices for stock structure studies as they have the capacity to supply the contemporary estimates of migration and can assess the relativity of individuals (Selkoe and Toonen, 2006). Microsatellites are short tandemly arrayed repeats of 1–6 nucleotides and occurred at high frequency in the nuclear genomes of most of the taxa. These repeat regions are flanked by regions of nonrepititive unique DNA sequences (Tautz, 1989). These neutral markers are also called as simple sequence repeats (SSR), variable number tandem repeats (VNTR) and short tandem repeats (STR). A microsatellite locus usually differs in length between 5 and 40 repeats, but longer strands of repeats are also possible. Dinucleotide, trinucleotide and tetranucleotide repeats are mostly preferred for molecular genetic studies. Microsatellites were first recognized in humans in 1981 by allele sequence analysis at the β globin locus and further found that these are naturally occurring and ubiquitous in prokaryotic and eukaryotic genomes (Toth *et al*., 2000).

Of late Microsatellite markers are widely used for studying genetic variation due to the differences in the number of repeated copies of a segment of DNA. Depending on the size of the repeat unit these be grouped into three subgroups: satellites, minisatellites and microsatellites (Bois, 2003; Ellegren, 2000)

• Microsatellites have a repeat-unit length of 1-6 bp. Replication slippage is the process behind the evolution of microsatellites and the recombination does not have an effect on microsatellite evolution (Ellegren, 2000).

• Minisatellites usually contains GC-rich repeat-unit with a size between 10 and >100 bp and their evolution is due to recombination and conversion-like mutations. These are commonly used in finger printing (Bois, 2003).

• Satellites have the largest repeat units, ranging in size between 100 to >1000 bp. They are mainly found in centromeric regions (Ma *et al*., 2012).

In most of the species, the majority of microsatellites are dinucleotide in nature. In coding regions tri and hexa nucleotide repeats are the most probable repeat classes due to their inability to produce frameshift mutations (Toth *et al*., 2000). The amplification of microsatellites with PCR (Polymerase chain reaction) is achieved by the binding of short stretches of DNA (oligonucleotides or primers) to the flanking region. Microsatellite repeat sequences mutate recurrently by slippage and proofreading errors during DNA replication which will alter the number of repeats and thus the length of the repeat string (Eisen, 1999). Many microsatellites are having very high mutation rates (between 10^2 and 10^6 mutations per locus per generation) that can produce the high levels of allelic diversity essential for genetic studies of processes acting on ecological time scales (Schlotterer, 2000). The alleles of a particular microsatellite locus can be amplified by PCR from small samples of genomic DNA and these alleles can be separated by using polyacrylamide gel electrophoresis (PAGE) as one or two bands and this information can be used for measuring the genetic variation within and between the populations of a species (O'Connell *et al*., 1997).

The utility of microsatellites in population studies is mainly due to their codominant expression (Muneer *et al*., 2009). These markers are also inherited in Mendelian fashion (O'Connell and Wright, 1997) like allozymes and are more sensitive as compared to allozymes for examining the population dynamics including demographic bottlenecks (Ramstad *et al*., 2004), population size

fluctuations and effective population sizes (Gold *et al*., 2001; Waples, 2002). The genetic diversity are generally measured by using parameters such as heterozygosity (the proportion of heterozygous individuals in the population), allelic diversity (number of alleles at a locus in the population), and the proportion of polymorphic loci (Pujolar *et al*., 2005). A noticeable decline in the observed heterozygosity and reduced number of observed alleles of tested SSRs might be responsible to the action of population genetic bottleneck. They are strong tool for analyzing recent and contemporary events because their evolution is faster than single-copy nuclear DNA (Ellegren, 2000).

Table 2.1 Classification of microsatellite markers (reproduced from Kalia *et al*., 2011)

TYPES	**EXAMPLE**
a) Based on the number of nucleotides per repeat	
Mononucleotide	(A)n
Dinucleotide	(CA)n
Trinucleotide	(AGA)n
Tetranucleotide	(ATGT)n
Pentanucleotide	(CGCGA)n
Hexanucleotide	(ATTGTT)n
b) Based on the arrangement of nucleotides in the repeat motifs	
Perfect or simple perfect	(CA)n
Simple imperfect	(AAC)n ACT (AAC)n+1
Simple compound	(CA)n (GA)n
Compound imperfect	(CCA)n TT (CGA)n+1
c) Based on location of SSRs in genome	
Nuclear	nuSSR
Chloroplastic	cpSSR
Mitochondrial	mtSSR

The major advantages of microsatellites include codominant transmission (ie, the heterozygotes can be distinguished from homozygotes), high polymorphism and variability, specificity, relative abundance with uniform genome coverage, higher mutation rate than standard and easy preparation of samples (Abdul Muneer, 2014). Small product sizes are generally compatible with degraded DNA and PCR enables recovery of information from small amounts of material. SSRs remain the markers of choice for the construction of linkage maps. Tetra nucleotide microsatellites are widely used for paternity and forensic examinations in humans. These beneficial characters of microsatellites have led to modern developments such as digital storage and automated detection and scoring systems like automated DNA sequences and fluorescent-imaging devices (O'Connell and Wright, 1997). These markers are the choice for population studies because of these advantages (Estoup *et al*., 1993). More variable microsatellite loci can be used to genome mapping and pedigree analysis and less polymorphic alleles can solve the taxonomic uncertainty in different taxonomic groups (Carvalho and Hauser, 1995). Due to the highly polymorphic nature these can be also used as genetic tags in aquaculture and fisheries biology (Muneer *et al*., 2009). They may verify predominantly valuable for stock identification and population genetics due to the high level of polymorphism compared with conventional allozyme markers (Muneer *et al*., 2012). They can also be employed in differentiating geographically isolated populations, sibling species and subspecies (Zardoya and Meyer, 1996).

The disadvantages of microsatellites include the appearance of shadow or stutter bands, occurrence of null alleles (existing alleles that are not visible using standard procedures); homoplasy; and many alleles at some loci that would demand very high sample size for analysis (Mohindra *et al*., 2001). Prior genetic information is needed for studies of microsatellites. In addition to this, microsatellite flanking regions (MFRs) sometimes show length mutations which eventually lead to the production of identical length variants that could compromise microsatellite population level links (and comparisons of levels of variation across species for homologous loci) and phylogenetic inferences as these length variants

in the flanking regions can potentially minimize allele length variation in the repeat region (Zardoya and Meyer, 1996).

There are numerous reports suggesting the usefulness of microsatellites in genetic variability and stock structure studies in marine and fresh water fishes (Estoup *et al*., 1993; Rico *et al*., 1996; Garcia de Leon *et al*., 1997; Appleyard *et al.,* 2002; Han *et al.,* 2004; Ball *et al.,* 2000; Kirankumar *et al*., 2002). Takagi *et al*., (1999) identified four microsatellite loci in tuna species of genus *Thunnus* and studied the genetic polymorphism at these loci in Northern Pacific populations. Gopalakrishnan *et al*., (2004) assessed population structure of red tailed barb, *Gonoproktopterus curmuca* using microsatellite markers in Indian waters. O'reilly *et al*., (2004) examined the effect of microsatellite polymorphism on the magnitude of genetic differentiation in walleye pollock, *Theragra chalcogramma* using 14 microsatellite loci among six putative populations. Sekino and Hara (2001) evaluated the utility of microsatellite markers for studying the stock structure of Japanese Flounder, *Paralichthys olivaceus*. Muneer *et al*., (2009) used microsatellite markers for genetic differentiation studies in *Horobagrus brachysoma* from three geographically isolated river systems in Kerala. Feldheim *et al*., (2001) demonstrated for the first time the efficacy of microsatellites to study population genetic structure and mating systems in the Chondricthyes. Schrey and Heist (2003) reported the weak genetic differentiation of short fin mako shark (*Isurus oxyrinchus*) using microsatellie markers. Multiplex panels of nuclear-encoded microsatellites were developed for three species of marine fishes including red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*) (Renshaw *et al*., 2006). Zeng *et al.,* (2012) developed polymorphic microsatellite primers for *Scomber japonicus*.

2.3.2.1 Cross species amplification

Microsatellite markers are very valuable tools for conservation genetics because of their polymorphism and the ease of genotyping (Balloux and Lugon-Moulin, 2002). But the development of species specific microsatellite primers is expensive and labour consuming because construction of genomic libraries,

screening of clones with microsatellite sequences and designing of microsatellite primers. Fortunately there are many reports which pointed out the flanking region similarities in closely related species so that primers developed for one species can be used to amplify homologous loci in related species that diverged as long as 470 million years ago (Sun and Kirkpatrick, 1996; Zane *et al*., 2002). Nowadays researchers often adapt information about microsatellite markers originally developed for one species (resource) for use in other, usually closely related species (target). The accomplishment rate of cross priming is related to many factors including the evolutionary divergence between the resource and the target species. Low annealing temperature will also increase the chance of successful cross priming and polymorphism (Primmer *et al*., 2005).

Since the microsatellite primers developed through cross priming has some problems including null alleles, amplification of non-orthologous loci, size homoplasy etc (Ellegren *et al*., 1995; Yue *et al*., 2010), we should take care in using the transferable SSR markers. The complication of the mutation process in SSRs as well as size homoplasy may make difficulty the understanding of SSR variations (Park *et al*., 2009). Anyway cross priming can reduce the preliminary work load for developing the PCR primers. Zardoya and Meyer, (1996) reported that microsatellite flanking region (MFR) contain the consistent phylogenic information in his study within the Family Cichlidae and other families of the suborder Labroidei.

Many efforts have been made to study the cross species amplification of microsatellite loci in fishes. Scribner *et al*., (1996) isolated 22 cloned microsatellites from salmon genomic libraries and used for cross-species amplification and population genetic applications in salmon species. Banks *et al*., (1999) described the isolation and characterization of 10 new microsatellite loci for the protected species chinook salmon (*Oncorhynchus tschawytscha*) for population discrimination. Neff *et al*., (1999) explained 10 microsatellite loci from bluegill (*Lepomis macrochirus*) and examine their utility within the Centrarchidae. Gopalakrishnan *et al*., (2002) sequenced microsatellite loci in an endemic cyprinid

of the Western Ghats (*Labeo dussumieri*) by cross-species amplification of *catla catla* G1 primer. Wilson *et al*., (2004) tested cross-species amplification of 48 existing aphid loci in species of the related genera. The cross priming of microsatellite in *Puntius denisonii* by using the primers of other cyprinid fishes was done by Lijo John (2009). Successful identification of polymorphic microsatellite markers for *Cirrhinus mrigala* and *Gonoproktopterus curmuca* was achieved through use of primers of other cyprinid fishes (Lal *et al.*, 2004; Gopalakrishnan *et al.,* 2004). Dubut *et al*., (2010) developed 55 novel polymorphic microsatellite loci for the critically endangered *Zingel asper* L. and cross amplified in five other percids. Muths and Bourjea, (2011) reported isolation and characterization of polymorphic microsatellite markers from the bluestriped snapper *Lutjanus kasmira* and successfull amplification in *L. Bengalensis* for population genetics studies. O'Bryhim *et al*., (2013) reported the development and characterization of twenty-two new microsatellite markers for the mountain whitefish, *Prosopium williamsoni* and cross-amplification in the round whitefish, *P. cylindraceum*, using paired-end Illumina shotgun sequencing.

Umino *et al*., (2013) isolated eleven polymorphic microsatellite loci for the endangered *Sillago parvisquamis* and these loci were tested for cross-amplification with *Sillago japonica* and shown a subset of seven polymorphic loci that were successfully amplified. The polymorphic microsatellite loci developed in this study can be utilized for population genetic studies in both species. Seyoum *et al*., (2013) isolated 18 microsatellite loci for the largemouth bass, *Micropterus salmoides* and these markers cross-amplified in seven other micropterid species, which suggests they would have utilized for studies of hybridization among other members of the genus. Chen and Cheng (2013) developed thirty-five novel polymorphic microsatellite markers in *Pseudosciaena polyactis* (Perciformes: Sciaenidae) and successfully cross amplified in closely related species, *Pseudosciaena crocea*. Musammilu *et al*., (2014) attempted a total of 40 microsatellite primers from closely related fish species in endemic red-tailed barb, *Gonoproktopterus curmuca*. Sajeela *et al*., 2015 developed 81 microsatellites in Indian white shrimp, *Fenneropenaeus indicus*, through cross species amplification from related species.

2.3.3 Combination of microsatellites and mitochondrial markers

Usually, a single type of marker has been used for the major phylogeographical studies (Avise, 2000). This approach sometimes causes problems which can lead to erroneous results and conclusions. One way to reduce the impact of such problems is to combine markers with different modes of inheritance and rates of evolution (Hewitt, 2004). The preference of marker used in phylogeographical studies also has important effects for the timescale over which events can realistically be inferred. Hyper variable markers such as microsatellites can be used for measuring the contemporary gene flow and are considered less suitable for phylogeographical inference due to their tendency towards homoplasious mutations (Hewitt, 2004). This is also a problem associated with rapidly evolving parts of the mitochondrion such as the control region, ATPase 6/8 region for which homoplasy can confuse genetic distance estimation and phylogenetic inference over timescales beyond tens of thousands of years (Avise, 2000). Dammannagoda *et al*., (2008) screened variation in yellowfin tuna (*Thunnus albacares*) from the north Indian Ocean around Sri Lanka using both the mitochondrial ATPase 6 and 8 region and three microsatellite loci. Dutton *et al*., (2013) studied the stock structure of leatherback turtles (*Dermochelys coriacea*) in the Atlantic using mtDNA control region and microsatellite markers.

2.4 Current status of research and development in pomfrets

Research on developing the culture technology for the silver pomfret was initiated for the first time during 1998 by the Mariculture and Fisheries Department (MFD) of Kuwait Institute for Scientific Research (KISR) and succeeded in larval rearing of this species with the eggs collected from the wild. Since then several investigations have been carried out at MFD, Kuwait, relevant to hatchery larval rearing feed requirement and growth under tank culture conditions, breeding under domesticated culture conditions and health management of this species. In more recent years, during 2005, East China Sea Fisheries Research Institute and Shanghai Fisheries University in China have initiated research on the culture of silver pomfret and has succeeded in the larval rearing of this species based on the

eggs collected from the wild. Other countries in Asia are also showing interest in developing the culture technology for this species because of its depleting wild stock, market demand and high price. Morgan (1985) analyzed the estimates of growth, mortality, and mean selection length for the stock assessment of *P. argenteus* in Kuwaiti waters. He also estimated the natural mortality (M) using the relationship between M, the growth parameters, and mean environmental temperature**.** Another study in *P. argenteus* was done by Ali and Mahmood (1993) in the north western Arabian Gulf and they employed the standard length (ST) and weight (W) relationship of this species for the detection of stocks. Population dynamics of *Parastromateus niger* (black pomfret) in Kuwaiti waters was assessed using length–frequency analysis by Dadzie *et al*., (2007). Ghosh *et al*., (2009) also reported the stock estimates of the silver pomfret landed by gill netters at Veraval (India) using the morphomeristic characters. An additional study of silver pomfret in the Northern Persian Gulf was done by Narges *et al*., (2011) based on the monthly data of length composition. Evaluations of genetic diversity are necessary in population genetics/conservation genetics of this species. Geneticists have primarily used genetic markers to infer the levels and patterns of genetic diversity in any population. Multiplex genotyping of novel microsatellites from silver pomfret (*P. argenteus)* and cross species amplification in other pomfret species were attempted by Yang and Li (2006). They have isolated eleven polymorphic microsatellites from *P. argenteus* with a view to bring out the distinct stocks in Pomfrets of Kuwait waters. Peng *et al*., (2009) used mitochondrial DNA (mtDNA) control region sequence data for assessing the genetic diversity of silver pomfret samples from China seas. Wei *et al*., (2009) isolated 10 polymorphic microsatellite loci in the dark pomfret (*P. cinereus*) and reported that these primers could be utilized for the population studies of *P. cinereus* and other related species. Golestani *et al*., (2010) identified genetically distinct populations of the silver pomfret in the Persian Gulf and the Sea of Oman using microsatellite markers. Zhao *et al*., (2011) investigated the population genetic structure of silver pomfret samples from the Yellow and East China Seas using amplified fragment length polymorphism (AFLP). Sun *et al*., (2012) examined the stock structure and historical demography of *P. argenteus* in three areas (the South China Sea, the

Arabian Sea and the Bay of Bengal) of the Indo-West Pacific using mitochondrial cytochrome *b* gene sequences. Qin *et al*., (2012) described the development of 13 new polymorphic microsatellite markers for silver pomfret and provided molecular information about this species. Genetic Structure of silver pomfret in Persian Gulf and Oman Sea was assayed using 11 microsatellite loci by Archangi *et al*., (2013). AlMomin *et al*., (2015) used the next generation sequencing facility to identify microsatellites for pomfrets and they developed matching primers in Arabian Gulf populations. They performed studies using 48 short-listed loci across multiple individuals from Iranian and Kuwait waters.

Since, there are no reports of stock structure analysis and microsatellite marker development in *P. argenteus* in Indian waters, the present study was initiated with the aim of revealing the genetic stock structure of the species using mitochondrial (ATPase 6/8 genes) and microsatellite markers which will be very helpful in proper conservation and management of this species.

Chapter III

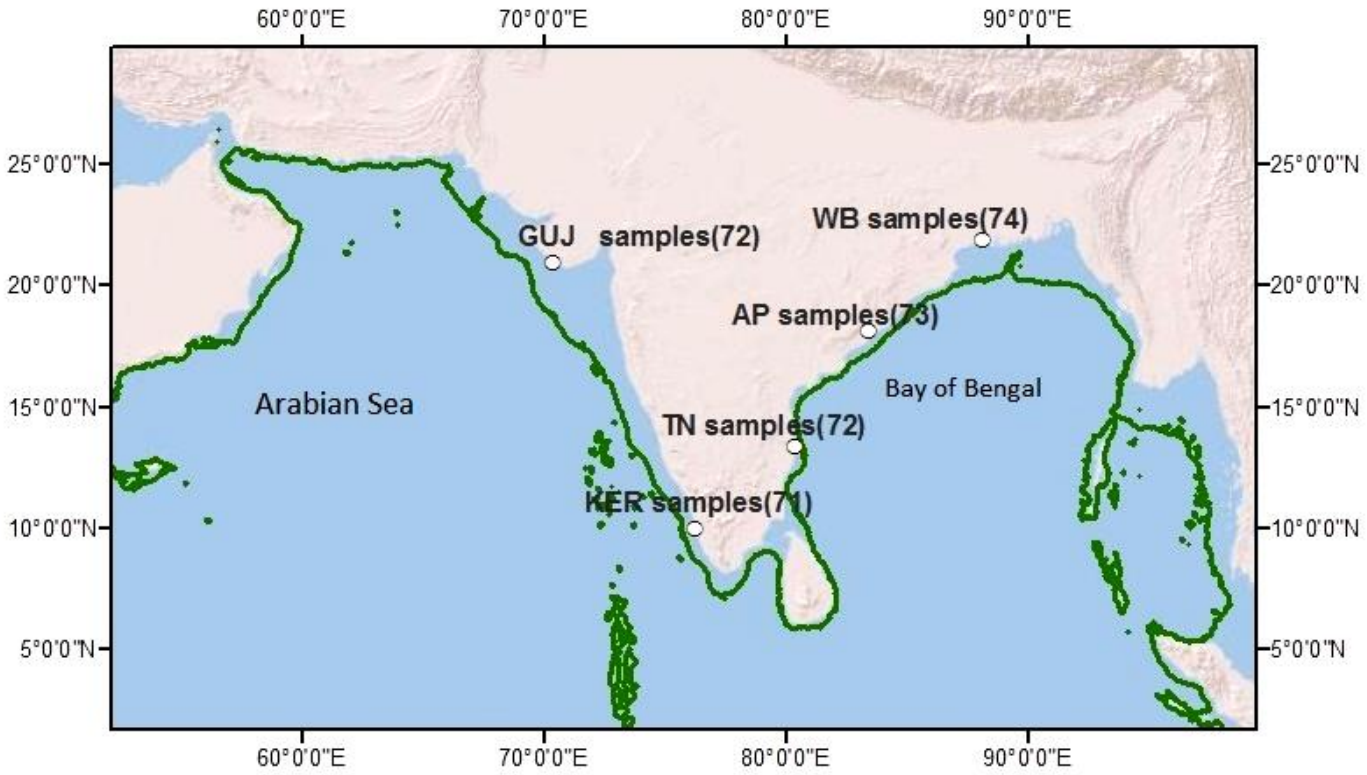
MATERIALS AND METHODS

3.1 Sample Collection

Fresh samples of *Pampus argenteus* (including juveniles and adults) were obtained from commercial trawl landings from Arabian Sea [Veraval, Gujarat in the north and Cochin, Kerala in the south] and from Bay of Bengal [Kakdwip, West Bengal, in the north and Chennai, Tamil Nadu, Vishakhapatnam, Andhra Pradesh in the south]. The mean length and weight of the individuals collected from both the coasts were 17 cm and 340 grams respectively. The details of sampling are given in Table 3.1 and Figure 3.1.

Table 3.1 Sample size and collection locations of *Pampus argenteus*

State	Sampling site	Location code	Geographic Location	Total no. of samples
Gujarat	Veraval	VER	20°53' N 73° 26' E	72
Kerala	Cochin	COC	9°59′05.80″N, 76°10′28.62″E	71
Tamil Nadu	Chennai	CHN	13°22′41.38″N, 80°17′57.27″E	72
West Bengal	Kolkata	KOL	21°53′46.23″N, 88°05′29.65″E	74
Andhra Pradesh	Vishakhapatnam	VP	17°68′N, 83°21′ E	73



200m contour **Figure 3.1 Map showing the sampling sites of *Pampus argenteus***

GUJ Gujarat (Veraval)
KER Kerala (Cochin)
TN Tamil Nadu (Chennai)
AP Andhra Pradesh (Visakhapattanam)
WB West Bengal (Kolkata)

3.2 Genomic DNA Extraction

For DNA extraction, a piece of tissue (fin clips or muscle of approximately 5x5 mm size) was excised and placed in 95% alcohol in screw cap vials. Total DNA was extracted from all the collected specimens following the salting out procedure of Miller *et al*., (1988) with slight modifications. Protocols for the preparation of different reagents are given below.

Reagents required:

Stock solutions:

1. 0.5M Tris Cl (pH-8.0)

Tris base - 3.028 g
Distilled water - 40 ml
Adjust pH to 8.0 using HCl.
Make up the volume to 50 ml, autoclave and store at 4°C.

2. 0.5M EDTA (pH-8.0)

EDTA - 9.31 g
Distilled water - 40 ml
Adjust pH to 8.0 using NaOH.
Make up the volume to 50 ml, autoclaved and stored at 4°C.

3. 10mM Tris Cl (pH-7.5)

Tris base - 0.030 g
Distilled water - 20 ml
Adjust pH to 7.5 using HCl.
Make up the volume to 25 ml, autoclaved and stored at 4°C.

4. RNAase buffer

10mM Tris Cl (pH 7.5)- 10 µl
15mM NaCl - 30 µl
Distilled water - 960 µl
Autoclaved and stored at 4°C.

Working Solutions

1. Solution 1:

Tris-HCl (pH8.0) - 50 mM
EDTA (pH8.0) - 20 mM
SDS - 2 %
Prepared in double distilled water.
Autoclaved and stored at 4°C.

2. Solution 2:

NaCl solution (saturated) - (6 M)
Prepared in double distilled water.
Autoclaved and stored at 4°C.

3. Proteinase K

Proteinase K – 20 mg/ml
Prepared in autoclaved double distilled water and store at -20°C.

4. TE buffer

Tris Cl (pH-8.0) - 10 mM
EDTA (pH-8.0) - 1 mM
Prepared in double distilled water. Autoclaved and stored at 4°C.

5. RNAase

RNAase - 10 mg/ml of RNAase buffer (autoclaved)

Total DNA isolation protocol:

Tissue stored in alcohol was washed with Tris buffer (pH 8.0) by spinning and then followed below listed steps.

- Placed tissue sample in 1.5 ml tube and added 500 µl **Solution 1**.
- Homogenized tissue sample with sterile homogeniser or melted filter tip.
- Added 5µl of **Proteinase K** (20 mg/ml)
- Incubated at 55°C in water bath for 2 hours (with occasional mixing).
- Chilled on ice for 10 minutes.
- Added 250 µl **Solution 2** and inverted several times for through mixing.
- Chilled on ice for 5 minutes.
- Centrifuged at 8000 rpm for 15 minutes.
- Carefully collected clear supernatant (~500 µl) with wide-bore filter tip into a newly labeled 1.5 ml tube.
- Added 1.5 µl RNase (final conc.20 µg/ml) and incubated at 37°C on heating block for 15 minutes.
- Added twice the volume (~1 ml) of ice cold 100 % molecular biology grade Ethanol to precipitate the DNA.
- Incubated overnight at -20°C.
- Next day, centrifuged at 11000 rpm for 15 minutes and removed supernatant.

- Rinsed DNA pellet in 250 µl of ice-cold 70% ethanol.
- Centrifuged at 11000 rpm for 5 minutes.
- Carefully removed supernatant and partially dry with lid off at room temperature.
- Resuspended partially dried DNA in 50-200 µl (depending on the size of pellet) of TE buffer (pH-8) by gently pipetting the sample with wide-bore filter tip until dissolved.
- The DNA samples were stored at -20^0C for further use.

3.3 Agarose gel electrophoresis and visualization of bands

The extracted DNA was checked through 0.7% agarose gel (10 x 4 cm) electrophoresis with Ethidium Bromide incorporated in 1X TBE buffer.

Reagents required:

1. TBE buffer 10X (pH-8.0)

Tris base - 10.8 g
Boric acid - 5.5 g
EDTA - 0.75 g
Made up the solution to 100 ml with double distilled water.
Autoclaved and stored at 4°C

2. Gel loading buffer

Bromophenol blue - 0.5%
Glycerol (mol. grade) - 30%
Prepared in 1X TBE
Stored at 4°C.

3. Agarose solution (0.7%)

Agarose - 0.21 g
10X TBE - 3 ml
Distilled Water - 27 ml

4. 1X TBE buffer

10X TBE - 10 ml
Distilled Water - 90 ml

3. Ethidium bromide solution

Ethidium bromide - 10 mg
Distilled water - 2 ml

Protocol:

According to the manufacturer instructions, we arranged the gel casting unit.

- The Agarose solution was boiled to dissolve agarose and cooled it down to approximately 50°C
- Added 3 µl of Ethidium bromide solution and mixed.
- Poured it in gel casting plate with already adjusted gel comb.
- Allowed it to solidify at room temperature for 30 minutes.
- Removed the comb and placed the gel in electrophoresis unit.
- 1X TBE buffer was poured into the electrophoresis unit as electrolyte.
- Loaded 4 µl of DNA solution and 2 µl of sample loading buffer in each wells.
- Electrophoresis was done at constant voltage (80 V) for 1 hr.
- After electrophoresis the gel was observed in ultraviolet light and documented using the gel documentation system Gel-Doc system (BIO-RAD, Molecular Imager, Gel Doc^{TM} XR).

3.3.1 DNA Quantification using spectrophotometer

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

3.4 Confirmation of sample identity with Mitochondrial COI region

Samples of each location collected for this study were randomly selected to authenticate the morphological identification of *P. argenteus* using mitochondrial COI region.

3.4.1 Gene amplification using PCR

The polymerase chain reaction (PCR) permits the synthetic amplification of a minute amount of DNA in millions of copies in a few hours (Mullis, 1990).

Employing specific universal primers Fish F1 (50-TCA ACC AAC CAC AAA GAC ATT GGC AC-30) and Fish R1 (50-TAG ACT TCT GGG TGG CCA AAG AAT CA-30) (Ward *et al*., 2005), amplifications of partial sequences of mitochondrial DNA region COI was performed in 25 µl reactions containing 1x assay buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) with 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 5 pmoles of each primer, 200 µM of each dNTP (Genei, Bangalore, India), 1.5 U *Taq* DNA polymerase and 20 ng of template DNA. To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. PCR reactions were carried out in Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA) following initial preheating at 95°C for 3 min, denaturation 94°C for 30 s, annealing 50^{o}C for 30 s, extension 72°C for 35 s, repeated for 29 cycles, followed by a final extension for 3 min at 72°C.

3.4.2 Test Run

PCR products (3 µl) along with marker (100 bp DNA ladder; Genei, Bangalore, India) were electrophoresed in 1.5% agarose gel (10 x 4 cm) using 1X TBE buffer for 90 minutes at constant voltage (80 V) and stained with ethidium bromide. The gel was visualized under UV transilluminator and documented using Image Master VDS (Pharmacia Biotech, USA).

3.4.3 Gene sequencing and analysis

The amplified products were sent for sequencing to the sequencing facility. The sequencing was done with the automated DNA sequencer ABI 3730 (Applied Biosystems, USA). The raw DNA sequences were edited using BioEdit sequence alignment editor version 7.0.5.2 (Hall, 1999). Sequences were used to search Genbank for similar sequences using the Basic Local Alignment Search Tool (BLAST) available on the NCBI website (http://www.ncbi.nih.gov/ BLAST/).

3.5 Analysis of mitochondrial ATPase 6/8 gene

3.5.1 Gene amplification of ATPase 6/8 region using PCR

PCR reactions were carried out in thermal cycler (Applied Biosystems) employing specific universal primers for amplifications of partial sequences of mitochondrial ATPase 6/8 genes. A total of 20 samples each from Veraval, Cochin and Chennai, Vishakhapatnam and Kolkata were amplified and sequenced. The ATP synthase 6 and 8 (ATPase 6/8) genes were PCR amplified using primers ATP8.2L8331 (5' – AAA GCR TYR GCC TTT TAA GC - 3') and COIII.2H9236 (5' – GTT AGT GGT CAK GGG CTT GGR TC - 3') (Page *et al*., 2004). The amplifications were performed in 25 µl reactions containing 1x assay buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) with 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 5 pico moles of each primer, 200 µM of each dNTP (Genei, Bangalore, India), 1.5 U *Taq* DNA polymerase and 20 ng of template DNA. To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. The following thermocycler conditions were used: initial preheat at 95°C for 3 minutes, denaturation 94°C for 30 seconds, annealing 55^{o}C for 30 seconds, extension 72°C for 45 seconds, repeated for 30 cycles, followed by a final extension for 5 minutes at 72°C.

3.5.1.1 Test Run

PCR products (3 µl) along with marker (100 bp DNA ladder; Fermentas) were electrophoresed in 1.5% agarose gel (10 x 4 cm) using 1X TBE buffer for 60 minutes at constant voltage (80 V) and stained with ethidium bromide. The gel was visualized under UV transilluminator and documented using software Image Lab 4.0 (Bio Rad, USA).

3.5.1.2 Purification of PCR products

The remaining PCR product was purified using PCR purification kit (Fermentas) according to the directions given by the manufacturer.

3.5.1.3 Gene sequencing

The cleaned up PCR products were used as the template for sequencing PCR. Nucleotide sequencing was carried out by the dideoxy chain-termination method (Sanger *et al*., 1977) using ABI Prism Big Dye Terminator v3.1 Cycle Sequencing kit, (Applied Biosystems, USA). Terminators are dideoxy nucleotides labelled with different coloured fluorescent dyes that will present different emission spectra on an electrophoresis gel illuminated by laser. In most cases, each PCR product was sequenced using both the forward and reverse primer.

3.4.1.3.1 Composition of reaction mix

Components	**Vol. per reaction**
BDT (Big Dye Terminator - kit)	1.0 µl
Buffer (Supplied with Cycle Sequencing kit.)	1.5 µl
DNA (10 - 25 ng/µl)	1.0 µl
Primer (forward or reverse; 10 pmol/µl)	0.5 µl
De ionized water	6.0 µl.
Total volume	10.0 µl

3.5.1.3.2 Cycle sequencing conditions

95^0C for 30 sec, 55^0C for 5 sec and 60^0C for 4 min repeated for 25 cycles and finally stored at 4^o C. A total of 100 sequences were generated in the current study.

3.5.1.3.3 Clean up for Sequencing

The following procedure was used for the cleaning up of fragments.

- 2 µl of 125 mM EDTA was added to each tube (PCR tube containing the PCR product) and mixed.
- 73 µl H_2O was added to this tube (final volume was adjusted to 100 µl)

- It was then transferred to a 1.5 ml tube and 10 µl 3 M sodium acetate (pH - 4.6) was added.
- 250 µl of 100% ethanol was added followed by gentle mixing.
- The tubes were incubated at room temperature for 15 min.
- Tubes were spun at 12,000 rpm, at room temperature, for 20 min.
- The supernatant was decanted and 250 µl of 70% ethanol was added.
- Tubes were again spun for 10 min and the ethanol was decanted.
- The above step (ethanol wash) was repeated again.
- The supernatant was decanted and the pellet was air dried for 25 – 30 min.

3.5.1.3.4 Electrophoresis and visualization of sequences

The cleaned up products were sent for sequencing to the sequencing facility. The dried products were dissolved in 10% formamide and electrophoresed in a poly acrylamide gel and visualized using an AB 3730 XL capillary sequencer (Applied Biosystems). The products were visualized by laser detection of fluorescence emitted by different emission spectra of fluorescent labelled terminators. The raw DNA sequence information was generated as electropherograms read using DNA Sequencing Analysis Software Version 3.3 ABI (Applied Biosystems).

3.5.2 Analysis of data

3.5.2.1 Sequence alignment

The raw DNA sequences were edited using BioEdit sequence alignment editor version 7.0.5.2 (Hall, 1999). Multiple alignments of sequences were performed using CLUSTAL X version 2 (Larkin *et al*., 2007) alignment editor. Alignment was checked manually and corrected. The different haplotypes after their confirmation were submitted in GenBank using a multiplatform submission programme called “Sequin” (ww.ncbi.nlm.nih.gov/Sequin/index.html)

3.5.2.2 Haplotype analysis

The numbers of haplotypes and haplotype frequencies among different populations were calculated using the software DnaSP 5.0 (Librado and Rozas, 2009). A minimum spanning haplotype network showing genetic relationships among the haplotypes was constructed using PopART 1.7 (Jessica Leigh, software available http://popart.otago.ac.nz).

3.5.2.3 Population genetic analysis

The obtained sequence information of all the five locations were grouped into two for further analysis based on the distribution of the species. Group 1 (West coast) included the populations from the distribution sites of *P. argenteus* in the states of Veraval and Cochin; and group 2 (East coast) consisted that of Chennai, Vishakhapatnam and Kolkata. Intrapopulation diversity was analyzed in Arlequin software by estimating gene diversity (*h*)-the probability that two randomly chosen haplotypes are different, and nucleotide diversity (π)–the probability that two randomly chosen homologous nucleotides are different (Tajima, 1983; Nei, 1987).

Estimation of average F_{ST} and determining whether the values are significantly different from zero; and calculation of pair-wise population F_{ST} values (θ) and their significance levels, were carried out using Arlequin software. This programme performs numerical resampling by bootstrapping (1000 times in the present study) and jack-knife procedures in order to estimate confidence intervals and the significance of values. F_{ST} values range from 0 to 1, the greater the value, the greater the differences among populations (Beaumont and Hoare, 2003).

Hierarchical genetic differentiation and the significance of group and population structure were tested using analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) and F-statistics (Wright, 1951) respectively. All individuals collected from the different sampling sites of each state were treated as a single population. This analysis was performed for three hierarchical groupings of the data. The first level compared the variation among individuals within each population. The second level examined genetic structure among populations of

each group. Finally, variation was determined by combining all geographical samples. This analysis provided insight into the proportion of genetic variation attributable to within-population (Φ_{ST}), within-group (Φ_{SC}), and among-group (Φ_{CT}) differences. Pairwise F_{ST}-values were also calculated among the different populations All population analyses were performed using Arlequin version 3.0 (Excoffier *et al*., 2005).

3.5.2.4 Phylogenetic and molecular evolutionary analysis

Phylogenetic and molecular evolutionary analysis was conducted using the software MEGA version 6 (Tamura *et al.,* 2013). Sequence data was subsequently analysed using Neighbour-Joining and Maximum Parsimony methods. The sampling error of Neighbour-Joining and Maximum Parsimony Trees was analyzed using bootstraps of 10,000 replicates where possible followed by the construction of Majority Rule Trees. To test the confidence level of each branch dendrogram, the data were bootstrapped 1000 times. Pairwise sequence divergence among populations was calculated according to Kimura two-parameter model (Kimura, 1980). The number and rate of transitions / transversions were also calculated using the program MEGA version 6 (Tamura *et al.,* 2013). Phylogenetic relationships based on pairwise F_{ST} values generated from mitochondrial DNA data among five populations of *P. argenteus* were made and dendrogram plotted, following boot strap method of unweighted pair group method using arithmetic averages using MEGA version 6 (Tamura *et al.,* 2013).

3.5.2.5 Inference on population demographic history

Molecular genetic data can provide information on the relationship among existent populations, but can also reveal information on recent evolutionary history such as past population size (Avise *et al.,* 1988). Demographic history was investigated by analyzing mismatch distributions of pairwise differences between all individuals of each population using the Arlequin software package version 3.0 (Excoffier *et al*., 2005). This kind of analysis can discriminate whether a population has undergone a rapid population expansion (possibly after a

bottleneck) or has remained stable over time. The mismatch distribution will appear unimodal (like a Poisson curve) if accumulation of new mutations is greater than the loss of variation through genetic drift, and multimodal if the generation of new mutations is offset by random genetic drift (Slatkin and Hudson, 1991; Rogers and Harpending, 1992). Unimodal distributions tend to indicate a population expansion, whereas bimodal or multimodal distributions indicated that the population has been stable over long time. Agreement between the observed distributions and expected distributions under a sudden-expansion model was tested following Schneider and Excoffier (1999). The validation of our results to the demographic expansion model was evaluated according to associated sums of squared deviations (SSD) and Harpending's raggedness index by Arlequin software.

Assuming neutrality, evidence of a population expansion was also tested using Tajima's (1989) D as implemented in Arlequin. Tajima's D statistic in a selective neutrality test decides whether the mean number of differences between pairs DNA sequences is compatible with the observed number of segregating sites in a sample. Significantly negative values of this statistics indicate an excess of new mutations relative to equilibrium expectations on the basis of the number of segregating sites. To evaluate the demographic history, Fu's Fs (Fu, 1997) tests were conducted to determine whether patterns of mitochondrial sequence variation were consistent with predictions of the neutral model.

3.6 Microsatellite analysis

3.6.1 Primers used for microsatellite PCR

Population genetic analysis was carried out using twelve identified polymorphic primers. A total of ten microsatellite primer pairs from *Pampus cinereus* (Wei *et al*., 2009) were used for cross-species amplification of microsatellite loci. Eleven polymorphic microsatellite primers were found in *P. argenteus* (Yang and Li, 2006). List of these 21 primers screened for identifying polymorphic primers are given in Table 3.2.

Table 3.2 List of 21 primers screened for identifying polymorphic primers in *Pampus argenteus*

Sl No	Locus	Sequence 5'- 3'	Reference
1	**Par 01**	F: ATT GTG ACA GGC TGC TCT TCA GA R: TGT CCT GTA TAG CAC CTG GAT GAC	Yang and Li, 2006
2	**Par 02**	F: CTG ACA TGT CGG ATA TGC TTA CA R: CTT GCG GTG AAG ATA CAG ATA CAT	
3	**Par 03**	F: TCC TCC CCC TAA AAT AAA GTA TCT R: TTG TTT TTG TAC ATT GCT TCA CAC	
4	**Par 05**	F: AAA CCT GGG GAA TAT GGG AGA G R: GGC GTA GGG GCG GGT AG	Yang and Li, 2006
5	**Par 06**	F: GTT TGG GAT TTG GGG AGG TAG TG R: AGG CCG TGG AAT CCT GGT GT	
6	**Par 08**	F: TTT TGC AAG TGG GAA CAC AGA CT R: CTG GGG CTA GTT TCA TCT TCA CTT	
7	**Par 12**	F:AGC AGG CGT AGC AAA ACA GAC AA R: TGG CCC ACA GAG TTC AAA GGT C	
8	**Par 15**	F: GAC GCC ATG CAG GAC CAC A R: CTA GAC TGC CGG GGG ACT CAC	
9	**Par 17**	F: TCC ATA AAG GCA GGA AAA TGT CTC R: AAA CGC TCC TCA A AC AGA ACG AC	
10	**Par 18**	F: TTC ACG ACC CCA GTG TTC ACC R:TGA TTT GTG GGG CTT AGA GGA GAG	
11	**Par 20**	F: AAT CCC CTG AGG GTT GAG AAA R: CTG CTG CCA CAC TGA GTC TTA TCT	
12	**P 6-2**	F: CAA CCC TCA TAC ACA CCT R: ATT GAA CCT TCT CAC ATC	Wei *et al*., 2009
13	**P15-2**	F: GCA AGC CTC TAA TTC ACT CC R: CTG CCT CTG TTT CTT CCT G	
14	**P72**	F: ACA CCC TAA ACA TGT CAG CAT C R: CAC AGC AGG AAT CAC TCA AAT A	
15	**P 85**	F: CGC ACA AAT CTC CAC CTA R: ATA CAG AGA CAG GGG AAG CCA A	

16	**P 106**	F:ATT CCA AAA CCG TGG CTA T R:GCA GAC ACC ATC CCA GAC T	
17	**P 119**	F: CCC TCT ATC CTT CAA ACC CT R: TGA CTC TCA CCT CTG CCA TC	
18	**P-189**	F: ATT CAA TAA CAA CTC CAC C R: TGT CTC ACC ACT CTT CAG C	
19	**P 191**	F: TGT CTG AAC CCC CTT TGA T R: CAC TTG TGT TGT GAT TGC C	
20	**P 229**	F: GAT GTC ACA GCC GTT CTT CG R: CGT CTG CTC ACC GTC TCT CC	
21	**P 230**	F: CCG TCC TCT TCC CTG TAA R: GCC AAG CAA GCC TCT AAT	

3.6.2 Amplification using PCR

PCR reactions were carried out in Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA employing the microsatellite primers. Amplifications were performed using standard protocols, in 25µL reaction mixture containing 1X reaction buffer (10mM Tris, 50mM KCl, 0.01% gelatin, pH9.0) with 1.5mM $MgCl_2$ (Genei, Bangalore, India), 10 pmoles of each primer, 200mM dNTPs, 2U Taq DNA polymerase (Fermentas Life Sciences) and 20 ng of template DNA. To check DNA contamination, a reaction set up omitting the DNA from the reaction mixture (negative control). The reaction mixture was pre-heated at 94^0C for 5 minutes followed by 25 cycles (94^0C for 30 seconds, annealing temperature depending upon the Tm value of primer (usually 50^0C - 60^0C) and 72^0C for 10 minutes).

The optimum annealing temperature to get scorable band pattern was calculated through experimental standardization for each primer pair and it was calculated using the following formula, Tm = {2 (A+T) + 4 (G+C)}, where Tm = melting temperature of the primer; A, T, G and C are the number of bases in the primer. The Tm values of both forward and reverse primers were calculated separately and the annealing temperature (Ta) for a primer combination was fixed 3-5^0C below the lowest Tm value obtained for the forward/reverse primer in that

combination. For standardization of annealing temperature, the amplified products were checked in 10% polyacrylamide gel electrophoresis (PAGE).

3.6.3 Polyacrylamide gel electrophoresis (PAGE)

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

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

Reagents required:

1. Acrylamide- Bis acrylamide Solution

Acrylamide (19:1) 30% stock	- 4 ml
Double distilled water	- 6 ml
10 x TBE	- 1.25 ml
10% Ammonium persulphate (freshly prepared)	- 80 µl
TEMED	- 8 µl

2. TBE buffer 10X (pH-8.0)

Tris base	- 10.8 g
Boric acid	- 5.5 g
EDTA	- 0.75 g

Made up the solution to 100 ml with double distilled water.

Autoclaved and stored at 4°C

3. Gel loading buffer

Bromophenol blue	- 0.5%
Glycerol (mol. grade)	- 30%

Prepared in 1X TBE

Store at 4°C.

4. 1X TBE buffer

10X TBE	- 10 ml
Distilled Water	- 90 ml

3.6.3.1 Visualization of microsatellite products

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

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

3.6.3.2 Calculation of the molecular weights of the bands

Molecular weights of the bands were calculated in reference to the molecular weight markers with the software Biorad Image Lab™. The alleles were designated according to PCR product size relative to molecular marker pBR322DNA/*Msp*I digest (Figure 3.2)

3.6.3.3 Confirmation of microsatellite repeats

The microsatellites loci were confirmed by PCR products sequencing of all the loci selected through cross species amplification in order to find out the repeat units. The sequencing reactions were carried out using forward and reverse sequencing primers according to manufacturer's instructions with the automated DNA sequencer ABI 3730 at Scigenom Pvt.Ltd, Cochin.

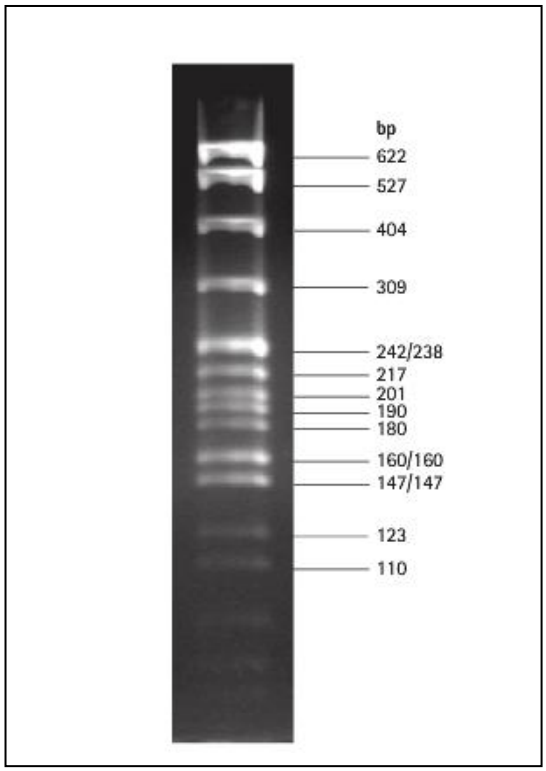


Figure 3.2 pBR 322/*Msp1* molecular ladder

3.6.3.4 Genbank submission of sequences

The Basic Local Alignment Search Tool (BLAST) available on the NCBI website (http://www.ncbi.nih.gov/BLAST/) was used to search Genbank for similar sequences. The DNA sequences were edited using BioEdit sequence alignment editor version 7.0.5.2 (Hall, 1999). The number of microsatellite repeats was identified using online software (http://www.biophp.org/minitools/ microsatellite_ repeats_finder/). Bad reads were checked manually, corrected and submitted to GenBank using a submission programme called "sequin" (www.ncbi.nlm.nih.gov/ Sequin/index.html).

3.6.4 Automated genotyping

Forward primers of the selected loci were labelled with 6-FAM fluorescent dye (Applied biosystem). After standardization procedure, the primers having amplified products were again evaluated with more number of individuals (60-70 individuals in five locations). PCR amplifications and genotyping have been completed with 12 primers in five populations of silver pomfret. The alleles were separated using capillary electrophoresis on an ABI Prism 3730 genetic analyzer (Applied Biosystems). Sized fragments obtained in the form of .fsa files were analyzed using GeneMapper software (version 4.0; Applied Biosystems Inc., California, USA) to generate genotype calls for each marker. Size matching was evaluated for each sample using Size Match Quality Indicator. Samples that passed the size standard quality checks were processed for binning. For each marker, samples were binned using the autobinning option present in GeneMapper software. Wherever required, bins were edited to cover all peaks with genotype quality above 0.1. Fragment sizing was carried out using the default analysis settings in the software, by comparison with the internal size standard ROX 400 or LIZ 500. This genotyping procedure was carried out at Rajiv Gandhi Center for Biotechnology (RGCB), Trivandrum. All genotypes were scored manually, and genotypes were verified independently by a second reader.

3.6.5 Data Analysis

372 individuals of *P. argenteus* from five different geographic locations were amplified using 12 polymorphic microsatellite primers. Data were analysed for homogeneity between data sets of different time interval within each population. The following parameters like genetic variability and departure from Hardy Weinberg equilibrium, bottleneck analysis, population differentiation and genetic distance, effective population size, gene flow and phylogenetic algorithms were estimated using different softwares.

3.6.5.1 Genetic variability analysis

To analyze variation at microsatellite loci, the mean number of alleles per locus; observed and expected heterozygosities (Ho and He), allele frequencies at each locus and percentage of polymorphic loci for overall and each population and F_{IS} (coefficient of inbreeding) were calculated using GENEPOP Ver. 4.1.1 (Rousset, 2008) and GenAlEx (Genetic Analysis in Excel) version 6.5, Peakall and Smouse, 2012). Allele richness and gene diversity were estimated using FSTAT software version 1.2 (Goudet, 2001). Polymorphic Information Content (PIC) was calculated using Cervus 3.0.6 (Kalinowski *et al*., 2007). F_{IS} refers to the Hardy-Weinberg distribution (or otherwise) of genotypes of individuals within sub-population and is defined as the correlation between homologous alleles within individuals with reference to the local population. It is a measure of deviations from Hardy-Weinberg proportions within samples. Positive values for F_{IS} demonstrate an excess of homozygotes (positive correlation between homologous allele) or conversely, a deficiency of heterozygotes, relative to the Hardy-Weinberg model. This could be due to inbreeding and this index is often labelled as "inbreeding coefficient". Microsatellite genotype data of the loci with known inbreeding coefficient ($+F_{IS}$) were tested for the expected frequency of null alleles according to Van Oosterhout *et al*., (2004) using MICRO-CHECKER version 2.2.3 (http://www.microchecker.hull.ac.uk/) and thereafter analyzed for population differentiation.

3.6.5.1.1 Linkage Disequilibrium (LD)

This parameter was tested using a contingency table test for genotype linkage disequilibrium between pairs of loci in a population, based upon the null hypothesis that genotypes at one locus are independent of genotypes at other loci. Calculations were performed using the GENEPOP Ver. 4.1.1 programme (Rousset, 2008) Arlequin version 3.5 software (Excoffier and Lischer, 2010) which performs a significance test using Markov chain procedures.

3.6.5.1.2 Hardy-Weinberg Equilibrium (HWE)

Exact P-tests for conformity to Hardy-Weinberg Equilibrium (probability and score test) were performed by the Markov Chain method using GENEPOP Ver. 4.1.1 programme (Rousset, 2008) with parameters, dememorization = 1000; batches = 10 and iterations = 100; and based upon a null hypothesis of random union of gametes. The significant criteria were adjusted for the number of simultaneous tests using sequential Bonferroni technique (Rice, 1989).

3.6.5.2 Estimation of population differentiation

The genetic differentiation between populations was investigated in a quantitative way to determine the extent of population subdivision among samples using F-statistics/Fixation indices (F_{ST}) (Wright, 1951), using GenAlEx (Genetic Analysis in Excel) version 6.5, (Peakall R. and Smouse P.E, 2012). The coefficient of genetic differentiation (F_{ST}) was also estimated. Estimation of average F_{ST} and determination of whether the values were significantly different from zero; and calculation of pair-wise population F_{ST} values (θ) and their significance levels, were carried out using GENEPOP with numerical resampling by bootstrapping (1000 times in the present study) to increase the significance of values. F_{ST} values range from 0 to 1, the greater the value, the greater the differences among populations (Beaumont and Hoare, 2003). As F_{ST} only employs allelic frequency data and does not make use of allele size differences, population differentiation was also estimated based on allele sizes of using Slatkin's (1995) pair-wise and overall R_{ST}, assuming a step wise mutation model (SMM) with Genepop ver. 3.3d

software. To correct for multiple simultaneous comparisons, sequential Bonferroni corrections were applied using a global significance level of 0.05 (Lessios, 1992).

The partitioning of genetic variation among and within populations and between groups of *P. argenteus* was calculated by hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) at 1000 permutations. The hierarchical components of genetic variation include (1) variance due to differences between individuals within a population; (2) variance due to differences among populations and (3) variance due to differences between population groups. The AMOVA calculations were performed using Arlequin v2.0 (Schneider *et al*., 2000; http://lgb.unige.ch/arlequin/) and GenAlEx (Genetic Analysis in Excel) version 6.5, (Peakall and Smouse, 2012). Population subdivision of silver pomfret within the Indian waters was initially explored without a priori assignment of samples to particular regions using the Bayesian approach in Structure version 2.3.3 (Pritchard *et al*., 2000). All samples with complete genotypes were included. Posterior probabilities for the existence of up to five silver pomfret subpopulations were evaluated with independent runs of K = 1–5. All runs were performed with ten iterations of a burn-in of 100,000 cycles and 200,000 MCMC repetitions with no prior information. Allele frequencies were assumed to be correlated and admixture was allowed. A likelihood ratio test was used to determine the most probable number of subpopulations and chain convergence was assessed by degree of stability in alpha values after the burn-in period. In subsequent analyses samples were assigned to their regional groupings. Further we used a ΔK metric suggested by to determine the statistically most supported number of clusters as implemented in Structure Harvestor (Earl, 2012). Principal Co-ordinate Analysis (PCoA) was performed in GenAlex. Isolation by distance (IBD) was evaluated by assessing the correlation between genetic distance measure F_{ST} / (1-F_{ST}) (Rousset, 1997) and geographic distance, with significance determined by the Mantel test in the program IBDWS 3.22 (Jensen *et al*., 2005). For the IBD analyses, geographic distances were calculated manually. Significance was based on 10000 permutation replicates.

Genetic identity and distance (Nei, 1978) between pairs of populations of *P. argenteus* were calculated using GenAlEx (Genetic Analysis in Excel) version 6.5 (Peakall and Smouse, 2012). For neutral markers, Nei's standard genetic distance between pairs of populations of co dominant data sets calculated under an infinite-allele-model as $D = -\ln(I)$ where I is Nei's Genetic Identity ($I = Jxy/\sqrt{(JxJy}$) (Nei, 1972, 1978).

Phylogenetic relationship based on Nei's genetic distance (Nei, 1978) was ascertained from allele data among five populations of *P. argenteus.* A dendrogram was plotted using the unweighted pair group method and arithmetic averages (UPGMA) using Treefit 1.0 software and FigTree v1.4.0. To test the confidence level of each branch dendrogram, the data were bootstrapped 1000 times using WinBoot (Yap and Nelson, 1996).

3.6.5.3 Bottleneck analysis

The bottleneck analysis was done to systematically screen for genetic bottlenecks in silver pomfret using microsatellite data from five populations collected from East and West coast of India. We also tested a series of population genetic simulations to assess the usefulness of M ratio (Garza and Williamson, 2001) for detecting the footprints of bottlenecks. This is crucial because the overall population of these silver pomfret has gone down rapidly during the past two decades.

3.6.5.3.1 Wilcoxon's test and mode-shift test

The catch data of the species in Indian waters shows sharp decline in the past two decades, so the bottleneck analysis was done to determine the genetic bottleneck undergone by the species. Genetic bottleneck is occurring if a population is passed through a harsh demographic decline followed by a recovery and the bottleneck operation theory is under the principle that if a population is experienced with a bottleneck, the allele numbers and the levels of heterozygosity will be reduced accordingly (Fine *et al*., 2013). To detect more recent genetic bottlenecks, two tests were used: the Wilcoxon's sign rank test which is based on heterozygosity excess and the mode-shift test which evaluates the allele frequency

distribution. These bottleneck impressions are detectable over a relatively short period. Both these bottleneck tests were performed using BOTTLENECK 1.2.02 (Piry *et al*., 1999) using both the stepwise mutation model (SMM) and the two-phase model (TPM) as recommended by Luikart and Cornuet (1998) using 1000 stimulations. It has been previously demonstrated that the mutation model has a significant effect on the estimation of heterozygosity excess, but not on the distortion of allele frequency distribution since rare alleles are expected to be abundant regardless of the mutation model (Nei *et al*., 1976). The values estimated using SMM was considered for the analysis as it is the more appropriate model for microsatellite evolution because microsatellite loci mutate at an extremely high rate and are generally thought to evolve through a stepwise mutation model (Balloux and Goudet, 2002).

Chapter IV
Results : Mitochondrial ATPase 6/8 gene Analysis

Morphological identification of *Pampus argenteus* was confirmed by mitochondrial Cytochrome C Oxidase subunit I region with randomly selected samples of each location collected for the study. The size of amplified products was approximately 655 bp.

4.1 Mitochondrial ATPase 6/8 gene analysis

The complete ATPase 6/8 genes were sequenced from 100 samples of *P. argenteus* from five different geographical locations of both the coasts of India. Out of a total of 842 bp of mitochondrial gene amplified (Figure 4.1) 168 bp fragments were of ATPase 8 and 684 bp of ATPase 6, with an overlapping region of 10 bp from 159-168. ATG was the start codon in ATPase 6/8 genes. TAA was the stop codon in ATPase 8 genes and incomplete stop codon of TA was found in ATPase 6 genes. A total of 24 haplotypes were identified among 100 individuals of five locations. DNA sequence of a representative haplotype is given in Figure 4.2.

4.1.1 ATPase 6/8 sequence variations

Out of the total 842 characters obtained, 781 (92.76%) were constant and 61 (7.24%) were variable, in which 51 (83.61%) were parsimony informative and 10 (16.39%) were singleton variable sites. The different nucleotide compositions were found to be A = 29.4%, C = 26.9%, G = 13.2% and T = 30.6%. As expected, most of the nucleotide variations were resulted from transitions (87.02%) followed by transversions (12.98%). The transition-transversion ratio for the ingroup was 6.7. According to codon position, the most informative was the second position (11 informative characters) followed by the third position (6 informative characters). The nucleotide sequence characteristics of ATPase 6 and 8 genes across five populations of *P. argenteus* given in Table 4.1. All haplotypes

representing ATPase gene were submitted to the GenBank (Accs. NoJX 293025-293034) and (JX 460972 - JX460982, JX 944218-944220).

4.1.2 Variation in amino acid sequences

Nucleotide base pairs of the different haplotypes from all locations were translated into amino acid residues. Out of 280 total residues observed, 239 (85.35%) were constant and 41 were variable characters (14.65%) among them 33 (36.84%) were parsimony informative sites (80.48%) and eight were of singleton (19.52%) across the groups. Not many variations were observed in amino acid sequences with in populations.

4.1.3 Nucleotide and Haplotype Diversity

The average nucleotide diversity (pi) for the all samples from five geographic locations was found to be 0.002672 while haplotype diversity (Hd) was found 0.838 (variance 0.00081± 0.028). Haplotype diversity was found to be high ranging from 0.182 (Vishakhapatnam) to 0.935 (Veraval) and the nucleotide diversity was low, ranged between 0.00022 (Vishakhapatnam) to 0.00407 (Veraval) (Table 4.2). The pattern of genetic diversity with high haplotype diversity and low to moderate nucleotide diversity may be attributed to a population expansion after a low effective population size caused by 'bottlenecks' (Grant and Bowen, 1998). This has an important direct influence on genetic variation, as populations with lower effective sizes are more affected by random genetic drift and gene flow tends to decrease.

4.1.4 Genetic relationship among haplotypes

The Minimum spanning network tree (Figure 4.3) and haploype network tree based on statistical parsimony clearly showed that all individuals from Kolkata and Vishakhapatnam clustered into one clade, whereas samples from Veraval, Cochin and Chennai formed a unit cluster network. As the samples from Kolkata and Vishakhapatnam showed a distinct clade, the genetic identity of specimens collected for the study from these locations were identified as *P.*

argenteus through amplification of Cytochrome C oxidase subunit I gene. The samples collected from Kolkata and Vishakhapatnam revealed average pair wise genetic distance of 1. 22% with samples collected from other localities of Indian waters. Each haplotype was connected with others by 1 to 4 mutational steps. Specific haplotypes was found in all the locations (Table 4.3). Maximum number of specific haplotypes was found in samples of Veraval, followed by Cochin. Mean pairwise distances between the haplotypes of each geographic location was in the range of 0.181818 (Vishakhapatnam) to 3.424837 (Veraval and Cochin) (Table 4.4)

4.1.5 Phylogenetic analysis

Phylogenies were constructed using the Neighbour joining (NJ) (Figure 4.4) and Maximum parsimony (MP) methods. Both trees showed almost the same topology. They consistently indicated that two clear major groups (VER, COC, CHN; one group and VP, KOL; second group) could be recognized in both analyses. There was no mixing up of haplotypes within the respective groups.

4.1.6 Phylogeographic relationships of populations

Estimates of genetic differentiation between all the five locations, using F-statistics, was calculated. Low level of genetic differentiation was observed among populations within the groups ranging between -0.00689 and 0.35241. The genetic difference between samples of Vishakhapatnam and Kolkata are absolutely nil (Pairwise F_{ST}= -0.00689). The level of genetic differentiation among groups showed high levels ranging between 0.95047 and 0.98047 indicated that significant genetic divergence among the *P. argenteus* populations along the Indian coast. The *p*-values associated with these comparisons were all significant (P<0.001).

The analysis of molecular variance (Table 4.5) for the *P. argenteus* indicated that a high proportion of the total variance (53.51%) was attributed to among populations within the group (F_{CT}=0.41349), which was significant and

also less variation within populations (5.14). The analysis also showed differences between the geographically isolated populations (F_{SC}=0.91239), which was significant (P <0.001).

4.1.7 Demographic history

Tajima D value and Fu's FS values were found to be negative and significant for all the five locations (Table 4.7). The significant negative values for these two tests imply that there were more nucleotide site variants than would be expected under neutral model of evolution (Read, 1996). Significant negative values of these statistics in this study indicated that *P. argenteus* of both the coasts had experienced population expansion. The pattern of genetic diversity with high genetic variation and low to moderate nucleotide diversity may be attributed to a population expansion after a low effective population size caused by 'bottlenecks' or 'founder events' (Grant and Bowen, 1998). Mismatch distributions were calculated and the smooth unimodal curves were obtained for all the locations matching the expected distributions under the sudden expansion model which explains the history of occurrence of rapid expansion in the populations of *P. argenetus.* The unimodal mismatch frequency distribution pattern based on the mtDNA sequence accorded well the predicted distribution under a model of population expansion (Figure 4.5). Tajima's D and Fu's Fs, corresponding P values and mismatch distribution parameter estimates are indicated in Table 4.7. In addition, non-significant raggedness index and the SSD values, sum of square deviations, hold up the null hypotheses of population expansion for all samples of *P. argenteus* across different collection sites (Table 4.6).

Figure 4.1 Amplified products of ATPase 6/8 genes separated on 1.5% agarose gel. Lane 1 to 11 – eleven samples and M – 100 bp DNA ladder.

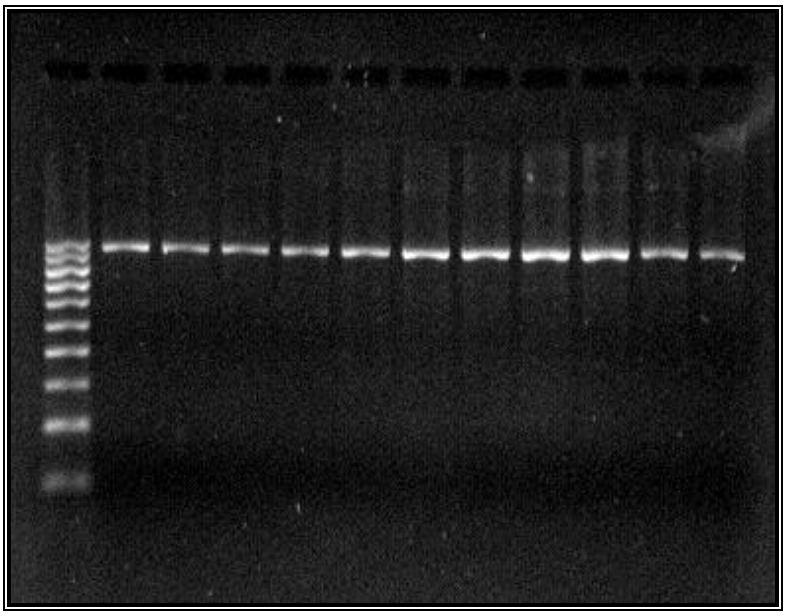
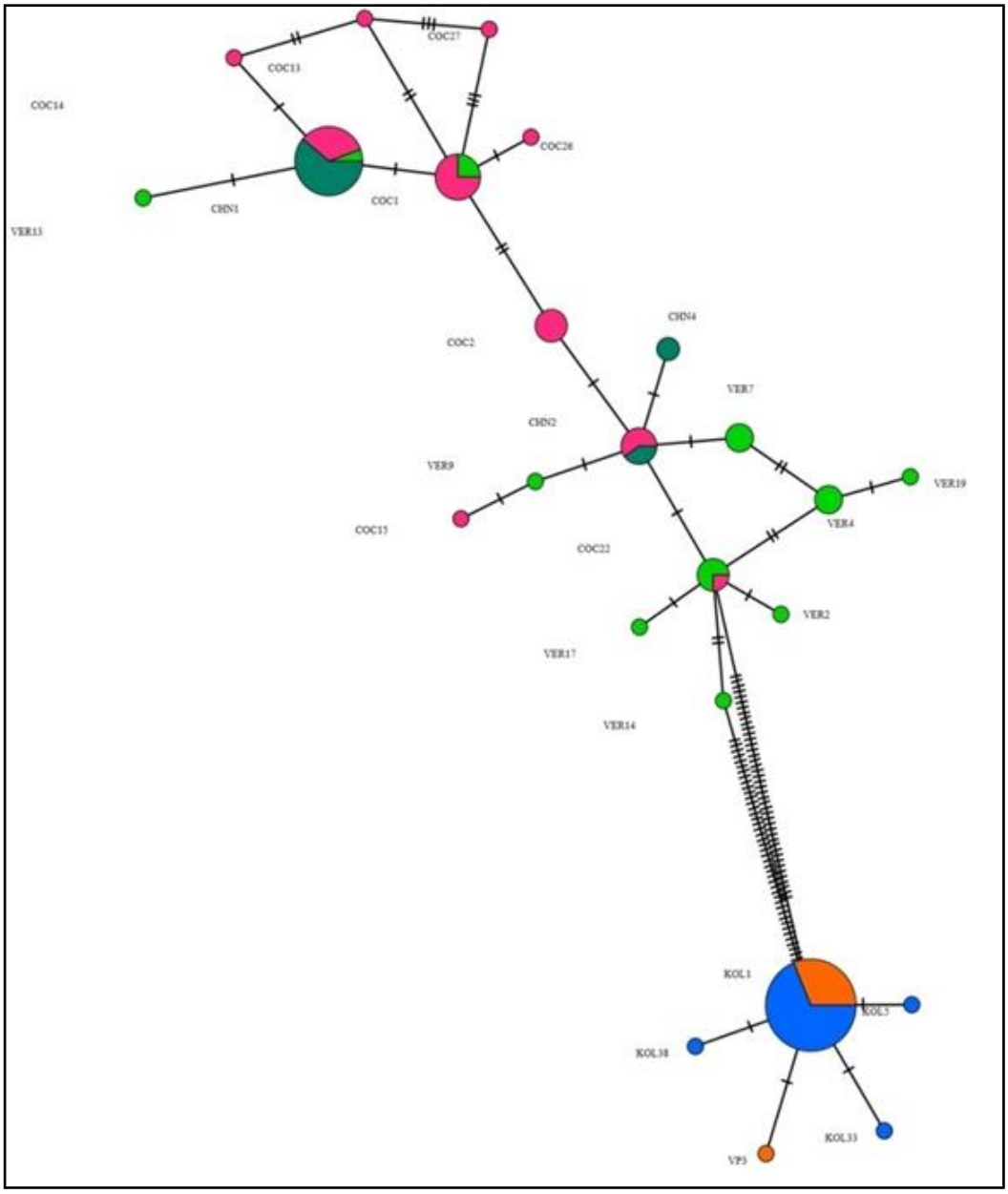


Figure 4.2 DNA sequence of ATPase 6/8 gene and translated protein sequences of a representative haplotype of *Pampus argenteus*. Underlined region is the stop codon of ATPase 8 gene and green highlighted region is the start codon of ATPase 6 gene.

```
001 ATGCCGCAGC TAGATCTGGA AATCTGATTT ATGATACTTA TTCACTCTTG ATTCGCCTTC
061 CTGGTAATTG TGGTACCTAA AGTGTTAGCC CATTCTTTCA CTAAAGTAAC CCTACTCAAA
121 AAAACCCAGA AAACAGAAAC AAAAACATGA GACTGACCTT GACACTAAGC CTGTTTAACC
181 AATTCGCACC CACATATATT TTATGTATCC CAAGTAGTAT AGTAGCCATT TTTATCCCAT
241 GAGTACTATT CCCTACCCCT ACAAATCGAT GATTAAACAA CCGTCTGGTT ACACTCCAAA
301 ACTGATACCT CGTTCAACTT CTGCGAGAAT TATTTTTACC TATAAAACCT GAAGGCCACA
361 AATGAGCTCT TATATTTATT TCCTTGGCAG TTTATTTAAC CTTCTTAAAC CTATTAGGAC
421 TCCTCCCATA CACTTTTACG CCCACTTCAC AACTATCCCA TAATTTAGGC TTTGCAATCC
481 CCTTCTGATT GGTAACAGTA ATTATTGGTT TCCAGAATAA ACCAAACGAA GCCCTGGCCC
541 ACCTTCTCCC AGAAGGAACC CCTACTCCTC TTATTCCCAT CCTTATTATC ATTGAGACAA
601 TTAGTCTCTT TATTCGTCCA CTGGCACTTG CAGTACGACT AACAGCTAAT ATTACAGCCG
661 GCCATCTTCT AATTCAATTA GTTGCAAAAG GCACACTTGC CCTACTATCA ATTATACCAG
721 CTGTAGCAGT CTTAACTATG ACTCTACTCC TCCTCTTAAC CCTCCTAGAA ATTGCCGTCG
781 CTATAATCCA GGCTTACGTA TTTGTTTTAT TACTAAGCCT ATACTTGCAA GAAAACGATT
841 AA
```

Figure 4.3 Minimum spanning network tree estimated among haplotypes of *Pampus argenteus* in Indian waters



Veraval (VER)

Cochin (COC)

Chennai (CHN)

Vishakhapatanam (VP)

Kolkata (KOL)

Figure 4.4 Neighbour-joining tree of *Pampus argenteus* inferred from mitochondrial ATPase 6/8 genes

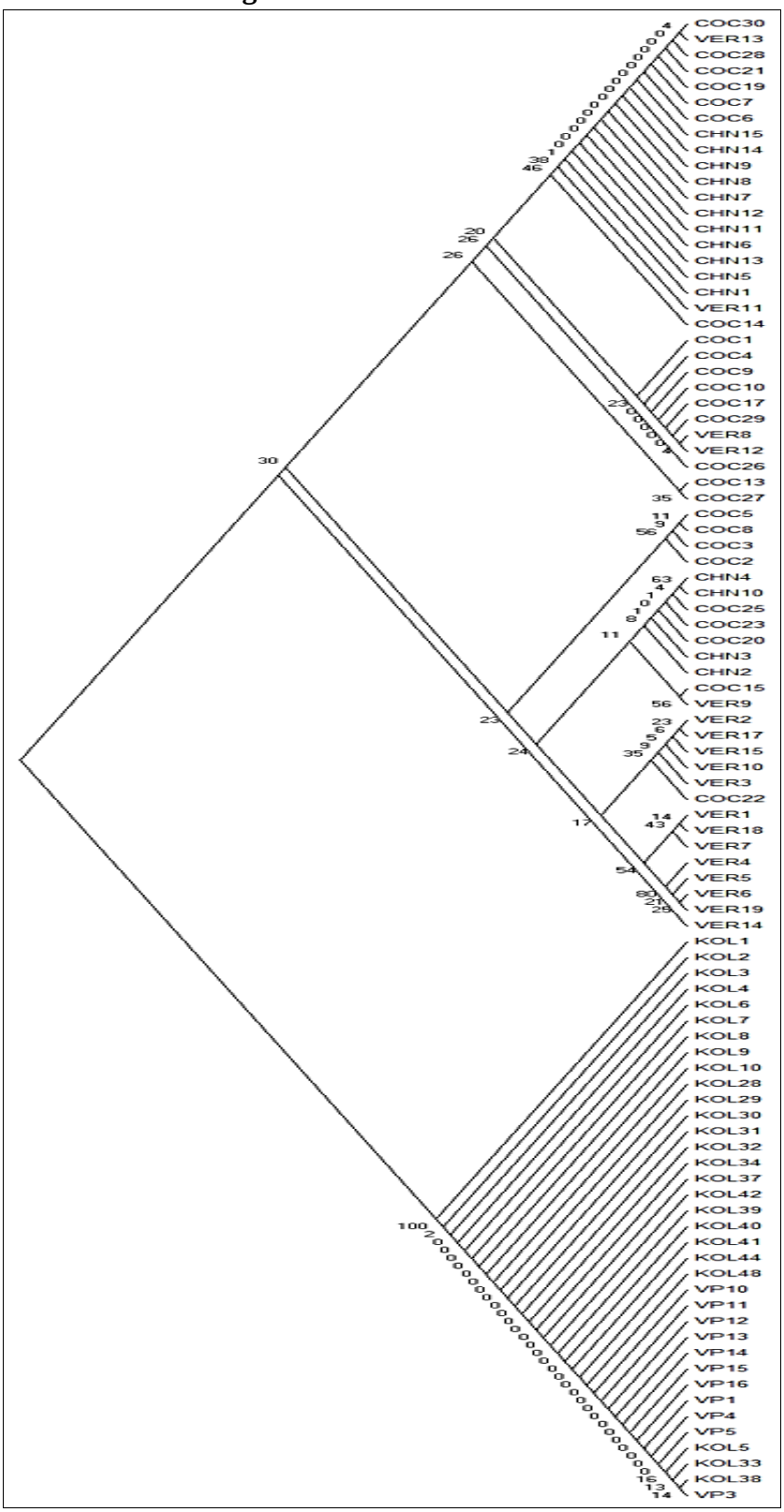


Fig. 4.5 Pairwise mismatch distributions (Rogers & Harpending 1992), simulated model of sudden expansion (Rogers 1995) for each geographic location of *Pampus argenteus*

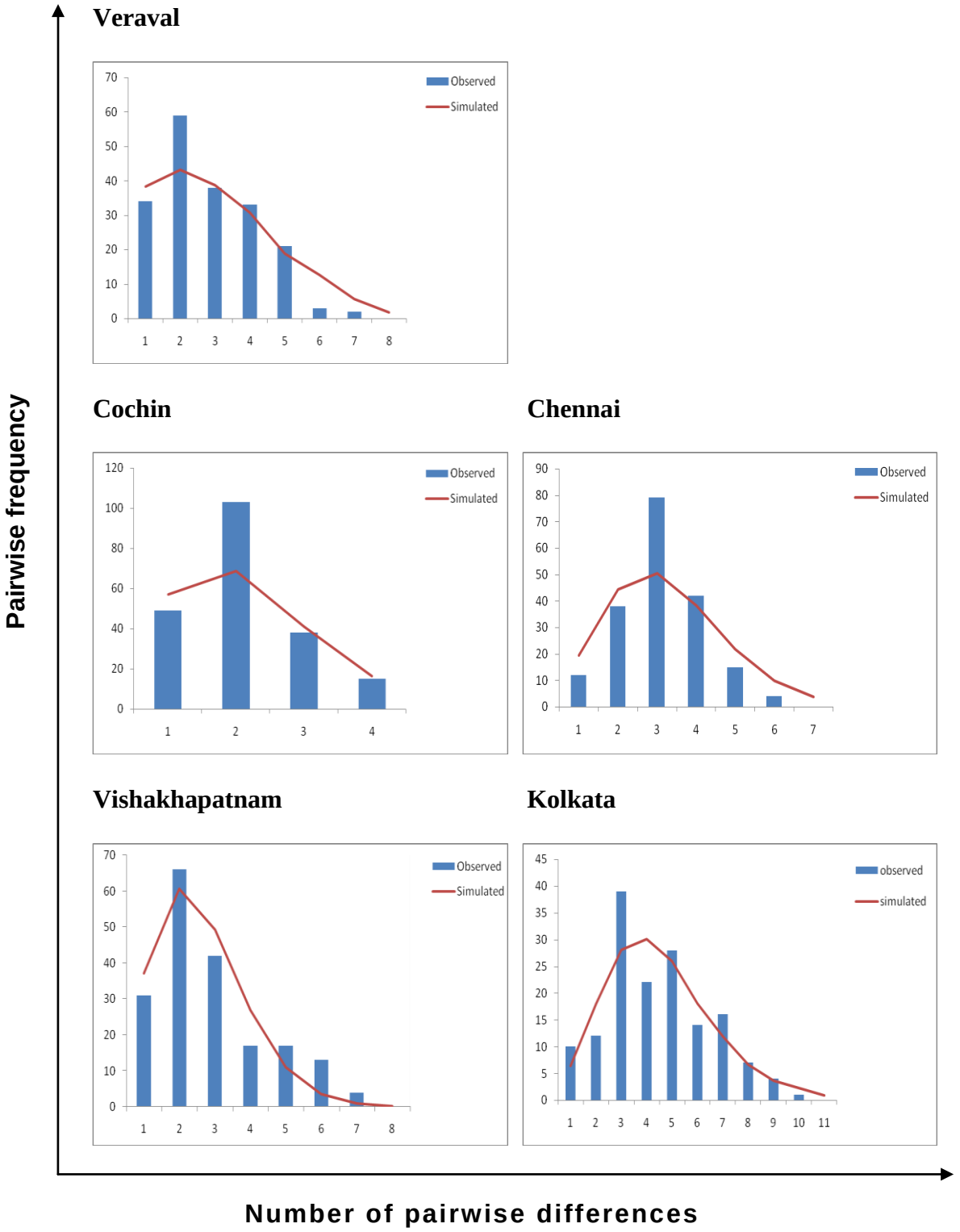


Table 4.1 Molecular diversity indices of ATPase 6/8 genes across five locations of *Pampus argenteus*

Statistics	VER	COC	CHN	VP	KOL	Mean	SD
No. of transitions	11	8	5	1	3	5.6	3.975
No. of transversions	2	2	0	0	0	0.8	1.095
No. of substitutions	13	10	5	1	3	6.4	4.98
No. of indels	0	0	0	0	0	0	0

Table 4.2 Intra-population haplotype diversities (h) and nucleotide diversities (π) for ATPase 6/8 region of *Pampus argenteus*

Location	Haplotype diversity (h)	Nucleotide diversity (π)
Veraval (VER)	0.935	0.00407
Cochin (COC)	0.870	0.00287
Chennai (CHN)	0.457	0.00228
Vishakhapatnam (VP)	0.182	0.00022
Kolkata (KOL)	0.230	0.00029

Table 4.3 Haplotype frequencies for ATPase 6/8 region of *Pampus argenteus*

Haplotype	VER	COC	CHN	VP	KOL
H1	0.0556	0.24	0.733	--	--
H2	--	0.12	0.133	--	--
H3	--	--	0.133	--	--
H4	--	--	--	0.909	0.88
H5	--	--	--	0.0909	--
H6	--	--	--	--	0.04
H7	--	--	--	--	0.04
H8	--	--	--	--	0.04
H9	0.0556	--	--	--	--

H10	0.167	0.04	--	--	--
H11	0.167	--	--	--	--
H12	0.167	--	--	--	--
H13	0.111	0.24	--	--	--
H14	0.0556	--	--	--	--
H15	0.0556	--	--	--	--
H16	0.0556	--	--	--	--
H17	0.0556	--	--	--	--
H18	0.0556	--	--	--	--
H19	--	0.16	--	--	--
H20	--	0.04	--	--	--
H21	--	0.04	--	--	--
H22	--	0.04	--	--	--
H23	--	0.04	--	--	--
H24	--	0.04	--	--	--

Table 4.4 Mean pairwise distances between the haplotypes of each location of *Pampus argenteus* based on ATPase *6/8* gene sequences

Locations	n_h	Mean pairwise distances
Veraval (VER)	11	3.424837 +/-1.835832
Cochin (COC)	10	3.424837 +/-1.835832
Chennai (CHN)	3	1.923810 +/-1.157585
Vishakhapatnam (VP)	2	0.181818 +/-0.253430
Kolkata (KOL)	4	0.240000 +/-0.284615

n_h : Number of haplotypes

Table 4.5 Results of the hierarchical Analysis of Molecular Variance (AMOVA) of *Pampus argenteus* based on mitochondrial ATPase 6/8 region

Source of Variation	d.f	Sum of squares	Variance components	Percentage of variation
Among groups	1	501.456	6.81776 Va	41.35
Among Populations within groups	3	469.211	8.82323 Vb	53.51
Within populations	96	75.407	0.84727 Vc	5.14
Total	100	1046.074	16.48826	100.00

Fixation Indices:

F_{SC} : 0.91239 F_{ST} : 0.94861 F_{CT} : 0.41349

Table 4.6 Tajima's D and Fu's FS tests, corresponding p value and mismatch distribution parameter estimates for *Pampus argenteus* from different geographic locations (P<0.01)

	Tajima D	P value	Fu's FS	P value	SSD	P Value	Raggedness index
Veraval	-0.35	0.02	-2	0.02	0.0119	0.34	0.05
Cochin	-0.28	0.02	-2.6	0.01	0.0049	0.57	0.02
Chennai	-0.84	0.01	-2	0.02	0.2983	0.08	0.34
Vishakhapatnam	-0.73	0.01	-2.4	0.02	0.083	0.38	0.08
Kolkata	-1.73	0.02	-3.4	0.01	0.0026	0.42	0.34

Chapter V
Results: Microsatellite Analysis

We had collected information from NCBI and published resources and a total of 21 microsatellite loci were selected from *Pampus cinereus and P. argenteus* for the present study. Screening of these selected primers was carried out using standard protocols with required modifications (particularly in annealing temperature and $MgCl_2$ concentration). A total of 17 loci amplified and produced bands. Out of these, 12 loci produced polymorphic bands were selected for further genotyping and location genetic structure analysis (Table 5.1). Remaining five loci were excluded from the study because of the presence of stutter bands. After sequencing, all the seven loci amplified through cross priming were confirmed as microsatellites containing the repeat sequences and submitted in Genbank (Accession Nos: JX872231- JX872237). Of the 17 amplified loci, all were perfect and contain di-nucleotide repeats. The tandem repeats of the seven microsatellite loci developed through cross priming contained repeat motifs differed from that of the resource species.

5.1 GENETIC VARIABILITY PARAMETERS

5.1.1 Allele diversity

A total of 382 individuals collected from five geographic locations viz., Kerala (Cochin), Gujarat (Veraval), Tamil Nadu (Chennai), West Bengal (Kolkata), and Andhra Pradesh (Vishakhapatnam) were analyzed with 12 microsatellite loci. The nonamplified reactions were repeated and genotyped again. The allele frequency from samples of a location collected at different periods were exhibited significant similarity after testing and resulted in five combined data sets representing all the collection sites in *P. argenteus*. Number of alleles per locus ranged from 28 (Par 17 and Par 06) to 64 (P-189) (Table 5.1). The locus wise and location wise allele frequency is given in Appendix1.

5.1.2 Allele Distribution patterns in microsatellite loci

In Par 01, 30 alleles were observed with 338-430 bp size range. The allele size 402 bp was present only in Veraval and 338 bp was found only in Chennai. All other alleles were dispersed among all the five locations.

In Par 02, a total of 39 alleles were observed. The size of the alleles ranged from 92 to 200 bp. The allele size of 154 bp was observed in Vishakhapatnam. There were no other location specific alleles for this primer.

In Par 03, 51 alleles were recorded. The size of the alleles ranged from 202-330 bp. The allele size of 226, 228 and 264 bp was found in Cochin whereas Chennai contains unique alleles of sizes 208, 234, 238 bp. The allele size of 246 bp was specific to Kolkata.

In Par 06, 28 alleles were noticed with size range of 112-198 bp. The allele, 198 bp was specific for Veraval and the fragment 138 bp was specific to Chennai .

In Par 08, there were 46 alleles and their sizes ranged from 200-448 bp. The fragment 206 bp was present only in Veraval. There were four specific alleles (212, 222, 240, 278 bp) to Kolkata. The fragments of 252, 254 and 400 bp were unique to Vishakhapatnam.

In Par 12, 42 alleles were recorded. The size of the alleles ranged between 232-368 bp. This locus developed one private allele for Chennai (250 bp) and five for Kolkata (240, 242, 268, 278 and 286 bp). The 312 and 368 bp alleles were observed only in Vishakhapatnam.

In Par 05, altogether 42 alleles were observed ranging from 200 bp-330 bp. This loci developed private alleles for the locations like 330 bp for Veraval; 302 bp for Cochin; 206, 230 and 248 bp for Vishakhapatnam. There were no specific alleles for Chennai and Kolkata.

In Par 17, a total of 28 alleles were recorded. The size of the alleles ranged from 92 to 194 bp. There was only one private allele for this locus ie, for Kolkata (112 bp).

In Par 18, 31 alleles were observed with a size ranged from 206-300 bp. There were no unique alleles to Veraval and Cochin. Three alleles were found to be specific for Chennai (234, 252 and 272 bp), two for Kolkata (238, 248, 260 bp). The allele size 266 bp was found only in Vishakhapatnam.

In Par 20, out of the 29 alleles recorded (200-300 bp) one allele (216 bp) was specific to Veraval. The allele fragment of 200 bp was found only in Cochin. There were no specific alleles for the East coast.

In P 15-2, there were 49 alleles ranging from 100-296 bp. Allele of size 296 bp was unique to Veraval and the allele fragment of 190 bp was specific to Cochin. In Kolkata also one private allele was observed (114 bp).

In P-189, 64 alleles were present with a size range of 100-296 bp. The highest number of alleles was recorded in this locus. In Veraval three private alleles were observed including 146, 156 and 172 bp. The allele fragments 180 and 186 bp was reported in Cochin. Specific alleles were absent for Chennai. 10 private alleles were observed in Vishakhapatnam (128, 130, 132, 134, 136, 138, 140, 240, 264 and 270 bp) and two in Kolkata (206 and 224 bp).

5.1.3 Allelic Patterns across the geographic locations

Veraval: The number of alleles ranging from a minimum of 5 alleles in Par 17 to a maximum of 23 in P 15-2. The average number of alleles in this location was 17.17. The effective number of alleles (Ne) was least in Par 17 (1.44) and the highest in P-189 (11.66) with an average value of 7.75 (Table 5.2).

Cochin: In this location the highest number of alleles was observed in Par 03 (34) and the least number was reported in Par 18 (13). The mean number of alleles in Cochin was 22.5. The effective number of alleles was ranged between 4.413 (Par 20) and 22.491 (Par 03). The average value of effective number of alleles was 11.334 (Table 5.2).

Chennai: In this location the number of alleles was ranged between 12 (Par 08) and 30 (Par 03). The average number of alleles was found to be 19.91. The effective number of alleles was found to be least in P 15-2 (3.16) and most in

22.124 in Par 03. The mean value of effective number of alleles was 9.028 (Table 5.2).

Vishakhapatnam: The lowest number of alleles was recorded with Par 18 and Par 20 (17) and the highest with P-189 (38). The mean number of alleles was 26.08. This location showed a mean effective number of alleles 12.76, ranged from a minimum of 5.441 (Par 01) to a maximum of 20.948 (P 15-2) (Table 5.2).

Kolkata: In this location the least number of alleles was reported in Par 06 (16) and the highest number in Par 03 (33). The mean value of number of alleles for all the loci was 22.92. The effective number of alleles was ranged from 4.24 (Par 01) to 23.38 (Par 03) with a mean value of 11.69 (Table 5.2).

Overall locations: Among all the locations with all the 12 microsatellite loci, the mean of observed number of alleles was 21.717 and the mean of effective number of alleles was 10.512 (Table 5.2). Allelic distribution patterns among the five locations graphically represented in Figure 5.1.

5.1.4 Linkage Disequilibrium (LD)

No significant linkage disequilibrium observed between any pair of microsatellite loci for any location ($P>0.05$). As a result it was assumed that allelic variation at microsatellite loci could be considered independent across all the 12 loci for all the five locations.

5.1.5 Frequency of null alleles

All the loci showed positive F_{IS} values in different locations were tested for presence null alleles using MICROCHECKER. The estimated null allele frequency was not significant ($P< 0.05$) at all 12 loci showed the nonexistence of null alleles and false homozygotes (Table 5.3). In addition, there was no instance of non-amplified samples in repeated trials with any of the primer pairs. So, for population genetic analysis, data generated from all the 12 loci was considered.

5.1.6 Observed (Ho) and expected (He) heterozygosities

In **Veraval**, the Ho showed a range of 0.184 (Par 17) to 1.000 (P-189) with a mean value of 0.614. The He values ranged from 0.305 (Par 17) to 0.914 (P-189) with a mean value of 0.811 (Table 5.4).

In **Cochin**, the Ho values were in a range of 0.327 (Par 18) to 1.000 (P-189) with a mean value of 0.718. The He values ranged between 0.773 (Par 20) and 0.956 (Par 03) with a mean value of 0.890 (Table 5.4).

In **Chennai**, the Ho values were ranged from 0.480 (Par 02) to 1.000 (multiple loci) with a mean value of 0.703. The He values showed a range from 0.683 (P 15-2) to 0.955 (Par 03) with a mean value of 0.851 (Table 5.4).

In **Vishakhapatnam**, the Ho values showed a range of 0.629 (Par 02) to 0.984 (P-189) with a mean value of 0.843. The He values ranged from 0.802 (Par 17) to 0.952 (Par 08) with a mean value of 0.904 (Table 5.4).

In **Kolkata**, the Ho values exhibited in a range of 0.817 (multiple loci) to 1.000 (P-189) with a mean value of 0.915. The He values ranged from 0.764 (Par 01) to 0.957 (Par 03) with a mean value of 0.894 (Table 5.4).

5.1.7 Polymorphic Information Content (PIC)

For overall location, PIC values for all the 12 loci ranged from 0.768 (Par 20) to 0.9792 (P-189) (Table 5.5).

5.1.8 Agreement with Hardy-Weinberg expectation

Test for agreement with Hardy-Weinberg expectations in the probability test (Chi-square test) showed that the observed allele frequencies in many loci manifested consistent significant deviations ($P<0.05$) from Hardy–Weinberg Equilibrium expectations (Table 5.4). Significant departure from Hardy Weinberg expectations indicates heterozygote deficiency which could have resulted from null alleles, small sample size or mixing of locations within samples. However, the

possibility of null alleles was ruled out by analyzing the data and sequential Bonferroni corrections using MICROCHECKER software. Wright (1978) fixation index (F_{IS}) is a measure of heterozygote deficiency or excess (inbreeding coefficient) and the significant values for each locus in five locations are given in Table 5.5. Positive F_{IS} indicating heterozygote deficiency was evident in all loci except Par 17 and P-189. F_{IS} values close to zero are expected under random mating, while substantial positive values indicate inbreeding or undetected null alleles. Negative values indicate excess of heterozygosity because of negative assortative mating.

5.2 GENETIC DIFFERENTIATION

5.2.1 Co-efficient of genetic differentiation

The co-efficient of genetic differentiation, F_{ST} ranged from 0.019 for the locus Par 05 to 0.095 for the locus Par 20, with a mean of 0.049. Pair-wise F_{ST} estimates between locations differed significantly ($P < 0.001$) from zero for all the pairs of five locations (Table 5.6). Pair-wise F_{ST} analysis showed three separate population clusters-Veraval-Cochin cluster; Chennai cluster and Vishakhapatnam-Kolkata cluster. Low differentiation was found among samples from Veraval and Cochin (0.025) as well as samples from Kolkata and Vishakhapatnam (0.028). The pairwise genetic distance was the highest between Veraval and Kolkata (0.085), which can be correlated with the geographical distance between two locations (Table 5.8). In addition to F_{ST}, location differentiation was measured using pair-wise and overall R_{ST} (Slatkin, 1995) based on the differences in repeat numbers (allele sizes) in microsatellite loci. Locus-wise R_{ST} varied from 0.005 (Par 03) to 0.426 (Par 01) with an overall value of 0.146. Pair-wise R_{ST} values were similar to that of F_{ST} in *P. argenteus* varying significantly higher between locations of east and west coasts of India and non-significant lower values were observed within locations of each coast (Table 5.6). Overall F_{ST} and R_{ST} values were 0.049 and 0.146 respectively, indicating that there is genetic differentiation among silver pomfret.

5.2.2 Nei's (1978) genetic distance and similarity

Nei's (1978) unbiased genetic distance estimated between pairs of five locations of *P. argenteus* is presented in Table 5.7. The genetic distance was high between Veraval and Vishakhapatnam(0.897) and lowest was found between Veraval and Cochin (0.374). The results agreed with the geographic distances between the locations.

5.2.3 Gene flow

The gene flow or migration rate (Nm) for each locus for overall location ranged from 2.368 (Par 20) to 12.863 (Par 05) with the mean value of 6.432 (Table 5.5). Gene flow among the locations of *P. argenteus* was also calculated (Table 5.8). The highest value for Nm was found between Kolkata and Vishakhapatnam (13.261) and the lowest between Veraval and Kolkata (5.568) and this is also in accordance with the results of co-efficient of genetic differentiation.

5.2.4 Analysis of molecular variance (AMOVA)

The AMOVA based on microsatellite data indicated significant genetic differentiation among *P. argenteus* locations (F_{ST} 0.049; P<0.001) (Table 5.9). The value 83.24% was observed within individuals whereas, 0.88% variation was observed among the groups.

5.2.5 STRUCTURE analysis

The Bayesian cluster analysis performed in STRUCTURE showed that three clusters could be identified among the samples (Figure 5.2), using the Delta K estimator. When k = 3, maximum value for log likelihoods lnPr (X/k) and the minimum variance in log likelihoods (varlnPr(X/k)) was noticed, thus fixing the number of underlying clusters as three (Figure 5.3). When the underlying clusters were three in Veraval and Cochin; Kolkata and Vishakhapatnam showed admixture, whereas Chennai formed a different cluster as indicated in the Bar plot (Figure 5.2). This result was confirmed both by treating each sample as a single entity and by pooling the samples into three "populations" (Veraval and Cochin; Kolkata and Vishakhapatnam; Chennai).

5.2.6 Dendrogram

Phylogenetic relationships based on genetic distance values generated from allele data among five locations of *P. argenteus* were made and based on the Nei's (1978) measure of genetic distance, dendrogram (Figure 5.4) was constructed using open source software Treefit 1.2 and FigTree v1.4.0. The dendrogram depicted three genetic stocks 1) Vishakhapatnam, Kolkata 2) Chennai (along Bay of Bengal in the east coast of India) and Veraval and Cochin (along Arabian Sea in the west coast of India). Samples collected from Chennai in Bay of Bengal were showing moderate and significant differences from samples of other localities in this region.

5.2.7 Principal Coordinates Analysis (PCoA)

Principal Coordinate Analysis (PCoA) or the Multidimensional scaling (MDS) based on the conversion of the distance matrix to a covariance matrix also showed distinct location clustering similar to the UPGMA dendrogram. The two dimensional PCoA plot shows that the first principal coordinate accounts for 50.19% of total variation and separates the locations of east (Kolkata and Vishakhapatnam) and west coast (Veraval and Cochin). PC2 forms 26.09% of variation and separates Chennai. The dispersion of genetic variability analyzed by the Principal Coordinate Analysis (PCoA) suggests that Chennai form a fairly distinct set from the Kolkata and Vishakhapatnam (Figure 5.5).

5.2.8 Isolation by Distance (Mantel test)

The pattern of isolation by distance (IBD) was supported when all the sampling locations were compared by Mantel test, the IBD observed was significant $p<0.05$; $r2 = 0.768$ (Figure 5.6).

5.2.9 Stock specific alleles

A total of 40 private alleles restricted to a specific stock observed in the study. The stock constituting West coast (Veraval and Cochin) had 14 specific alleles while Chennai stock contain 9 private alleles and the third stock (Kolkata

and Vishakhapatnam) contain 17 specific alleles. The occurrence of these private alleles can give the genetic signatures for a Particular location (Table 5.10).

5.3 BOTTLENECK ANALYSIS

5.3.1 Wilcoxon's test and mode-shift test

The Wilcoxon signed rank test is the most suitable and authoritative method for bottleneck detection if we are analyzing fewer than 20 loci (Piry*et al*., 1999). Here we employed SMM and TPM methods because these are generally more appropriate when testing microsatellite data (i.e. dinucleotide repeat loci) (Luikart and Cornuet, 1998). In this study, Wilcoxon test were not significant under any of the two models. P values were found to be 0.99915, 0.99768, 0.99768, 1.00000, and 1.00000 in Veraval, Cochin, Chennai, Kolkata and Vishakhapatnam respectively under SMM method. Under TPM method, it was calculated as 0.96143, 0.88330, 0.99963, 0.99915 and 0.99829 in Veraval, Cochin, Chennai, Kolkata and Vishakhapatnam respectively (Table 5.12). This analysis show that heterozygosity is very high hence, the null hypothesis of the Wilcoxon's test (no significant heterozygosity excess on average across loci) is accepted and thus it suggests that there is no satisfactory evidence for a bottleneck in the silver pomfretlocation.

The Mode-shift indicator test was also utilized as a second method to detect potential bottlenecks, as the non bottleneck populations that are near mutation-drift equilibrium are expected to have a large proportion of alleles with low frequency. This test discriminates many bottlenecked locations from stable locations (Luikart and Cornuet, 1998). The distribution followed the normal L-shaped form and this clearly indicates that the studied location has not experienced a recent bottleneck (Figure 5.6).

5.3.2 M ratio test

Many studies reported the overexploitation of this species and we took a more conservative approach and calculated the G-W statistics and it was found to be very low, 0.20389 ± 0.11548, 0.21655 ± 0.06485, 0.20410 ± 0.06262, 0.20801 ±

0.05763, 0.23121 ± 0.07883 for Veraval, Cochin, Chennai, Vishakhapatnam and Kolkata respectively. In bottlenecked locations this Parameter is supposed to be very low and this result suggests a decline in the investigated silver pomfret population, contrary to the results obtained using the test for heterozygosity excess test and mode shift test (Table 5.13).

Table 5.1 Characteristics of polymorphic microsatellite loci in *P.argenteus*

Sl No	Locus	Primer sequence	Repeat motif	Ta (^{0}C)	No. of alleles	Size range (bp)
1	Par 01	F: ATT GTG ACA GGC TGC TCT TCA GA R: TGT CCT GTA TAG CAC CTG GAT GAC	(CT)n	55	30	338-430
2	Par 02	F:CTG ACA TGT CGG ATA TGC TTA CA R: CTT GCG GTG AAG ATA CAG ATA CAT	(GT)n	54	39	92-200
3	Par 03	F: TCC TCC CCC TAA AAT AAA GTA TCT R: TTG TTT TTG TAC ATT GCT TCA CAC	(GT)n	52	51	202-330
4	Par 05	F:AAA CCT GGG GAA TAT GGG AGA G R: GGC GTA GGG GCG GGT AG	(CA)n	55	42	200-330
5	Par 06	F:GTT TGG GAT TTG GGG AGG TAG TG R: AGG CCG TGG AAT CCT GGT GT	(CA)n	57	28	112-198
6	Par 08	F:TTT TGC AAG TGG GAA CAC AGA CT R: CTG GGG CTA GTT TCA TCT TCA CTT	(CA)n	54	46	200-448
7	Par12	F:AGC AGG CGT AGC AAA ACA GAC AA R: TGG CCC ACA GAG TTC AAA GGT C	(AC)n	55	42	232-368
8	Par 17	F:TCC ATA AAG GCA GGA AAA TGT CTC R: AAA CGC TCC TCA A AC AGA ACG AC	(TG)n	53	28	92-194
9	Par 18	F:TTC ACG ACC CCA GTG TTC ACC R:TGA TTT GTG GGG CTT AGA GGA GAG	(CA)n	57	31	206-300
10	Par 20	F:AAT CCC CTG AGG GTT GAG AAA R: CTG CTG CCA CAC TGA GTC TTA TCT	(GA)n	55	29	200-300
11	P 15-2	F:GCA AGC CTC TAA TTC ACT CC R: CTG CCT CTG TTT CTT CCT G	(AC)n (TG)n	53	49	100-296
12	P-189	F:ATT CAA TAA CAA CTC CAC C R: TGT CTC ACC ACT CTT CAG C	(GT)n	56	64	100-296

Table 5.2 Observed (Na) and Effective (Ne) number of microsatellite alleles in locations of *P.argenteus*

Locations		P15-2	Par 01	Par 02	Par 03	Par 06	Par 08	Par 12	Par 17	Par 18	Par 20	Par 05	P-189
Veraval (VER)	**Na**	23	22	18	22	17	17	19	5	11	12	20	20
	Ne	7.708	6.086	10.088	11.090	5.842	8.294	10.791	1.439	9.547	2.614	7.846	11.655
Cochin (COC)	**Na**	26	19	25	34	17	21	23	20	13	18	26	28
	Ne	14.103	6.062	14.828	22.491	5.879	12.764	14.508	6.224	8.705	4.413	13.152	12.872
Chennai (CHN)	**Na**	21	23	17	30	15	12	22	17	20	20	21	21
	Ne	3.159	8.547	6.281	22.124	3.981	9.921	12.195	3.378	9.158	9.398	11.086	9.107
Vishakhapatnam (VP)	**Na**	34	20	26	34	19	32	27	18	17	17	31	38
	Ne	20.948	5.441	6.220	20.126	9.152	20.667	15.722	5.038	9.946	5.864	14.672	19.268
Kolkata (KOL)	**Na**	29	14	19	33	16	30	23	23	20	17	26	25
	Ne	12.414	4.240	5.858	23.377	10.169	15.618	10.542	7.973	8.955	7.835	17.955	15.352
Overall Locations	**Na**	26.600	19.600	21.000	30.600	16.800	22.400	22.800	16.600	16.200	16.800	24.800	26.400
	Ne	11.666	6.075	8.655	19.841	7.005	13.453	12.752	4.811	9.262	6.025	12.942	13.651

Table 5.3 Summary statistics of null allele frequencies of microsatellite loci in *Pampus argenteus*

LOCUS	Locations showing positive F_{IS} values	Null allele frequency* (from MICRO-CHECKER)		
		Van Oosterhout	Chakraborty	Brooksfield
P 15-2	Veraval	0.047	0.044	0.044
	Cochin	0.040	0.049	0.051
	Chennai	0.045	0.048	0.048
	Kolkata	0.038	0.035	0.035
Par 01	Veraval	0.013	0.044	0.043
	Cochin	0.028	0.048	0.047
	Chennai	0.038	0.049	0.045
	Kolkata	0.045	0.041	0.039
Par 03	Veraval	0.042	0.047	0.046
	Cochin	0.036	0.050	0.050
	Chennai	0.013	0.014	0.014
	Kolkata	0.034	0.031	0.031
Par 06	Chennai	0.053	0.053	0.053
	Kolkata	0.047	0.041	0.041
Par 08	Veraval	0.038	0.035	0.035
	Cochin	0.042	0.036	0.036
	Chennai	0.029	0.041	0.046
	Vishakhapatnam	0.032	0.030	0.030
	Kolkata	0.017	0.016	0.016
Par 12	Veraval	0.046	0.040	0.040
	Cochin	0.020	0.018	0.018
	Chennai	0.042	0.037	0.037
	Vishakhapatnam	0.057	0.053	0.053
	Kolkata	0.033	0.031	0.031
Par 18	Veraval	0.051	0.050	0.050
	Cochin	0.046	0.042	0.042
	Chennai	0.026	0.025	0.025
	Vishakhapatnam	0.038	0.042	0.032
Par 20	Veraval	0.050	0.047	0.047
	Cochin	0.043	0.044	0.043
	Chennai	0.058	0.049	0.047
	Kolkata	0.039	0.035	0.035
Par 05	Cochin	0.019	0.021	0.019
	Chennai	0.003	0.003	0.002
	Kolkata	0.002	0.002	0.002
Par 17	Veraval	0.031	0.029	0.029
	Cochin	0.036	0.034	0.034
	Vishakhapatnam	0.050	0.040	0.040
	Kolkata	0.053	0.052	0.052

Table 5.4 Summary of genetic variation and heterozygosity statistics of 12 microsatellite loci in *Pampus argenteus*

LOCATIONS		P15-2	Par 01	Par02	Par 03	Par 06	Par 08	Par 12	Par 17	Par 18	Par 20	Par 05	P-189
VERAVAL	**Na**	23	22	18	22	17	17	19	5	11	12	20	20
	Ne	7.708	6.086	10.088	11.090	5.842	8.294	10.791	1.439	9.547	2.614	7.846	11.655
	Ho	0.551	0.531	0.735	0.653	0.816	0.551	0.735	0.184	0.347	0.388	0.878	1.000
	He	0.870	0.836	0.901	0.910	0.829	0.879	0.907	0.305	0.895	0.617	0.873	0.914
	Probability	0.000	0.000	0.000	0.000	0.000	0.000	0.041	0.000	0.000	0.000	0.000	0.236
	Fis	0.367	0.365	0.184	0.282	0.015	0.373	0.190	0.398	0.612	0.372	-0.006	-0.094
Cochin	**Na**	26	19	25	34	17	21	23	20	13	18	26	28
	Ne	14.103	6.062	14.828	22.491	5.879	12.764	14.508	6.224	8.705	4.413	13.152	12.872
	Ho	0.873	0.655	0.727	0.655	0.782	0.782	0.891	0.636	0.327	0.455	0.836	1.000
	He	0.929	0.835	0.933	0.956	0.830	0.922	0.931	0.839	0.885	0.773	0.924	0.922
	Probability	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.957
	Fis	0.061	0.216	0.220	0.315	0.058	0.152	0.043	0.242	0.630	0.412	0.095	-0.084
CHENNAI	**Na**	21	23	17	30	15	12	22	17	20	20	21	21
	Ne	3.159	8.547	6.281	22.124	3.981	9.921	12.195	3.378	9.158	9.398	11.086	9.107
	Ho	0.560	0.800	0.480	0.700	0.560	0.760	0.720	1.000	0.520	0.620	0.720	1.000
	He	0.683	0.883	0.841	0.955	0.749	0.899	0.918	0.704	0.891	0.894	0.910	0.890
	Probability	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.013
	Fis	0.181	0.094	0.429	0.267	0.252	0.155	0.216	-0.420	0.416	0.306	0.209	-0.123
VISHAKHAPATNAM	**Na**	34	20	26	34	19	32	27	18	17	17	31	38
	Ne	20.948	5.441	6.220	20.126	9.152	20.667	15.722	5.038	9.946	5.864	14.672	19.268
	Ho	0.887	0.742	0.629	0.903	0.774	0.823	0.919	0.806	0.935	0.806	0.903	0.984
	He	0.952	0.816	0.839	0.950	0.891	0.952	0.936	0.802	0.899	0.829	0.932	0.948
	Probability	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.050	0.000
	Fis	-0.033	-0.156	-0.146	-0.010	-0.054	-0.015	0.098	-0.048	0.043	0.064	0.012	-0.070
KOLKATA	**Na**	29	14	19	33	16	30	23	23	20	17	26	25
	Ne	12.414	4.240	5.858	23.377	10.169	15.618	10.542	7.973	8.955	7.835	17.955	15.352
	Ho	0.950	0.883	0.950	0.967	0.950	0.950	0.817	0.917	0.850	0.817	0.933	1.000
	He	0.919	0.764	0.829	0.957	0.902	0.936	0.905	0.875	0.888	0.872	0.944	0.935
	Probability	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Fis	0.068	0.091	0.250	0.050	0.131	0.136	0.018	-0.006	-0.04	0.028	0.031	-0.038

Table 5.5 F-Statistics, gene flow and estimates of polymorphism information content over all locations for each Locus

Locus	F_{IS}	F_{IT}	F_{ST}	R_{ST}	N_m	PIC
P15-2	0.123	0.187	0.073	0.197	3.164	0.9339
Par 01	0.127	0.182	0.064	0.426	3.686	0.8758
Par 02	0.189	0.235	0.056	0.349	4.177	0.9102
Par 03	0.18	0.196	0.02	0.005	12.368	0.9647
Par 06	0.076	0.122	0.05	0.109	4.777	0.8724
Par 08	0.157	0.19	0.038	0.116	6.315	0.9529
Par 12	0.112	0.132	0.022	0.022	11.124	0.9336
Par 17	-0.005	0.075	0.08	0.192	2.868	0.9324
Par 18	0.332	0.363	0.046	0.215	5.176	0.9153
Par 20	0.226	0.3	0.095	0.055	2.368	0.7677
Par 05	0.068	0.086	0.019	0.031	12.863	0.9327
P-189	-0.081	-0.05	0.029	0.034	8.299	0.9792

Table 5.6 Pair-wise F_{ST} (below diagonal) and R_{ST} (above diagonal) values among five locations of *Pampus argenteus* with 12 microsatellite loci.

	Veraval	Cochin	Chennai	Vishakhapatnam	Kolkata
Veraval	0.000	0.073	0.228*	0.138*	0.225*
Cochin	0.025	0.000	0.216*	0.081*	0.175*
Chennai	0.064*	0.049*	0.000	0.116*	0.093*
Vishakhapatnam	0.074*	0.036*	0.035*	0.000	0.062
Kolkata	0.085*	0.049*	0.055*	0.028	0.000

* Significant after Bonferroni adjustment (P<0.001)

Table 5.7 Nei's (1978) genetic identity (above diagonal) and genetic distance (below diagonal) using microsatellite markers in *Pampus argenteus*

	Veraval	Cochin	Chennai	Vishakhapatnam	Kolkata
Veraval	****	0.815	0.602	0.486	0.413
Cochin	0.205	****	0.578	0.608	0.491
Chennai	0.507	0.547	****	0.689	0.538
Vishakhapatnam	0.721	0.497	0.372	****	0.681
Kolkata	0.885	0.712	0.620	0.384	****

Table 5.8 Gene flow (Nm) among five locations of *Pampus argenteus* with 12 microsatellite loci.

	Veraval	Cochin	Chennai	Vishakhapatnam	Kolkata
Veraval	****				
Cochin	9.928	****			
Chennai	3.667	4.863	****		
Vishakhapatnam	3.135	6.775	6.971	****	
Kolkata	2.701	4.838	4.321	8.680	****

Table 5.9 Analysis of Molecular Variance (AMOVA) based on microsatellite markers in five locations of *Pampus argenteus*

Source ofvariation	d.f.	Sum ofsquares	Variancecomponents	Percentageof variation
Among groups	2	85.514	0.04918 va	0.88
Among locations Within groups	2	69.991	0.28693 vb	5.12
Among individualsWithin locations	248	1456.466	0.60342 vc	10.76
Within individuals	253	1180.5	4.66601 vd	83.24
Total	505	2792.47	5.60554	100

F_{IS} : 0.11451
F_{SC} : 0.05164
F_{CT} : 0.00877
F_{IT} : 0.16761

***P<0.001; Significance test after 1000 permutations

Table 5.10 Stock specific alleles and their frequencies in microsatellite loci of *Pampus argenteus*

Locus	Allele size (bp)	Stock 1	Stock 2	Stock 3
P15-2	296	0.021		
Par 01	402	0.031		
Par 06	196	0.031		
	198	0.031		
	138		0.020	
Par 08	206	0.061		
	222			0.033
	278			0.033
	252			0.040
	254			0.024
Par 20	216	0.051		
Par 05	330	0.020		
	230			0.024
	248			0.024
P-189	146	0.020		
	156	0.031		
	172	0.031		
	180	0.027		
	186	0.036		
	128			0.065
	130			0.048
	136			0.024
	138			0.073
	140			0.040
	270			0.032
	224			0.025
Par 03	228	0.045		
	208		0.020	
	234		0.020	
	238		0.060	
Par 18	228	0.055		
	234		0.040	
	252		0.020	
	272		0.020	
	260			0.033
Par 01	338		0.050	
Par 12	250		0.020	
	242			0.033
	278			0.033
Par 02	154			0.024

Table 5.11 Summary of the results of probability values for Wilcoxon's test using BOTTLENECK software in *Pampus argenteus*

Location	Two phase model	Stepwise mutation model
Veraval	0.96143	0.99915
Cochin	0.88330	0.99768
Chennai	0.99963	0.99768
Kolkata	0.99915	1.00000
Vishakhapatnam	0.99829	1.00000

Table 5.12 Garza–Williamson index (M-ratio) for all locations and loci in *Pampus argenteus*

	VER	COC	CHN	VP	KOL	Mean	SD
P15-2	0.1326	0.18705	0.16935	0.15287	0.19858	0.16809	0.02637
Par 01	0.48889	0.21176	0.25806	0.32258	0.40816	0.33789	0.11209
Par 02	0.26087	0.30000	0.19101	0.17284	0.26582	0.23811	0.05384
Par 03	0.30667	0.35052	0.23622	0.22481	0.35065	0.29377	0.0606
Par 06	0.1954	0.19753	0.2000	0.21053	0.20000	0.20069	0.00582
Par 08	0.06939	0.08550	0.07273	0.10526	0.15183	0.09694	0.03376
Par 12	0.17117	0.21212	0.19008	0.16814	0.19259	0.18682	0.01787
Par 17	0.05155	0.20792	0.17172	0.19802	0.13131	0.1521	0.06355
Par 18	0.20339	0.19718	0.21053	0.27869	0.19753	0.21746	0.03465
Par 20	0.17647	0.24742	0.31646	0.20253	0.23288	0.23515	0.05309
Par 05	0.15504	0.2233	0.27273	0.21239	0.2381	0.22031	0.04300
P-189	0.23529	0.17834	0.16031	0.24742	0.2071	0.20569	0.03684
Mean	0.20389	0.21655	0.2041	0.20801	0.23121	0.21275	0.01152

Figure 5.1 Allelic Patterns across five geographiclocations of *Pampus argenteus*

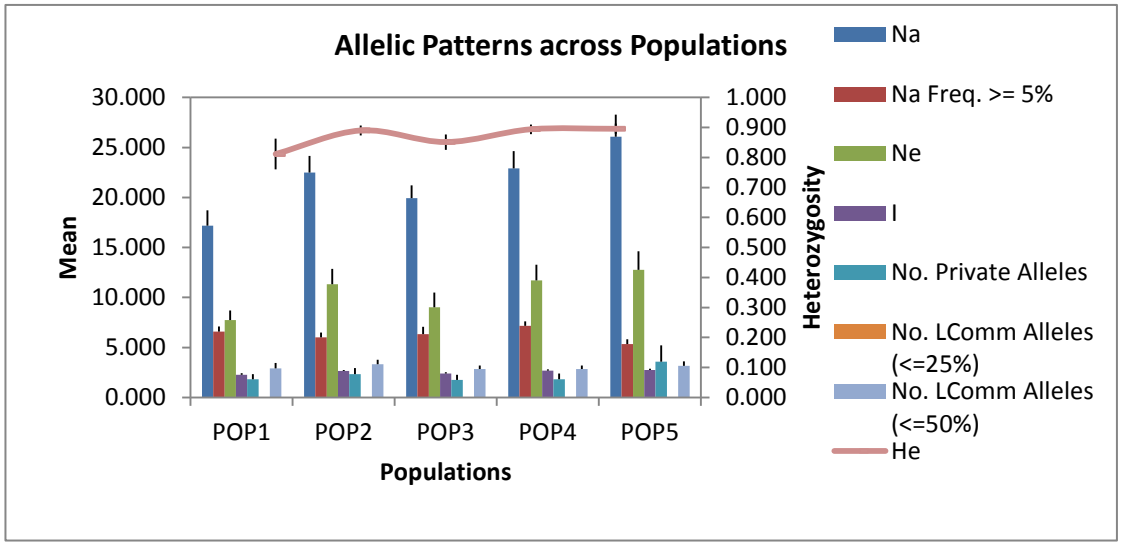


Figure 5.2 STRUCTURE bar plot of membership co-efficients (K = 3), for *Pampus argenteus* from five geographical regions along Indian Coast(1-Veraval, 2-Cochin, 3-Chennai, 4-Vishakhapatnam, 5-Kolkata)

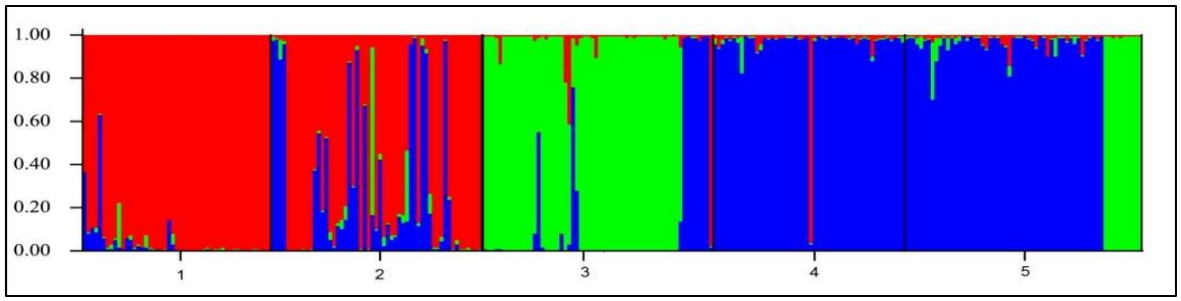


Figure 5.3 Plot of Delta K for the microsatellite data. (Peaks at 3 suggest that populations are hierarchically grouped into 3 groups)

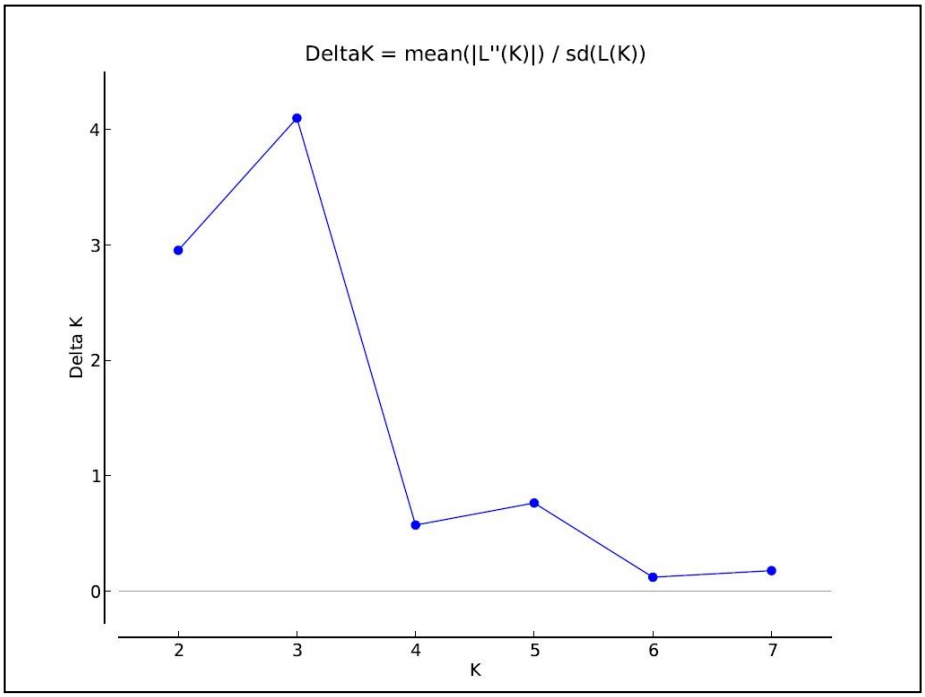


Figure 5.4 UPGMA tree of different locations of *Pampus argenteus* based on Nei's Genetic Distance (1978)

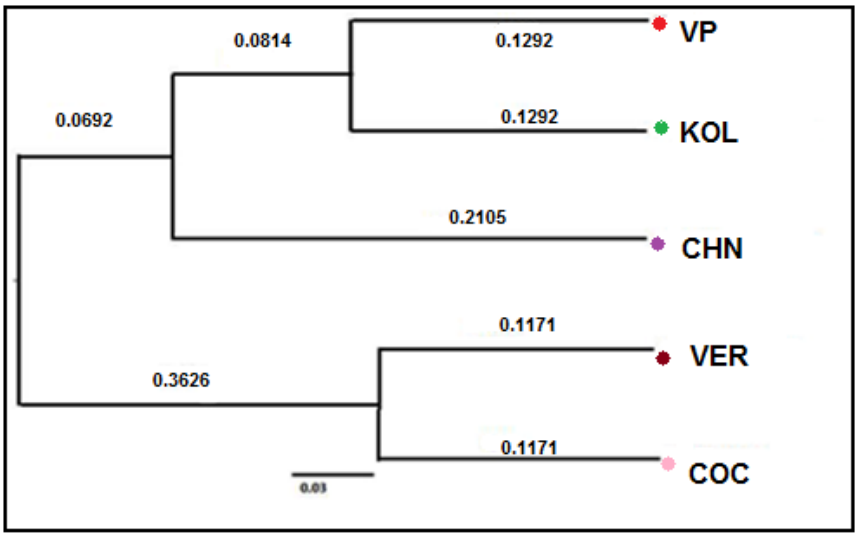


Figure 5.5 Scatter diagram based on PCoA of significant distance variables between locations of *Pampus argenteus*

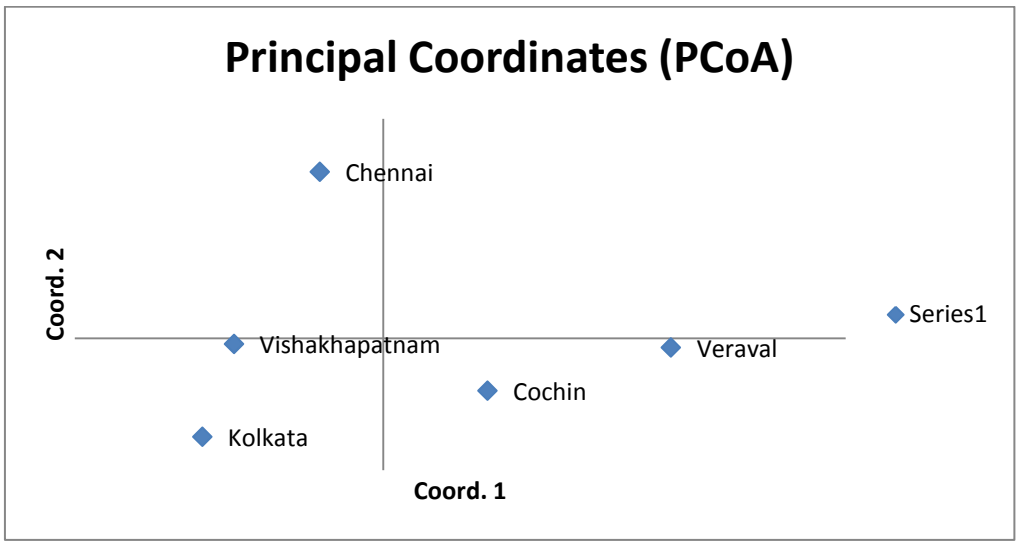


Figure 5.6 Correlation between pairwise F_{ST} values and geographic distance among five locations (Mantel test)

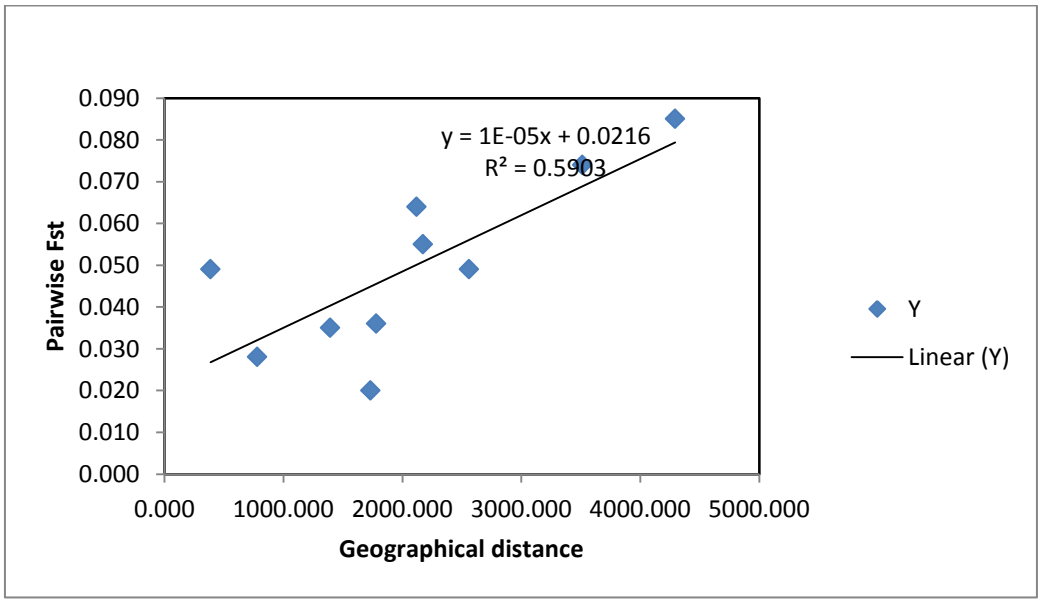
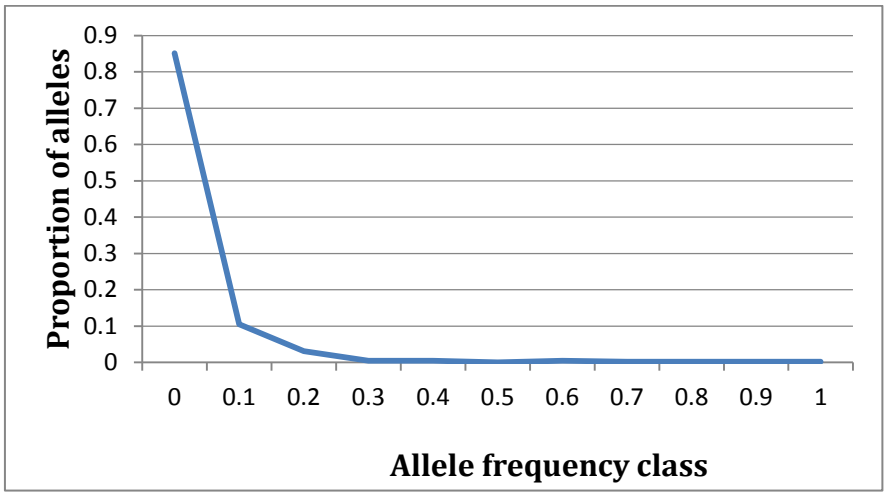


Figure 5.7 Allele frequency distribution for 12 microsatellite loci in the *Pampus argenteus* population. The distribution is L-shaped, as expected for a stable population under mutation-drift equilibrium.



Chapter VI

DISCUSSION

In the present study combined analysis of polymorphic microsatellites and mitochondrial (ATPase 6/8) markers was done for the genetic stock structure and demographic detection of *P. argenteus* from east and west coasts of India. This is the first reported work to assess the population diversity of the *P. argenteus* from a wide bio geographical region of India including Bay of Bengal and Arabian Sea using microsatellites and mitochondrial ATPase 6/8 gene.

5.1 Mitochondrial ATPase 6/8 Gene Analysis

5.1.1 ATPase 6/8 gene characteristics in *P. argenteus*

The size of ATPase 6/8 gene amplicon obtained in this study was 842 bp with the overlapping sequence of 10 bp; and contained 22 variable sites with nine parsimony informative sites. A 10 bp overlapping sequence was reported in some other fishes like American Cichlid *Andinoacara coeruleopunctatus* (McCafferty *et al*., 2012); *Channa marulius* (Habib *et al*., 2012); *Coilia dussumieri* (Kathirvelpandian *et al*., 2014). Most nucleotide variations were resulted from transitions followed by transversions (transition-transversion ratio for the in-group was 6.7). Similar results were obtained in *Channa marulius* (Habib *et al*., 2012); *Coilia dussumieri* (Kathirvelpandian *et al*., 2014); *Scomberomorous commerson* (Vineesh *et al*., 2016) and *Rachycentron canadum* (Linu *et al*., 2016). Nucleotide composition was found to be A = 29.4%, C = 26.9%, G = 13.2% and T = 30.6%. The anti G bias (13.33%) exhibited by ATPase 6/8 gene is the characteristic for the mitochondrial genome (Meyer, 1993). The nucleotide frequencies were found to be A+T rich like many other previous studies (Habib *et al*., 2012; Kathirvelpandian *et al*., 2014).

5.1.2 Population differentiation

Estimates of genetic differentiation between all the five locations were estimated. Low pairwise F_{ST} values was reported between samples from Veraval, Cochin and Chennai indicating low genetic differentiation among these locations; however statistically significant levels of genetic structuring was found for the samples between VER, COC, CHN and KOL, VP ($P< 0.001$). The analysis of molecular variance (AMOVA) (Table 4.5) also showed that 41.35% out of the total variance was attributed to differences between the groups (F_{CT}=0.41349), which was significant and also less variation within populations (5.14%) and among population (53.51%) (Table 4.5). The analysis also showed differences between the geographically distinct populations (F_{SC}= 0.912), which was significant ($P <0.001$). Similar type of stock structuring using mitochondrial genes was reported earlier by Horne *et al*., 2011 in *Eleutheronema tetradactylum*; Habib *et al*., 2012 in *Channa marulius*; Weng *et al*., 2013 in Chinese horseshoe crab; Rajeev *et al*., 2014 in *Labeo calbasu*; Kathirvelpandian *et al*., 2014 in *Coilia dussumieri*. Haplotype network tree was constructed using PopArt software and this can be used to infer population level genealogies when divergences are low (Gerber and Templeton 1996). The observed pattern and distribution of genetic variation was supported by the statistical parsimony based haplotype network tree and NJ tree analysis. Haplotype networks recreate the genealogical history of haplotypic variation and elucidate the evolutionary relationship among unique haplotypes. According to coalescent principles, the most common haplotype and that occupy central position in a network are assumed ancestral, while tip haplotypes are considered as more recently derived types (Crandall, 1996; Luharia *et al*., 2014). So the haplotype h01 from Kolkata must have been ancestral and precursor to other haplotypes including the population exclusive haplotypes. The haplotype network tree clearly indicated that there were two separate clusters consisted of both coasts of India. Interestingly no haplotypes were shared between Kolkata and Vishakhapatnam with the other locations. This indicates that there may be a barrier to gene flow between these geographic locations. This suggests a certain degree of reproductive isolation of *P. argenteus* populations leading geographical

structuring with limited or no gene flow between populations of silver pomfret in Indian waters. Minimum spanning network tree constructed by using PopArt software also replicated similar results.

5.1.3 Demographic history and population expansion

In conservation genetics, population demography is considered when inferring patterns of population structure. Molecular genetic data can offer the informations regarding the relationship among existent populations and can also disclose information on recent evolutionary history such as past population size (Avise *et al*., 1988). Mitochondrial DNA is maternally inherited and haploid and its effective population size is around one-fourth of nuclear DNA, resulting in increased rates of genetic drift and a faster approach to equilibrium between drift and migration in populations that do not have highly skewed sex ratios or extreme male polygyny (Birky *et al*., 1983). Mitochondrial DNA (mtDNA) has been extensively used to evaluate the intra-specific partition of the genetic polymorphism and to infer the evolutionary and demographic history of populations and species (Ballard and Whitlock, 2004). For mtDNA, the insight into the historical demography of *P. argenteus* was achieved by mismatch distributions (i.e. the observed number of differences between pairs of haplotypes), and classical statistical tests of neutrality (Tajima, 1989; Fu, 1997) over observed mtDNA haplotypes. A mismatch distribution is usually multimodal for populations at demographic equilibrium (i.e. stationary), and unimodal for populations that experienced a recent demographic expansion (Rogers and Harpending, 1992; Excoffier, 2004). The observed pair wise mismatch distributions for all the five locations of *P. argenteus* were not significantly different from the expectations predicted under a sudden population expansion model and these types of the results were reported in some other fishes like tasselfish (*Polynemus sheridani*) (Chenoweth and Hughes, 2003); West African estuarine fishes (*Sarotheradon melanotheron* and *Ethmalosa fimbiata*) (Durand *et al*., 2005); cobia (Linu *et al*., 2016); Seer fish (Vineesh *et al*., 2016). Harpending's (1994) raggedness test (RI for 'raggedness index') was used to

determine if the observed mismatch distribution was drawn from an expanded or a stationary population (small versus large RIs, respectively). SSD (sum of the square deviations) indicating the statistical significance between the two mismatch distributions was also measured. The nonsignificant values for SSD and raggedness index values also indicated that the values do not depart from that expected under the model of expansion.

Selective neutrality tests like Tajima D (Tajima, 1989) and Fu's FS (Fu, 1997) were also estimated because these tests are good signs of occurrence of demograohic events (Garcez *et al*., 2011). Significantly negative values of Tajima D anf Fu's Fs indicate an excess of low frequency polymorphism compared to that expected for null neutral hypothesis while the significantly positive values point out the signatures of genetic subdivision (Delrieu-Trottin *et al*., 2014). These tests are considered as the additional measures for tracing population growth expansion. Significant negative values of Tajima D and Fu's Fs in this study indicated that *P. argenteus* of both the coasts had experienced recent population expansion. A negative Tajima's D (-0.52994) as observed in this study signifies an excess of low frequency polymorphisms indicating population size expansion after a bottleneck (Peng *et al*., 2009). The negative values with significant *P*- values for Fu's F_S statistic (-1.51847) in silver pomfret populations indicated that there is departure from equilibrium which can be linked to large number of unique haplotypes or selection. Similar results were observed in *Rachycentron canadum* (Linu *et al*., 2016) and *Scomberomorous commerson* (Vineesh *et al*., 2016).

Haplotype diversity is used to quantify the uniqueness of a particular haplotype in a given population (Nei and Tajima, 1981). According to Nei (1978) nucleotide diversity is a measure of genetic variation, which is used to measure the degree of polymorphism within a population. In the present study, haplotype diversity for the entire population of *P. argenteus* was high. The origin of new haplotypes as observed from high haplotype diversity is possible as ATPase 6/8 gene is reported to have high mutation rate of 1.3% per million years (My) (Sun

et al., 2012). It needs special mention that populations along west coast are genetically more diverse than east coast as evidenced by more number of haplotypes. Similar results were reported in silver pomfrets by earlier workers (Sun *et al*., 2012). Based on different combinations of haplotype diversity (h) and nucleotide diversity (π) magnitudes of mtDNA sequence, marine fishes can be classified into four categories defined by Grant and Bowen (1998). Based on the above criteria, low nucleotide (π <0.5%) and lower haplotype (h<0.5) diversity (category I), which was found in pomfrets from east coast hints recent population bottle neck or founder event by single or few mitochondrial lineages and the low nucleotide diversity is the characteristics of populations with shallow genetic structure due to the rapid lineage sorting between small founder populations. Similarly, low nucleotide diversity (π <0.5%) and larger haplotype diversity (h>0.5) (category II), hints population bottle neck followed by rapid growth and accumulation of mutations which was found in pomfret samples from west coast. Similar pattern of nucleotide and haplotype diversities were noted in marine species like bill fishes (h=0.68–0.85, π=0.0018), Spanish sardines and haddocks (h=0.79–0.98, π=0.0029) (Grant and Bowen, 1998), *Coilia dussumieri* (h=0.8211-0.9368, π=0.0012-0.0025) (Kathirvelpandian *et al*., 2014); *Scomberomorous commerson* (h=0.809, π=0.0021) (Vineesh *et al*., 2016) and *Rachycentron canadum* (h=0.785, π=0.002) (Linu *et al*., 2016).

5.1.4 Phylogenetic relationships

Testing evolutionary hypotheses in a phylogenetic context becomes more reliable because reconstruction methods based on more realistic models of molecular evolution are available. Phylogenies from molecular data are often computed by pair-wise genetic distance based (numerical) methods like Neighbor Joining (NJ) tree, in which branch lengths are proportional to the amount of divergence. Neighbor joining takes as input a distance matrix specifying the distance between each pair of taxa. We can construct the phylogenetic trees based on "discrete methods" that operate directly on sequences like the Maximum Parsimony (MP) tree under which the phylogenetic tree that minimizes the total

number of character-state changes is to be preferred. In the present study both the numerical (NJ) and discrete (MP) tree making methods are used as in the analysis of most of the species. Both trees showed almost the same topology. They reliably indicated that two clear major groups –VER, COC and CHN as one group and KOL and VP as the second group. Haplotypes were mixed within the corresponding group but haplotypes were not sharing between the two groups. This may be due to the overall level of genetic exchange may be below that required to homogenise populations, or there may be a barrier to gene flow between these populations. This recommends some degree of reproductive isolation of *P. argenteus* populations leading geographical structuring with limited or no gene flow between these two clusters along the Indian coast. The result obtained from these two phylogenetic trees was in concordance with the pairwise F_{ST} values. Similar phylogeny pattern was also reported in previous studies like Finnerty and Block, 1992; Rosel and Block, 1996; Martínez *et al*., 2005; Chiang *et al*., 2008.

Based on the present results, it seems instinctive that the wild populations of (i) Veraval, Cochin, Chennai and (ii) Kolkata and Vishakhapatnam coasts of India should be managed separately. From a resource conservation and management perspective, the conclusion of the study also strongly indicates the need for adoption of stock specific rehabilitation programmes for *P. argenteus* from Indian waters.

5.2 Microsatellite analysis

5.2.1 Microsatellite development through cross species amplification

Microsatellites have been isolated and characterized in a large number of fish species and have wide range applications in population genetics (Dunham, 2004). Primer development is a crucial component in the success of SSR markers in population study. Many microsatellite loci are having extremely fast rates of repeats evolution and quite conservative in their flanking regions and hence can persist for long evolutionary time spans much unchanged. Due to this, primers

developed for a species from the flanking regions of a microsatellite locus can be used to amplify the same locus in other related species. For population genetic analysis, cross-species amplification has been a useful tool to identify set of markers without developing specific primers for each species. This is a cost effective method for identifying polymorphic microsatellite loci compared to other methods like NGS. In this study, a total of 12 polymorphic primers were used; out of which 10 were developed in own species and two were developed through cross priming from the related species *Pampus cinereus*. Primers developed for a species by this method have been effectively tested for cross-species amplification on its related species. (Lal *et al*., 2004; Gopalakrishnan *et al*., 2004; Ma *et al*., 2011; Sajeela *et al*., 2015; Luo *et al*., 2015). From this study, it is inferred that the availability of the cross polymorphic loci presented here will be helpful for identifying the stock structure and diversity assessment of *P. argenteus* and other related species. These loci also have potential as DNA markers in various genetic studies such as pedigree analysis and quantitative trait locus mapping to elevate breeding technology. The optimum annealing temperature to get scorable band in *P. argenteus* differed from that reported for the respective primer pair in the resource species. Galbusera *et al*., 2000 and Zardoya *et al*., 1996 reported necessity of optimization of PCR conditions for the study species in cross-amplification tests.

In *P. argeneteus,* seven amplified microsatellite loci were sequenced and all loci were confirmed to contain microsatellites and dinucleotide repeats. All of the microsatellites sequenced were perfect and the base pair falls within the range which was similar to the loci developed in *P. cinereus*. But, the type of repeat motif in the resource species and *p. argenteus* differed in some loci. This can be due to the extremely fast rates of repeat evolution that may differ among loci, but keeping the highly conservative flanking regions unchanged, as reported by Zardoya *et al*. (1996) in other perciform fishes. The study found GT and CA rich microsatellites abundant in *P. argenteus* which is in conformity with the published reports (Na-Nakorn *et al*., 1999; Neff and Gross, 2001; Watanabe *et al*., 2001; Usmani *et al*., 2003). Weber, 1990 reported that usually most of

dinucleotide alleles are always visible as a ladder of bands rather than a single discrete product due to slipped-strand mispairing during PCR. Bezault *et al.,* (2012) reported that the phylogenetic relationships and evolutionary distance between the different groups used for cross amplification from the target species reflect the success of cross-species amplifications.

5.2.2 Genetic variability and deviation from Hardy-Weinberg Equilibrium

The number of alleles for *P. argenteus* using microsatellite loci in present study is 28–64 alleles per locus. Primers P-189 exhibited maximum allele number (64) compared to other primers (28 alleles for Par 17 and Par 06). Mean number of alleles per locus ranged around 21.717 for 12 microsatellite loci. This finding is similar to the results reported by Ruggeri *et al*., 2013 in another marine pelagic fish *Sardina pilchardus* sampled from European waters where the average number of alleles per locus ranged from 12-52. The number of alleles per loci reported in this study is higher than what was reported in earlier studies in silver pomfrets by other researchers (13-17 alleles/loci; Archangi *et al*., 2013 and 3-10 alleles/loci; Qin *et al*., 2013). Unlike the earlier studies, more number of alleles noted in this study may be attributed due to the usage of automated genotyping for allele separation and increased sample size (Lindqvist *et al*., 2016). DeWoody and Avise (2000) and Neff and Gross (2001) explained that compared with freshwater species, marine species have greater microsatellite allele variation and this was dependable with the increased evolutionary effective population sizes of marine species. More number of alleles in the microsatellite loci of *P. argenteus* was in similar line with general higher number of microsatellite allele variation in marine fishes (Neff and Gross, 2001). High microsatellite allele variation was also reported in a number of marine species such as oyster (3–57 alleles/locus; Yu *et al*., 2005); Atlantic salmon (5–41 alleles/locus; Nikolic *et al*., 2009); whale shark (3–34 alleles/locus; Schmidt *et al*., 2009); red sea bream (16-32 alleles/locus; Takagi *et al*., 1999) and in Atlantic cod (8-46 alleles/locus; Bentzen *et al*., 1996).

In the present study, the reported range of H_o and H_e was from 0.764 - 0.997 and 0.705–0.946 respectively, whereas the microsatellites study for silver pomfret from Chinese waters reported H_{obs} and H_{exp} in the range of 0.53–0.94 and 0.66–0.91 respectively (Qin *et al*., 2013). Our studies showed that observed heterozygosity of silver pomfret in Indian waters was less than expected heterozygosity. At P<0.0001, significant deviation from HWE, indicating heterozygote deficiency, was detected at most loci, in the sampled populations. Wright (1978) fixation index (F_{IS}) is a measure of heterozygote deficiency or excess (inbreeding coefficient) and the significant values for each locus in five geographic locations are given in Table 5.5. Positive F_{IS} indicating heterozygote deficiency was evident in all loci except Par 17 and P-189. Similar findings were reported by Archangi *et al*., 2013 for marine species. Deviations from HW equilibrium can be due to several reasons (Beaumont and Hoare, 2003): due to less sample size analyzed (Na-Nakorn *et al*., 1999), null alleles (Gopalakrishnan *et al*., 2009), selection (Langen *et al*., 2011), or grouping of gene pools (Wahlund effect), reduction in effective breeding populations, inbreeding or non-random mating (O'Leary *et al*., 2013). Small sample size can be a reason for failure in detecting all alleles in the populations. In the present study sample size was 70 per location. According to Silva and Russo (2000) sample sizes more than 30 and Ruzzante (1998), sample sizes of 50 ≤N≥100, were generally necessary for precise estimation of genetic distances and this value depended on number of loci, number of alleles, and range in allele size. We consider the sample size in the present study was sufficient enough for the analysis.

Usually another possibility for increased homozygosity is due to null alleles, large allele drop outs and stutter bands. Null alleles are the alleles which do not amplify during PCR due to mutation events changing the DNA sequence in one of the primer sites (mostly in 3' end), which causes the primer no longer to anneal to the template DNA during the PCR (Van Oosterhout *et al.*, 2004). Null alleles arise when mutations occur within the primer-binding sequences, which block the primer from annealing and amplifying one or both alleles in an individual. In such a situation, heterozygotes can be misinterpreted as

homozygotes biasing estimates of allele frequencies and finally the observed heterozygote deficiency will happen in the population (Pemberton *et al*., 1995). It is challenging to identify the presence of null alleles because panmixia and non-violation of any H-W assumption have to be assumed. In most of the cases, absolute identification of a null allele is not possible but it can be done by pedigree analysis for instance as in Callen *et al*., 1993. Once more, the presence of "stutter bands" can lead to scoring inaccuracies revealed by an apparent deficiency of heterozygotes (O'Reilly and Wright, 1995). But, in the current study, the analysis of data using micro-checker indicated, occurrence of null alleles in all the five populations is very unlikely for the 12 primer pairs. Stutter bands were practically absent in the present study, hence the possibility of changes in allele sizes due to stuttering can also be ruled out. Large alleles normally do not amplify as efficient as small alleles, leading to large allele dropouts (Van Oosterhout *et al.*, 2004). In the present investigation, all the amplified products were dinucleotide repeats and generally large alleles were not encountered. Moreover, the possibility of null alleles, large allele dropouts and stutter bands was ruled out by analysing the data using MICRO-CHECKER software (Van Oosterhout *et al*., 2004). Due to overexploitation, inbreeding can happen, which might result in deficiency of heterozygotes and deviation from HWE, as reported by Beaumont and Hoare (2003). Similar situation was reported in various fish species that showed decline in catches (Gopalakrishnan *et al*., 2009). The Wahlund effect (Walhund, 1928) is also a reason for deficiency of heterozygotes and this can occur if a sampled population is divided into smaller reproductive groups. Heterozygote deficiency is removed when populations are panmitic with large effective sizes, no barriers to dispersal and random mating.

Thereby, it is assumed from three inferences: dominance of expected heterozygosity than observed heterozygosity; majority of the loci deviating from Hardy Weinberg equilibrium (10/12microsatellite loci) and the positive F_{IS} value obtained in all loci, that the homozygosity excess exists in the silver pomfret population distributed in Indian waters. It gives indication that *P. argenteus* populations in Indian waters are being exploited at a higher level than the

optimum (Ghosh *et al*. 2009). According to them, the length at first capture (Lc) for pomfret was found to be 8.20 cm, which was very low when compared to the length at first maturity in silver pomfrets (Lm) of 27.5 cm indicating that majority of them were caught before they matured and spawned at least once in their life. Boopendranath *et al*., 2012 suggests that the pomfret stock in Indian waters is under more fishing pressure warranting immediate decrease in fishing effort for the optimal management of this species due to higher exploitation ratio and as the species Maximum Sustainable Yield (MSY) being lower than their annual catch. Excessive trawling and use of small meshed trawl were leading to depletion of the pomfret stock in Indian waters. Due to this sort of overexploitation, inbreeding can happen, which might result in deficiency of heterozygotes and deviation from HWE, as reported by Beaumont and Hoare, 2003. Similar situation was reported in various fish species that showed decline in catches due to overexploitation (Gopalakrishnan *et al*., 2009). Hence, the possible causes for the excess homozygosity levels can be speculated as overexploitation of the species over the years (FAO, 2009) due to habitat alteration by trawling and use of undersized nets, leading to reduction in catches, ending with inbreeding. According to Menezes *et al*., (1998), intense fishing of marine fishes including pomfrets in certain habitats, cause the elimination of distinct, locally adapted stocks, resulting in loss of diversity and the adaptive potential of that species. There were no significant associations indicative of linkage disequilibrium between any pair wise combination of microsatellite alleles in *P. argenteus* (after Bonferroni correction). It is therefore assumed that the allelic variation recorded at all the microsatellite loci could be independent as observed in many fishes (Na-Nakorn *et al*., 1999; Scribner *et al*., 1996; Usmani *et al*., 2003). These kind of results were reported in many fish species. In Korean rose bitterling (*Rhodeus uyekii*), out of the 18 loci, six loci showed significant deviation from Hardy-Weinberg equilibrium after Bonferroni's correction (Kim *et al*., 2014). In *Siniperca chuatsi* twelve microsatellite loci deviated significantly from Hardy-Weinberg equilibrium (Tian *et al*., 2014). Gonzalo *et al*., 2015 used 12 markers for assessing the populations of *Seriola lalandi* and most of these loci showed

deviations from Hardy-Weinberg equilibrium with moderate inbreeding. In *Epinephelus akaara* among the ten loci, three loci deviated from Hardy-Weinberg equilibrium after sequential Bonferroni's correction (Xie *et al*., 2015).

5.2.3 Genetic Differentiation

The population delineation using genetic markers requires a noticeable level of genetic differentiation (Ward *et al*. 1994). In the marine environment, many studies have failed to identify statistically significant population structuring because of low level of differentiation, especially over small geographical distances (Árnason *et al*., 1992). Wright's F-statistics helps to conclude the monitoring of genetic differentiation by describing the hierarchically subdivided populations as "the correlation between random gametes within a population, relative to gametes of the total population" (Wright, 1965). Three indices F_{IS}, F_{IT} and F_{ST} (depending on the hierarchical level being compared) are used in F-statistics. F_{ST} is a measure of overall population subdivision and has a value between zero (no differentiation) and one (complete differentiation). The present findings of genetic analysis with microsatellite markers in *P. argenteus* suggest that propagation assisted stock enhancement program may well be implemented to protect stocks from the ill-effects of overexploitation. Pairwise and overall F_{ST} and R_{ST} values (0.050 and 0.1392) (P<0.001) among five populations indicating the existence of three distinct genetically structured management units of *P. argenteus* along the Indian waters *i.e.* west coast populations of India 1. (VER and KOC) and east coast populations 2. (CHN); 3. (VP & KOL). Sun *et al*., 2012 found high genetic divergence between pomfrets from Mumbai, India (Arabian Sea) and Ngao, Thailand (Bay of Bengal). R_{ST}, an analogue of F_{ST}, provides a convenient approach for estimating levels of genetic differentiation from microsatellite data. The R_{ST} statistic has been proposed by Slatkin, (1995) based on the assumption that in microsatellites allele size depends on the size of its ancestor, assuming the stepwise mutation model contrary to the Infinite Allele Model assumed in F_{ST}. Higher R_{ST} values compared to F_{ST} were obtained in *P. argenteus*. According to Hardy *et al*., 2003 the R_{ST} and F_{ST} are generally similar

when the level of significant genetic differentiation is comparatively low, whereas R_{ST} is often higher than F_{ST} when differentiation is high. The present study involved a deeper understanding of the population structure of *P. argenteus* along Indian waters and it revealed that *P. argenteus* distributed along Bay of Bengal was genetically distinct with respect to specimens from Arabian Sea. Similar kind of stock structuring of marine species using microsatellites has been reported from Indian waters (Menezes *et al*., 1998, Karan *et al*., 2012; Mandal *et al*., 2012). AMOVA (Analysis of Molecular Variance) (Excoffier *et al*., 1992) is another useful method for detecting population genetic structure. It will allow the hierarchial analysis by grouping the total variance into covariance components at the level of individuals within populations, sub-populations or demes within regions and then between populations between geographic regions. The analysis of molecular variance (AMOVA) for the *P. argenteus* showed that 41.35% out of the total variance was attributed to differences between the groups (ie, east and west coast of India) (F_{CT}=0.41349), which was significant and also less variation within populations (5.14%) and among population (53.51%). The analysis also showed differences between the geographically isolated populations (F_{SC}= 0.912), which was significant (P <0.001). The statistically significant differentiation parameters are indication of their reproductive isolation and recommend that they belong to genetically dissimilar stocks which do not interbreed (Allendorf, 1979). So the *P. argenteus* populations along Indian coast are differentiated into separate gene pools and therefore these three populations should be treated as separate management units.

The connectivity assessments based on gene flow (Nm) have also being used to inform the design of marine protected area networks (Palumbi, 2003) because it can influence the potential for differentiation and also influence local population persistence (Avise *et al*, 1988). However population genetics cannot always differentiate between contemporary and historical gene flow. Standard estimates of migration among populations are increasingly imprecise at scales where there may be limited population differentiation (Hedgecock *et al*., 2007). Locus wise gene flow obtained for *P. argenteus* populations was moderate

ranged from 2.368 (Par 20) to 12.863 (Par 05). The very low genetic differentiation between samples collected within the west coast and VP-KOL stock in the east coast suggests high levels of gene flow. Low levels of differentiation in marine organisms is mainly because of the extensive gene flow (Waples, 1998; Avise, 2000) and do not necessarily imply that structuring does not exist, but that more powerful means are required to detect them (Waples, 1998). Marine organisms are poorly differentiated on a small geographical scale, usually show clear indication of differentiation over larger distances, probably because the long distance acts as an isolation mechanism. Gene flow can happen at either the egg or larvae stage by passive drift along with the ocean current, by active migration, or both. Passive transport of egg or larvae seems the most likely explanation because transport over long distances is possible in a short time (Knutsen *et al*., 2003). On one hand, gene flow was extremely limited between east and west coast populations of *P. argenteus*. The result was not surprising because the populations are located in different ocean basins separated by over 1,000km. Low gene flow between populations caused by physical, hydrographical and historical demographic factors are playing a chief role in shaping the genetic structure of the species at this regional scale. Genetic identity and distance based on Nei (1978) between pairs of populations of *P. argenteus* (Table 5.7) showed discrete population structure among the species. The highest genetic distance and the least genetic identity were found between Cochin and Kolkata populations, indicating high genetic differentiation between them.

The Bayesian modeling approach has several advantages over other approaches, such as multiple regression models (Cressie *et al*. 2009). It allows sampling or measurement error to be separated from actual variation in underlying abundances, which can progress measurement of the underlying biological processes (Clark, 2005) and this method allow considerable flexibility in modeling of biological processes, so a wide variety of process models can be formulated and fitted within a common framework. The findings from our STRUCTURE analysis identified three distinct population clusters; Veraval and Cochin comprised one cluster, Chennai a separate cluster while the other two

collections comprised the third cluster (K=3). Hierarchical STRUCTURE analysis revealed no further substructure. The apparent lack of genetic mixing between these three genetic clusters suggests that a barrier to either natural dispersal or reproduction exists between them. To confirm the results obtained from STRUCTURE, we conducted two additional analyses enabling us to visualise distribution of individuals. First, we performed Principal Coordinate Analysis (PCoA). PCoA is a scaling or ordination method that starts with a matrix of similarities or dissimilarities between a set of individuals. It facilitates valuations of the dimensionality of the data, as well as the major patterns of deviation within and between populations (Cresswell *et al*., 2001). The two dimensional PCoA plot shows that the first principal coordinate accounts for 50.19% of total variation and separates the populations of east (Kolkata and Vishakhapatnam) and west coast (Veraval and Cochin). PC2 forms 26.09% of variation and separates Chennai. The dispersion of genetic variability analyzed by the Principal Coordinate Analysis (PCoA) suggests that Chennai form a somewhat distinct set from the Kolkata and Vishakhapatnam. Similar types of structuring using PCoA was studied earlier in many marine fishes (Christie *et al*., 2010 in coral reef fish; Schunter *et al*., 2011 in Mediterranian fish; beneteau *et al*., 2012 in greenside darter and Antilla *et al*., 2014 in Atlantic salmon). Second, a dendrogram was constructed according to Nei's (1978) genetic distance and UPGMA method. Nei's unbiased genetic distances are considered suitable for long evolutionary processes, with population divergences due to genetic drift and mutational events (Weir, 1990). Among various agglomerative hierarchical methods, the UPGMA (Unweighted Paired Group Method using Arithmetic averages) (Sneath and Sokal, 1973) is the most commonly adopted clustering algorithm. UPGMA dendrograms have tended to predominate by in past literature and this method is generally stable with regard to the allocation of clusters when different types and number of characters were used (Mohammadi and Prasanna, 2003). UPGMA clustering reflects the grouping of populations according to geographical origin. In this work three main clusters can be observed in the dendrogram similar to the results of STRUCTURE and PCoA.

Population structure can result from limited gene flow between groups of individuals within a metapopulation. This phenomenon is known as isolation-by-distance (IBD) and is a simple consequence of limited dispersal across space. IBD was evaluated by testing for a statistically significant association between genetic differentiation as measured by Fst/(1 – Fst) (Rousset, 1997) and geographic distance among the five sample locations. Although high levels of gene flow are found in marine organisms, isolation-by-distance (Wright, 1943) has been reported for a number of fishes and invertebrates (Benzie, 1999; Mamuris *et al*., 1999; Huang *et al*., 2009). There is some evidence that relationships between geographical and genetic distances depend on sampling scale. For example, high levels of genetic divergence are observed in Pacific urchins (Palumbi *et al*., 1997) and coconut crabs (Lavery *et al*., 1996) but only over large geographical scales (> 5000 km). In the present study, the IBD observed was significant $p<0.05$; $r2 = 0.768$. Significant IBD was evident in other species like American Shad (Hasselman *et al*., 2013); *Alosa pseudoharengus* (McBride *et al*., 2015) etc.

In this study, there were many alleles specific to the particular stock. Presence of private alleles is an indication for non mixing of the gene pools between the populations of each coast (Table 5.10). Scribner *et al*., (1996) reported 22 stock specific alleles in three populations of Chinook salmon (*Oncorhynchus tshawytscha)* in Canada. 34 stock specific alleles were obtained in three spotted seahorse, *Hippocampus trimaculatus* in India using four microsatellite loci (Thangaraj *et al.*, 2012). Basak *et al*., (2014) also reported the stock specific alleles in two populations of *Catla catla* in Bangladesh waters. Musammilu *et al*., (2014) also reported the presence of nine private alleles in four loci in three populations of *Gonoproktopterus curmuca*. In *Argyrosomus inodorus*, Henriques *et al*., (2015) reported one to seven private alleles in seven loci in two populations. The stock specific microsatellite markers (private alleles) can be used as "genetic tags" for mixed stock analysis and these can be employed to detect the level of genetic differentiation (Appleyard and Mather, 2002). In the current study, the stock specific microsatellite markers would be useful in

developing stock specific management programmes and also in traceability studies.

5.2.4 Possible reasons for population genetic structure in *P. argenteus*

Mostly, lower levels of genetic differentiation are reported in marine fishes due to higher dispersal potential during planktonic egg, larval or adult by life history stages, coupled with an absence of physical barrier to movement between ocean basins (Mandal *et al*., 2012). The continuously changing coastal current pattern during different seasons may result in exchange of larvae along the Indian coast, resulting in low population genetic differentiation (Mandal *et al*., 2012). Sun *et al*., 2012 found high genetic divergence between pomfrets from Mumbai, India (Arabian Sea) and Ngao, Thailand (Bay of Bengal) and concluded that the mainland of India blocks genetic exchange between Bay of Bengal and Arabian Sea. The reason may be attributed for genetic differentiation of pomfret in the Northern part of both the coasts of India. Low mean sea surface temperature, mixing of freshwater from the peninsular rivers and the continental shelf pattern differentiates the profile of Bay of Bengal from that of Arabian Sea (Jaswal *et al*., 2012). Some other dissimilarities are also present between the two basins. The winds over the two basins are different, this difference being most striking during the summer monsoon (Figure 6.1) (Shenoi *et al*., 2002). This is because the Arabian Sea being surrounded on its west by the highlands of East Africa. These highlands serve as the western boundary for the low-level atmospheric flow, which will lead to the formation of a "western boundary current," just like in the oceans (Anderson, 1976) known as the Findlater Jet (Findlater, 1969), makes the winds over the Arabian Sea more than twice as strong as those over the Bay of Bengal, which does not experience a similar western boundary effect (Shenoi *et al*., 2002). In addition to the differences in horizontal distribution of salinity between the Arabian Sea and Bay of Bengal, the vertical distribution of salinity also differs considerably between the two basins (Prasad, 2004). Such strikingly varied oceanographic features in the North Bay of Bengal region may be playing an influential role for the large scale differences in the genetic diversity of silver pomfret populations of Kolkata coast.

The average genetic divergence in silver pomfret using ATPase 6/8 genes exceeds the usual intra specific range and it has to be suspected that *P. argenteus* distributed in the Indo-west Pacific region might have diverged at the population /sub species level. Dispersal distances and directions have a profound effect on gene flow and genetic differentiation within species (Froukh and Kochzius, 2007). However, this hypothesis can be proved only by a correlative study of these factors with larval/adult migration pattern of this fish.

During the south west monsoon season (Figure 6.2), the coastal currents are following a movement towards equator. With the spawning occurring during south west monsoon, the mixing of stocks is possible between Veraval and Cochin coasts. There can be a possible recruitment from Oman coast up to Cochin where the stocks are of similar nature. The differentiation between Cochin and Chennai may be partially attributed to recirculation cells observed in the Western boundary of bay of Bengal as described by Durand *et al*., 2009. The currents along the east coast of India seems to have less effect on the larval dispersion. As the southern tip, prominantly known as the coramandel coast has a unique stock of pomfret. This may be probably due to less mixing of gene pool in the Gulf of Mannar and Palk Bay ecosystems and by coastline areas which are shallower and does not allow a free flow of the currents. Gulf of Mannar region is rich in corals, and the bottom is generally rocky and sandy with narrow gullies of muddy bottom. Further north in the east coast, the stratified Bay of Bengal coastal waters also indicate the presence of a unique stock. Within the stratified waters of Bay of Bengal, the possibility of a mixing is ruled out from the southern coastal waters.

Figure 6.1 The geography of the north Indian Ocean. The two major rivers, Ganga and Brahmaputra, that debouch into the northern Bay of Bengal, are indicated by RG and RB, respectively. (Courtesy: Shenoi *et al*., 2002)

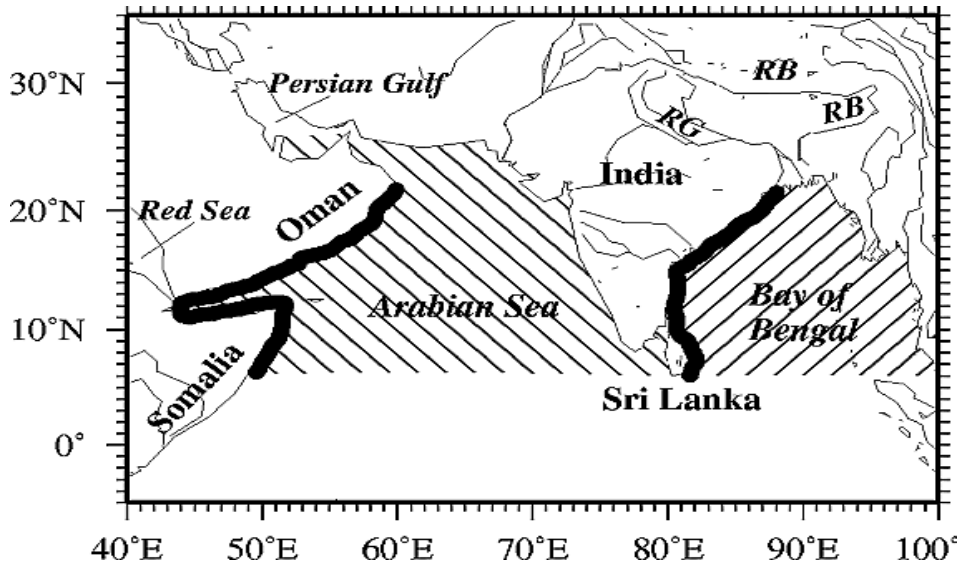
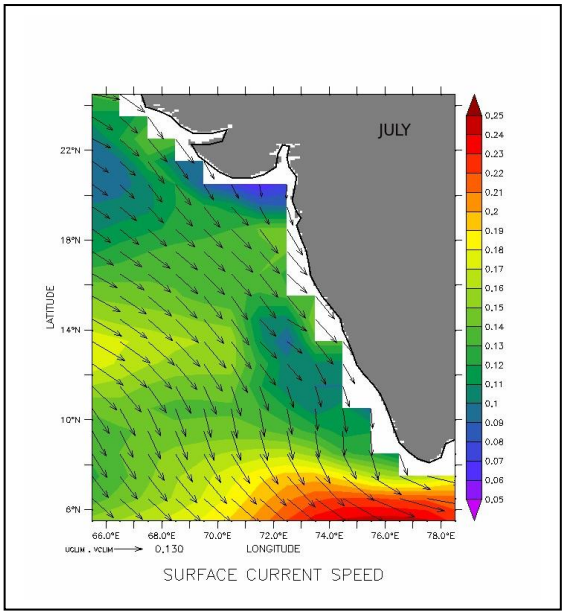
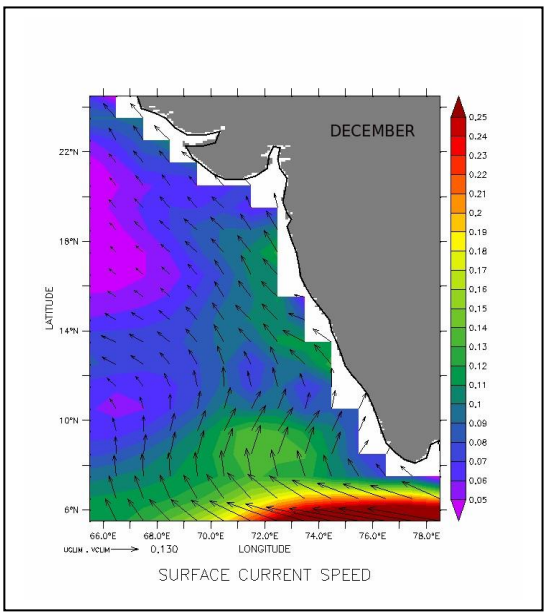


Figure 6.2 Climatology of currents [speed (m/s^2) and direction] during south west monsoon and north east monsoon along Indian coast





5.2.5 Bottleneck analysis

Population size reductions are often occurring in nature by several orders of magnitude and it is one of the important issues in consevation biology. This population size depletion causes the loss of genetic diversity (Frankham 1996) and allele fixation (Lande, 1994) that can lower the probability of short-term population existence and it will reduce the ability of a population to respond to environmental changes (Frankham, 1995). As a result, identification of drastic population declines remains one of the important issues in conservation biology. An increasing number of studies have detected such bottleneck footprints in various species groups including insects (Dhuyvetter *et al*., 2005), molluscs (Bouza *et al*., 2007), birds (Lambert *et al*., 2005), fish (Vasema¨gi *et al*., 2005; Va¨ha¨ *et al*., 2007) and mammals (Lucchini *et al*., 2004). This genetic bottleneck produces obvious genetic impression in extreme cases only (Luikart and Cornuet, 1998).

The bottleneck analysis investigates the level of genetic diversity in the silver pomfret population along the Indian coast and a possibility of population size contraction, since there are indications about the catch decline in the last two decades (CMFRI, 2014-15) which might have caused reductions in the effective population size. The results obtained in this study do not indicate reduction of genetic diversity in the population of silver pomfret in all the five geographic location measured by 12 microsatellite markers, since all the loci were highly polymorphic with a large number of alleles, high allelic richness and heterozygosities. When comparing these results with the results of investigations of genetic diversity of other silver pomfret populations or stocks (Archangi *et al*., 2013), a high level of genetic diversity in the investigated Indian waters is confirmed. This may be due to large sample size and automated genotyping used in this study.

The evidence for a genetic bottleneck in the silver pomfret population was confusing. The Bottleneck analysis using Wilcoxon test and Mode shift test did not identify adequate confirmation for a bottleneck. The Wilcoxon signed rank test is the most suitable and authoritative method for bottleneck detection if we

are analyzing fewer than 20 loci (Piry *et al*., 1999). Here we employed SMM and TPM methods because these are generally more appropriate when testing microsatellite data (i.e. dinucleotide repeat loci) (Luikart and Cornuet, 1998). However, all loci will follow a mutation model somewhere in-between the two extreme models (Piry *et al*., 1999). When testing both the extreme models, the null hypothesis of the Wilcoxon's test (no significant heterozygosity excess on average across loci) cannot be rejected and thus it suggests that there is no satisfactory evidence for a recent bottleneck in the silver pomfret population. Furthermore, analysis of allele frequency distribution failed to distinguish a mode-shifted distribution of allele frequencies, also suggesting that a bottleneck is not likely to have occurred in the recent past. But, the low values of M ratio using Arlequin software suggested a recent bottleneck in the silver pomfret population. The inconsistency between the results of the bottleneck analysis and M ratio test led us to question our sample size. We did genotyping of 70 individuals per each population with 12 microsatellite loci. Piry *et al*., (1999) suggested typing at least 10 polymorphic loci and sampling at least 30 individuals in order to achieve a convincingly high statistical power. So in this case we concluded that our sample size is enough to meet the requirements. Luikart and Cornuet, 1998 suggested that the bottlenecks can go unnoticed if they were either not very severe or were very recent. In addition the sampled individuals were not representative of the bottlenecked population (Piry *et al*., 1999). With the putative evidence of past studies (Mohamed *et al*., 2010) revealing silver pomfret decline in mind, we are more willing to accept the evidence for a recent bottleneck as evidenced by the M ratio test. As a result, we suggest managing the current level of silver pomfrets and cautious monitoring of the population in the future.

5.3 Combination of both markers in *P. argenteus*

In the present work we examined the fine-scale population structure in *P. argenteus* collected from five geographical locations in the Northern Indian Ocean using polymorphic microsatellite and mitochondrial (ATPase 6/8)

markers. Fast evolving mitochondrial genes like ATPase 6/8 and microsatellite markers could be considered as random indicators to discriminate the populations of *P. argenteus*. It is needed to compare the results obtained through these two markers for delineating genetic stock structure. The variation due to microsatellites is expected to be a more sensitive indication of the mutation rate compared to mitochondrial DNA markers. Furthermore, mitochondrial genes are frequently criticized as not being representative of the actual species tree because of problems associated with random lineage sorting, introgression, and sex-biased gene flow (Moore, 1995). Commonly, morphological, mitochondrial, and nuclear gene trees are discordant (Chan and Levin, 2005). Thus, analysis of multiple independent loci can be more informative than single gene trees. To date only few studies have compared the results of mitochondrial ATPase 6/8 genes with microsatellites (Dammannagoda *et al*., 2011; Broderick *et al*., 2011). Interpreting the relative roles of historical and contemporary processes in determining genetic structure of natural populations remains a persistent challenge in marine population genetics and conservation. In this study lack of congruence between nuclear and mitochondrial-based variation is found and may be due to the divergence was much more pronounced revealed by microsatellites compared to mitochondrial DNA. This may reflect increased rate of differentiation by genetic drift at the nuclear genome compared with the mitochondrial genome and microsatellite markers can give clues about recent and contemporary events in populations due to their high mutation rates. Microsatellite markers are highly variable, and this can increase the overall resolution power to detect genetic differentiation (Waples, 1998). Similar results also been previously reported, for example in cartilaginous fish, the blacktip shark (Keeney *et al*., 2005) and teleost, the European bitterling (Bryja *et al*., 2010). This inconsistency between mt DNA and microsatellite data in these studies was thought to be due to male-biased gene flow which would lead to homogenization of nuclear alleles while the maternally inherited such as mitochondrial markers would remain restricted to native localities (Nyakaana and Arctander 1999).

5.4 The utility of the approach employed

This study is unique in providing the first comprehensive genetic survey of *P. argenteus* within the Indian waters using mitochondrial ATPase 6/8 gene and microsatellites. Effective management of fisheries resources requires critical information on the population or stock structure of the exploited species. Therefore, the present study has important implications for the conservation of genetic diversity in *P. argenteus*. Analysis of *P. argenteus* using both mitochondrial ATPase 6/8 genes and nuclear microsatellite markers revealed a high level of genetic variation between populations of geographically isolated habitats of the Indian waters. The findings of the present study have direct relevance to the definition of conservation units for this species. Management Units (MUs) represent sets of populations that are currently demographically independent and are recognized by significant divergence of allele frequencies at nuclear or mitochondrial markers (Moritz, 1994). Each stock may behave biologically diverse manner in adapting to the environment with distinguish evolutionary pattern. Under this criterion, all the three stocks from the current study can be proposed as distinct Management Units, which require separate monitoring and management. So this information on genetic variation and stock structure of silver pomfr*et al*ong Indian coast can be used for genetic upgradation, aquaculture, fisheries management and conservation programmes in Indian waters.

SUMMARY

Silver pomfret (*Pampus argenteus*) (Euphrasen, 1788) belonging to the family Stromateidae is a potential fin fish species for mariculture in Asian countries. Silver pomfret, *P. argenteus* is widely distributed throughout the Indo-West Pacific: from the Persian Gulf to Indonesia, Japan, West and Southwest of Korea and Eastern parts of China. Along the Indian coast, percentage of silver pomfret landings reduced from 2.18 to 1.61 since 1950 to 2010 (Mohamed *et al*., 2010). The landings are mostly from Gujarat and Maharashtra coasts along the North West and Orissa and West Bengal on the north east coast of India. This species can grow up to 4 - 6 Kg. But the pomfret caught were mostly undersized (less than 1 Kg) due to over utilization of the stock. This overfishing can reduce the chance of successful reproduction and this may result in changes in species genetic variability and population structure. In the current scenario, to enhance productivity (through any culture practice), it is highly essential to standardize the breeding and hatchery technology of pomfrets and it is being attempted and found successful in Kuwait waters (Almatar *et al*., 2000). In India, this species is prioritised for hatchery development and mariculture. So, there is an urgent need to study to develop the baseline information on genetic stock structuring of this species throughout its distribution range, which may help in the near future for selective breeding programmes as well as useful to the planners and fishery resource managers for stock specific conservation programmes. So far, there is very little information about the genetic stock structure of *P. argenteus*. Against this background, the present work was carried out to delineate the stock structure analysis of *P. argenteus* using mitochondrial ATPase6/8 gene and nuclear microsatellite markers.

Fresh samples of *P. argenteus* (including juveniles and adults) were obtained from commercial trawl landings from Arabian Sea [Veraval, Gujarat in the north and Cochin, Kerala in the south] and from Bay of Bengal [Kakdwip, West Bengal, in the north and Chennai, Tamil Nadu, Visakhapatnam, Andhra

Pradesh in the south]. Total DNA was extracted from the tissue samples following the procedure of Miller *et al*., (1988) with minor modifications.

The complete ATPase 6/8 genes were sequenced from 100 samples of *P. argenteus* from five different geographical locations of both the coasts of India. Out of a total of 842 bp of mitochondrial gene amplified, 168 bp fragments were of ATPase 8 and 684 bp of ATPase 6, with an overlapping region of 10 bp from 159-168. A total of 24 haplotypes were identified among 100 individuals of five populations. Out of the total 842 characters obtained, 781 (92.76%) were constant and 61 (7.24%) were variable, in which 51 (83.61%) were parsimony informative and 10 (16.39%) were singleton variable sites. The different nucleotide compositions were found to be A = 29.4%, C = 26.9%, G = 13.2% and T = 30.6%. The significant pairwise F_{ST} values and the AMOVA values between samples indicated the occurrence of distinct population structure in silver pomfret between north east (Kolkata and Vishakhapatnam) and remaining populations. Mostly, lower levels of genetic differentiation are reported in marine fishes due to higher dispersal potential during planktonic egg, larval or adult by life history stages, coupled with an absence of physical barrier to movement between ocean basins. The continuously changing coastal current pattern also may result in exchange of larvae along the Indian coast, resulting in low population genetic differentiation (Mandal *et al*., 2012). Sun *et al*., 2012 found high genetic divergence between pomfrets from Mumbai, India (Arabian Sea) and Ngao, Thailand (Bay of Bengal) and concluded that the mainland of India blocks genetic exchange between Bay of Bengal and Arabian Sea. None of the haplotypes from Kolkata and Vishakhapatnam were shared by haplotypes from other regions. The average genetic divergence in silver pomfret using ATPase 6/8 genes exceeds the usual intra specific range and it has to be suspected that *P. argenteus* distributed in the Indo-west Pacific region might have diverged at the population /sub species level.

In conservation genetics, the basic thing is to detect the historical demography of a population. Mismatch distribution analyses were used to evaluate probable events of population expansion and decline. Smooth unimodal

curves were obtained for all the populations matching the expected distributions under the sudden expansion model which explains the history of occurrence of rapid expansion in the populations of *P. argenteus*. Along with this, haplotype and nucleotide diversity values (high *h* and low π), together suggested a history of genetic bottleneck events, with subsequent population expansion in *P. argenteus* and formation of new haplotypes which are found in low frequencies (Grant and Bowen, 1998). A negative Tajima's D as observed in this study signifies an excess of low frequency polymorphisms indicating population size expansion after a bottleneck.

All microsatellite loci identified for this study were polymorphic and the PIC value for each locus ranged from 0.7677 (Par 20) to 0.972 (P-189). Mean value of expected heterozygosity (He - 0.868) for each population was high compared to observed heterozygosity (Ho - 0.759). The total number of alleles per locus ranged from 28 (Par 17 and Par 06) to 64 (P 189) with the allele size of 92 to 448 bp. Positive F_{IS} indicating heterozygote deficiency was evident in all loci except Par 17 and P-189. Significant departure from Hardy Weinberg expectations were also observed in single locus exact tests after applying sequential Bonferroni correction. For the Bottleneck analysis, Wilcoxon signed rank tests and Mode shift test were not significant and indicates that the studied population has not experienced a recent bottleneck. But, as the species is highly significant commercially and overexploited, we took a more conservative approach and analyzed the M ratio test/ G-W statistic to understand the exploitation level and it was found to be in range 0.204 -0.231. Samples from species which have experienced a recent bottleneck exhibits a low M-ratio(< 0.29; Garza and Williamson, 2001). All the above findings gives indication that *P. argenteus* populations in Indian waters are being exploited at a higher level than the optimum. Higher exploitation ratio and Maximum Sustainable Yield (MSY) of the species lower than their annual catch was also reported. Excessive trawling and the use of small meshed trawl were considered to be the major reason for depletion of the pomfret stock in Indian waters. Since the pomfret

stock in Indian waters is under more fishing pressure, they suggest immediate decrease in fishing effort for the optimal management of this species.

Pair-wise F_{ST} analysis showed three separate population clusters-VER-COC cluster; CHN cluster and VP-KOL cluster. In addition to F_{ST}, population differentiation was measured using pair-wise and overall R_{ST} (Slatkin, 1995) based on the differences in repeat numbers (allele sizes) in microsatellite loci. Pair-wise R_{ST} values were similar to that of F_{ST} in *P. argenteus* varying significantly higher between populations of east and west coasts of India and non-significant lower values were observed within populations of each coast. AMOVA analysis was conducted to determine the variation explained by genepools and subgroups as well as to compare variability. The AMOVA indicated significant genetic differentiation among *P. argenteus* populations (F_{ST} 0.049; $P<0.001$). The value 83.24% was observed within individuals whereas, 0.88% variation was observed among the groups. The Bayesian cluster analysis performed in STRUCTURE 2.3.3 showed that three clusters could be identified among the samples using the Delta K estimator. When k = 3, maximum value for log likelihoods lnPr(X/k) and the minimum variance in log likelihoods (varlnPr(X/k)) was noticed, thus fixing the number of underlying clusters as three. The two dimensional PCoA plot shows that the first principal coordinate accounts for 50.19% of total variation and separates the populations of east (VP and KOL) and west coast (VER and COC). PC2 forms 26.09% of variation and separates CHN population. The pattern of isolation by distance (IBD) was supported when all the sampling locations were compared by Mantel test, the IBD observed was significant $p<0.05$; r2 = 0.768. It may be assumed that populations of silver pomfret from Arabian Sea were significantly different from that of Bay of Bengal region. Low mean sea surface temperature, presence of freshwater received from the peninsular rivers, weak winds and the continental shelf pattern differentiates the profile of Bay of Bengal and Arabian Sea (Jaswal *et al*., 2012). Such strikingly varied oceanographic features in the Bay of Bengal region may be playing an influential role for the large scale differences in the genetic diversity of silver pomfret populations of Indian coast. However, this

hypothesis can be proved only by a correlative study of these factors with larval/adult migration pattern of this fish. Among the samples distributed in Bay of Bengal region, Vishakhapatnam and Kolkata were quite similar, whereas moderate differentiation was observed with samples from Chennai. A restricted larval dispersal towards the Bay of Bengal Sea due to wide geographical separation and local recirculation patterns may be the cause of the significant genetic differentiation of samples from this region as reported by Durand *et al*., 2009.

This is the first comprehensive study using molecular markers on silver pomfret from Indian coast. The species-specific molecular signatures generated by various markers in the present study can help further investigations regarding the evolution and biogeography of these valuable and declining resources. The results of the stock structure studies signified the strong genetic differentiation between *P. argenteus* from Northern Indian Ocean with relevance to the definition of conservation units for this species. Management Units (MUs) represent sets of populations that are currently demographically independent and are recognized by significant divergence of allele frequencies at nuclear and mitochondrial loci. So, eventual development of management policies is needed for preserving the genetic stock structure of *P. argenteus* with proper monitoring. The three highly divergent management units of *P. argenteus* identified from five geographic locations in the present study indicates their independent evolution from other stocks and therefore, demands for non-mixing of the units for any reasons.

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Appendix

Appendix 1

Microsatellite alleles and allele frequencies in *Pampus argenteus* from five marine populations and overall populations.

Locus	Allele/n	Veraval	Cochin	Chennai	Vishakhapatnam	Kolkata
P 15-2	**100**	---	---	---	0.024	0.075
	102	---	0.027	---	0.048	0.067
	104	---	---	---	0.008	0.050
	108	---	---	---	0.008	---
	112	---	0.009	---	---	---
	114	---	---	---	---	0.017
	116	---	---	0.010	---	---
	118	0.010	0.027	0.130	0.065	0.008
	120	---	---	---	0.008	0.017
	122	---	---	---	0.008	---
	134	0.020	---	---	0.032	0.192
	138	0.286	---	0.540	0.032	0.067
	139	---	---	0.020	---	0.008
	140	0.010	0.018	0.030	0.032	---
	142	---	0.009	---	---	---
	148	---	---	0.010	---	0.008
	160	---	---	---	0.024	0.092
	166	---	---	0.010	---	---
	182	---	---	0.010	---	---
	184	---	---	0.030	---	0.017
	188	---	---	0.020	---	0.008
	190	---	0.018	---	---	---
	194	---	0.009	---	---	---
	196	---	0.018	---	0.016	---
	198	0.010	---	---	0.008	0.025
	200	0.031	0.073	0.010	---	0.017
	202	0.031	0.055	---	0.008	---
	204	0.071	0.091	0.010	0.024	---
	206	0.020	0.036	---	0.048	---
	208	0.020	---	---	0.040	---
	210	0.010	0.018	0.010	0.024	---

	212	---	0.027	---	0.008	---
	214	0.020	0.036	---	0.040	0.092
	216	0.051	0.036	0.010	0.113	0.008
	218	0.010	0.073	0.050	0.081	0.017
	220	0.010	0.082	---	0.056	0.008
	222	0.031	0.018	0.030	0.016	0.050
	224	---	---	0.030	0.048	0.008
	226	---	0.009	0.010	0.016	0.008
	228	0.020	0.036	---	0.032	0.008
	230	0.010	0.018	---	0.040	0.008
	232	0.041	---	0.010	0.024	0.025
	234	0.163	0.173	0.010	0.016	0.008
	236	0.031	0.018	---	0.016	---
	238	---	0.018	---	0.016	0.058
	240	0.071	0.045	0.010	0.008	---
	246	---	---	---	0.008	0.025
	256	---	---	---	---	0.008
	296	0.020	---	---	---	---
Par 01	**338**	---	---	0.050	---	---
	346	---	0.009	0.060	---	---
	348	---	---	0.010	---	---
	354	---	---	0.010	---	---
	378	---	---	---	0.008	---
	380	---	---	0.010	0.008	---
	382	---	---	0.020	0.032	0.008
	384	---	---	0.040	0.016	0.033
	386	0.010	---	0.020	---	0.025
	388	0.020	0.100	0.170	0.218	0.200
	390	0.010	0.036	0.060	0.073	0.058
	392	0.010	0.027	0.090	0.024	0.075
	394	0.051	0.227	0.250	0.347	0.417
	396	0.020	0.027	0.020	0.024	0.025
	398	0.041	0.018	0.030	0.016	0.008
	400	0.031	0.009	0.010	0.032	---
	402	0.031	---	---	---	---
	404	0.082	0.055	0.020	0.008	0.008
	406	0.020	0.009	0.030	0.056	0.100
	408	0.020	0.009	0.040	0.032	0.008
	410	0.010	---	---	0.040	0.017

	412	0.010	0.009	0.010	0.008	---
	414	0.010	0.009	0.020	---	0.017
	418	0.020	0.018	---	---	---
	420	0.020	0.009	0.010	0.008	---
	422	0.051	0.055	0.010	0.016	---
	424	0.061	0.055	---	0.008	---
	426	0.082	---	---	0.024	---
	428	0.367	0.300	---	---	---
	430	0.020	0.018	0.010	---	---
Par 02	**92**	---	---	---	0.008	0.008
	96	---	---	---	0.073	0.350
	98	---	0.045	---	---	0.050
	100	---	0.018	---	0.008	0.050
	102	---	---	---	0.016	0.117
	104	---	---	---	0.008	0.025
	108	---	0.009	---	---	---
	110	---	0.009	---	---	---
	112	---	---	0.010	---	---
	114	0.010	0.009	---	0.016	---
	116	0.143	0.045	---	0.008	---
	118	---	0.036	---	0.040	---
	120	---	---	---	0.016	0.025
	122	---	0.045	0.020	0.032	---
	124	0.020	0.018	---	0.016	---
	128	---	0.009	---	0.008	---
	130	---	---	---	0.008	---
	134	0.031	0.055	0.130	0.121	0.133
	136	0.020	0.009	0.060	0.016	0.067
	138	0.020	0.027	0.120	0.081	0.025
	140	0.133	0.136	0.320	0.355	0.067
	142	---	0.036	---	0.024	---
	144	0.041	0.027	0.030	0.024	0.008
	146	0.092	0.036	0.100	0.008	0.008
	148	---	0.009	0.030	---	0.017
	154	---	---	---	0.024	---
	156	---	0.064	0.010	0.008	---
	158	0.143	0.109	0.030	0.040	---
	160	0.020	0.027	---	---	0.008
	162	0.010	---	---	---	---

	166	---	---	0.010	0.016	0.008
	168	0.041	0.027	0.010	0.008	---
	170	0.133	0.109	0.090	0.016	0.017
	172	---	---	---	---	0.008
	174	0.010	---	---	---	---
	178	0.041	0.027	0.010	---	---
	180	0.082	0.055	0.010	---	---
	182	0.010	---	---	---	---
	200	---	---	0.010	---	0.008
Par 03	**202**	---	---	0.010	---	0.017
	208	---	---	0.020	---	---
	210	---	---	0.020	0.016	0.083
	212	---	---	---	0.008	0.025
	214	---	---	---	0.008	0.042
	226	---	0.018	---	---	---
	228	---	0.045	---	---	---
	230	---	0.009	---	---	---
	234	---	---	0.020	---	---
	238	---	---	0.060	---	---
	242	---	0.009	---	---	---
	244	0.010	---	---	---	---
	246	---	---	---	---	0.017
	248	---	0.009	---	---	---
	250	0.010	0.018	---	0.048	---
	254	0.020	0.009	---	---	0.008
	256	0.020	0.073	0.040	0.016	0.042
	258	0.010	0.027	---	0.024	0.025
	260	---	0.064	0.020	0.073	0.025
	262	0.071	0.027	---	---	---
	264	---	0.018	---	---	---
	266	0.143	0.064	0.090	0.024	0.017
	268	0.071	0.036	---	0.032	0.008
	270	0.163	0.027	---	0.032	0.042
	272	0.041	0.027	0.030	0.048	0.050
	274	0.031	0.018	0.020	0.008	0.050
	276	0.010	0.036	0.050	0.016	0.017
	278	0.010	0.045	0.020	0.016	---
	280	0.020	0.045	0.060	0.073	---
	282	0.071	0.036	0.050	0.016	0.083

	284	0.133	0.055	0.060	0.097	0.067
	286	---	0.009	0.020	0.089	0.033
	288	---	---	---	0.024	0.025
	290	---	---	0.040	0.024	0.050
	292	---	0.018	0.050	0.040	0.025
	294	---	0.027	---	0.073	0.008
	296	0.051	0.100	0.040	0.032	0.033
	300	0.041	0.018	0.010	---	0.008
	302	0.010	0.018	0.010	0.024	---
	304	0.010	---	0.010	0.016	0.008
	306	---	0.018	0.040	0.016	0.017
	308	0.031	0.018	0.050	0.008	0.025
	310	---	---	0.060	0.040	---
	312	---	0.009	0.020	0.008	---
	316	---	0.018	0.030	0.016	0.017
	318	0.020	---	0.020	0.008	0.033
	320	---	0.018	---	0.008	0.025
	322	---	0.009	0.020	---	0.017
	326	---	---	---	0.008	---
	328	---	---	0.010	---	0.033
	330	---	---	---	0.008	0.025
Par 06	**112**	0.061	0.100	---	---	---
	114	0.010	---	---	---	---
	118	0.286	0.118	0.270	0.065	0.042
	120	0.031	0.036	0.150	0.065	0.033
	126	---	0.009	0.020	0.008	---
	128	0.010	---	---	0.008	0.025
	138	---	---	0.020	---	---
	140	0.265	0.345	0.390	0.258	0.067
	142	---	0.036	---	0.073	---
	144	---	---	---	0.008	0.025
	158	0.010	0.027	---	0.024	0.125
	160	---	---	---	0.016	0.100
	162	0.051	0.127	0.010	0.040	0.008
	164	---	0.009	0.010	0.040	0.100
	166	---	0.027	0.010	0.024	0.067
	168	0.061	0.064	---	0.081	0.100
	170	---	---	0.030	0.040	0.183
	172	0.010	---	0.020	0.040	0.058

	174	0.010	0.009	0.010	0.016	0.050
	178	---	0.009	---	---	---
	184	0.010	---	---	---	---
	186	---	0.009	0.010	---	---
	188	---	---	0.030	0.089	0.008
	190	0.051	0.036	0.010	0.073	0.008
	192	0.051	0.018	0.010	0.032	---
	194	0.020	0.018	---	---	---
	196	0.031	---	---	---	---
	198	0.031	---	---	---	---
Par 08	**200**	0.010	---	---	---	---
	202	0.041	---	---	---	0.017
	204	---	0.027	---	---	0.017
	206	0.061	---	---	---	---
	212	---	---	---	---	0.017
	222	---	---	---	---	0.033
	240	---	---	---	---	0.017
	252	---	---	---	0.040	---
	254	---	---	---	0.024	---
	256	0.051	0.009	---	0.024	---
	258	---	0.036	---	0.065	---
	260	0.010	0.145	---	0.048	---
	262	---	0.055	---	0.040	---
	264	0.020	0.027	---	0.048	---
	266	---	0.082	---	0.048	---
	268	0.071	0.091	---	0.032	0.033
	270	---	0.055	---	0.024	---
	272	---	---	0.080	0.016	0.017
	274	---	0.009	---	0.008	---
	276	---	0.009	---	0.008	0.017
	278	---	---	---	---	0.033
	282	---	---	0.060	0.008	0.033
	284	---	---	0.060	---	0.008
	348	0.010	---	---	---	---
	400	---	---	---	0.016	---
	408	0.112	0.082	---	0.040	0.050
	410	---	---	---	0.040	0.025
	412	0.265	0.091	---	0.032	0.025
	414	---	---	0.160	0.008	0.025

	416	---	0.009	0.170	0.105	0.158
	418	0.041	0.073	0.070	0.056	0.092
	420	0.112	0.091	0.080	0.081	0.100
	422	0.010	0.009	0.070	0.048	0.033
	424	0.061	0.064	0.070	0.008	0.017
	426	---	---	---	0.040	0.033
	428	0.051	0.018	0.040	0.008	0.050
	430	---	---	---	0.016	0.025
	432	---	---	0.060	0.016	0.008
	434	---	---	---	---	0.008
	436	---	0.009	0.080	0.008	0.008
	438	---	---	---	0.024	0.058
	440	---	---	---	---	0.008
	442	---	---	---	0.008	0.008
	444	0.020	0.009	---	---	---
	448	---	---	---	0.008	0.025
Par 12	**232**	0.031	---	0.080	---	0.033
	234	0.041	---	0.060	0.032	0.017
	236	---	---	---	0.008	---
	240	---	---	---	---	0.017
	242	---	---	---	---	0.033
	250	---	---	0.020	---	---
	254	---	---	0.010	---	0.008
	256	---	0.009	---	---	---
	268	---	---	---	---	0.017
	270	---	0.009	---	---	---
	278	---	---	---	---	0.033
	280	0.122	0.027	---	---	0.017
	282	0.010	0.082	0.010	0.016	0.033
	284	---	---	---	0.008	---
	286	---	---	---	---	0.017
	290	0.010	---	---	---	---
	308	0.031	0.018	---	0.008	---
	310	0.031	0.036	---	0.024	---
	312	---	---	---	0.016	---
	314	---	0.027	0.010	0.040	---
	316	0.031	0.027	0.080	0.016	0.033
	318	0.112	0.091	0.070	0.089	0.075
	320	0.153	0.127	0.080	0.145	0.042

	322	0.051	0.055	0.040	0.073	0.033
	324	0.010	0.045	0.040	0.024	0.083
	326	0.031	0.100	0.020	0.081	0.092
	328	0.102	0.091	0.060	0.040	0.067
	330	0.082	0.036	0.130	0.081	0.233
	332	0.122	0.073	0.050	0.024	0.017
	334	---	0.036	0.030	0.056	---
	336	---	0.009	0.010	0.032	---
	338	0.010	0.018	0.160	0.032	0.067
	340	0.010	0.018	---	0.016	0.017
	342	0.010	0.018	---	0.032	---
	344	---	0.036	---	0.040	---
	346	---	---	0.010	0.024	---
	348	---	---	0.010	0.016	---
	350	---	---	0.010	---	0.008
	352	---	---	0.010	---	0.008
	354	---	0.009	---	---	---
	362	---	---	---	0.008	---
	368	---	---	---	0.016	---
Par 17	**92**	0.020	0.027	0.010	---	0.017
	94	0.092	0.045	0.050	---	0.017
	96	0.827	0.364	0.440	0.323	0.308
	98	---	0.009	---	---	---
	100	---	---	0.010	---	0.017
	102	---	0.018	0.020	0.008	0.033
	112	---	---	---	---	0.017
	118	---	---	---	0.008	0.025
	120	---	0.018	0.010	---	0.008
	122	---	---	0.010	---	0.008
	134	---	0.027	0.040	---	0.017
	136	---	---	0.010	---	0.017
	148	---	---	0.010	0.169	---
	154	---	---	---	0.008	---
	160	0.051	0.045	0.310	0.242	0.033
	168	---	0.018	0.020	0.008	0.042
	170	---	0.073	---	0.024	0.050
	174	---	0.009	0.010	---	0.008
	176	---	0.027	0.010	0.008	0.033
	178	---	0.045	---	0.016	0.025

	180	---	0.064	0.010	0.048	0.075
	182	---	0.064	---	0.040	0.042
	184	---	0.027	---	0.024	0.042
	186	---	0.018	---	0.008	---
	188	0.010	0.036	0.020	0.016	0.050
	190	---	0.055	0.010	0.032	0.083
	192	---	0.009	---	0.008	0.033
	194	---	---	---	0.008	---
Par 18	**206**	---	---	0.010	---	---
	214	---	---	0.010	---	---
	220	---	---	---	0.008	---
	224	---	---	0.010	---	---
	228	---	0.055	---	---	---
	234	---	---	0.040	---	---
	238	---	---	---	---	0.017
	240	---	---	0.050	0.089	0.017
	242	0.010	---	0.090	0.226	0.017
	244	---	---	---	0.065	0.258
	246	---	---	0.010	---	0.017
	248	---	---	---	---	0.017
	252	---	---	0.020	---	---
	260	---	---	---	---	0.033
	262	---	---	0.020	0.016	0.017
	264	---	---	0.010	---	0.017
	266	---	---	---	0.016	---
	272	---	---	0.020	---	---
	274	---	0.009	0.010	---	---
	276	---	---	0.030	0.032	0.008
	278	---	0.036	0.200	0.040	0.025
	280	0.041	0.018	0.150	---	0.058
	282	0.092	0.091	0.150	0.081	0.075
	284	0.102	0.109	0.030	0.081	0.100
	286	0.133	0.127	---	0.081	0.025
	288	0.112	0.091	---	0.032	0.058
	290	0.102	0.018	---	0.056	---
	292	0.071	0.064	---	0.073	0.067
	294	0.133	0.182	0.020	0.065	0.058
	296	0.102	0.164	0.040	0.032	0.100
	300	0.102	0.036	0.080	0.008	0.017

Par 20	**200**	---	0.018	---	---	---
	212	---	---	---	0.008	0.042
	216	0.051	---	---	---	---
	222	---	---	0.010	0.016	---
	226	0.010	0.018	0.010	---	0.008
	230	---	---	0.010	---	---
	236	---	0.027	0.080	---	---
	240	0.602	0.445	0.100	0.024	0.025
	244	0.041	0.055	0.050	0.016	0.092
	248	---	0.018	0.010	0.081	0.108
	250	0.061	0.100	0.060	0.161	0.092
	252	0.051	0.082	0.240	0.347	0.275
	255	---	---	0.070	0.056	0.050
	258	---	---	---	0.073	0.108
	260	---	0.018	0.010	0.056	0.008
	263	---	0.009	0.010	0.040	---
	266	---	0.018	---	0.032	0.025
	268	---	---	---	0.008	0.025
	270	---	0.018	---	0.040	---
	274	0.020	0.018	---	---	---
	276	0.020	0.036	---	---	---
	280	0.010	0.036	0.020	---	---
	284	0.041	0.018	0.010	---	0.008
	286	---	---	0.040	0.016	0.058
	288	---	---	0.040	0.008	0.025
	292	---	---	0.030	---	0.017
	294	---	---	0.040	0.016	---
	296	0.010	0.045	0.110	---	0.033
	300	0.082	0.018	0.050	---	---
Par 05	**200**	---	0.009	---	---	---
	202	0.031	0.027	---	0.032	0.075
	204	---	---	---	0.008	0.058
	206	---	---	---	0.016	---
	208	---	0.009	---	---	0.017
	214	---	---	---	0.008	0.042
	228	---	0.018	0.010	0.008	0.008
	230	---	---	---	0.024	---
	238	---	0.027	---	0.008	---
	240	0.031	---	---	0.048	---

	242	0.020	---	---	0.040	0.067
	248	---	---	---	0.024	---
	250	0.010	0.009	0.020	0.024	---
	252	0.010	0.009	0.030	0.032	---
	254	---	---	0.040	0.008	0.017
	256	---	0.009	0.030	0.065	---
	258	---	0.082	0.150	0.040	0.033
	260	0.224	0.136	0.150	0.185	0.083
	262	0.020	0.027	0.030	0.056	0.058
	264	0.061	0.045	0.110	0.056	0.067
	266	0.041	0.127	0.080	0.056	0.058
	268	0.235	0.136	0.110	0.065	0.100
	270	0.031	0.018	0.010	0.024	0.067
	272	0.020	0.027	0.080	0.024	---
	274	---	0.018	0.020	0.056	0.025
	276	---	0.045	0.020	---	0.008
	278	0.071	0.036	0.030	0.008	0.033
	280	0.051	0.045	---	---	0.025
	282	0.010	0.027	0.010	0.032	0.033
	284	0.010	0.045	0.010	0.008	0.017
	286	0.051	0.027	---	---	---
	288	---	0.009	---	---	---
	290	---	---	0.010	0.008	---
	294	0.020	0.009	0.020	---	0.017
	296	---	---	---	0.008	0.025
	300	0.031	---	---	0.008	0.025
	302	---	0.018	---	---	---
	304	---	---	0.030	---	0.008
	306	---	---	---	0.008	---
	308	---	---	---	0.008	0.025
	314	---	---	---	---	0.008
	330	0.020	---	---	---	---
P-189	**102**	---	---	---	0.008	---
	108	---	---	---	0.008	---
	114	---	---	---	0.008	---
	128	---	---	---	0.065	---
	130	---	---	---	0.048	---
	132	---	---	---	0.016	---
	134	---	---	---	0.016	---

	136	---	---	---	0.024	---
	138	---	---	---	0.073	---
	140	---	---	---	0.040	---
	142	0.082	0.045	---	0.065	0.150
	144	0.020	0.009	---	0.016	0.025
	146	0.020	---	---	---	---
	148	0.061	0.027	0.010	0.016	0.050
	150	0.112	0.091	0.190	---	0.083
	152	0.031	0.018	0.120	0.137	0.050
	154	---	---	---	0.016	0.033
	156	0.031	---	---	---	---
	158	0.010	---	---	---	---
	160	0.010	0.009	---	0.016	0.042
	162	0.163	0.064	0.020	0.040	0.050
	164	0.071	0.055	0.030	0.024	0.075
	166	0.010	---	---	---	---
	168	---	0.009	---	---	---
	172	0.031	---	---	---	---
	176	---	---	---	0.008	---
	180	---	0.027	---	---	---
	182	0.010	0.009	---	---	---
	186	---	0.036	---	---	---
	188	0.020	0.027	0.010	---	---
	190	0.071	0.109	0.110	---	0.025
	192	0.061	0.082	0.100	0.065	0.058
	194	0.122	0.182	0.150	0.008	---
	196	---	0.018	0.020	0.016	---
	198	---	0.018	---	---	0.008
	200	0.051	0.036	0.100	---	---
	202	---	---	0.010	---	---
	204	---	---	0.010	---	0.008
	206	---	---	---	---	0.017
	208	---	0.018	0.010	0.024	0.058
	210	---	---	0.010	0.040	0.033
	212	---	---	---	0.008	---
	214	---	0.018	0.010	---	0.017
	216	---	0.009	0.010	---	0.017
	218	---	---	0.020	0.016	0.008
	220	---	---	---	0.008	---

	222	---	0.009	0.010	0.008	0.017
	224	---	---	---	---	0.025
	226	0.010	0.036	---	0.016	0.033
	228	---	---	---	0.040	0.083
	230	---	0.009	---	---	---
	234	---	---	---	0.008	---
	236	---	0.009	---	---	0.017
	238	---	---	0.040	---	0.017
	240	---	---	---	0.016	---
	252	---	---	---	0.008	---
	254	---	---	---	0.008	---
	260	---	---	---	0.008	---
	262	---	---	---	0.008	---
	264	---	---	---	0.016	---
	270	---	---	---	0.032	---
	276	---	0.009	---	---	---
	278	---	---	0.010	---	---
	296	---	0.009	---	---	---