POPULATION GENETIC STRUCTURE OF INDIGENOUS ORNAMENTAL TELEOSTS, *PUNTIUS DENISONII* AND *PUNTIUS CHALAKKUDIENSIS* FROM THE WESTERN GHATS, INDIA

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by

LIJO JOHN
(Reg. No. 3100)

National Bureau of Fish Genetic Resources Cochin Unit
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
(Indian Council of Agricultural Research)
P.B. No. 1603, Kochi – 682 018, Kerala, India.

December, 2009.

Declaration

I do hereby declare that the thesis entitled **"Population genetic structure of indigenous ornamental teleosts, *Puntius denisonii* and *Puntius chalakkudiensis* from the Western Ghats, India"** is the authentic and bonafide record of the research work carried out by me under the guidance of Dr. A. Gopalakrishnan, Principal Scientist and SIC, National Bureau of Fish Genetic Resources (NBFGR) Cochin Unit, Central Marine Fisheries Research Institute, 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 (Lijo John)

16th December 2009

राष्ट्रीय मत्स्य आनुवंशिक संसाधन ब्यूरो
NATIONAL BUREAU OF FISH GENETIC RESOURCES
NBFGR Cochin Unit, CMFRI Campus, P.B. No. 1603, Cochin-682 018, Kerala, India
Fax: (0484) 2395570; E-mail: nbfgrcochin@eth.net

Dr. A. Gopalakrishnan,
Principal Scientist, Officer-in-Charge &
Supervising Teacher

Date: 16.12.2009

Certificate

This is to certify that this thesis entitled, **"Population genetic structure of indigenous ornamental teleosts, *Puntius denisonii* and *Puntius chalakkudiensis* from the Western Ghats, India"** is an authentic record of original and bonafide research work carried out by **Mr. Lijo John (Reg. No. 3100)** at National Bureau of Fish Genetic Resources (NBFGR) Cochin Unit, Central Marine Fisheries Research Institute, under my supervision and guidance in partial fulfilment 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.

(A. Gopalakrishnan)

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Preface

Our riverine system, especially the torrential streams and rivers originating in the Western Ghats are known to have variety of ornamental fishes. Except for traditional taxonomic description we have little information on these species, their ecology, biology, life habits, life history and population structure. There is large scale depletion of habitats due to anthropogenic factors which affect the ecosystems of these streams. At the same time, a growing trade in ornamental fish resulting in the exploitation of the resources from the wild stock threatening their existence.

On account of this, Government of Kerala State has promulgated a ban on the capture from wild of one of the species *Puntius denisonii* (Day) during its peek breeding season. The importance of learning more about the species such as *P. denisonii* and its associated species has motivated me to take-up the present study, the subject of this thesis entitled "Population genetic structure of indigenous ornamental teleosts, *Puntius denisonii* and *Puntius chalakkudiensis* from the Western Ghats, India." My study has brought out some important findings on *P. denisonii* species complex which I am sure will help towards developing management strategies for conservation and well being of the species.

The thesis is presented in five chapters excluding summary and conclusion and references. I have adopted updated techniques both in analyzing meristic and morphometric data and genetic data relating to mitochondrial DNA to arrive at conclusions. This work has carried out under the guidance of Dr. A. Gopalakrishnan, Principal Scientist and SIC, NBFGR Cochin Unit, CMFRI, Cochin.

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Contents

	Page No.
Declaration	ii
Certificate	iii
Acknowledgements	iv
Preface	vi
List of abbreviations	x
List of tables	xii
List of figures	xv
Chapter 1. **Introduction**	1-11
1.1. Background and scope of the study	1
1.2. Objectives of the study	5
1.3. Characterization of the study area	5
1.4. Description of the species	6
1.4.1. *Puntius denisonii*	6
Taxonomic status	7
Distinguishing Characters	7
1.4.2. *Puntius chalakkudiensis*	8
Taxonomic status	9
Distinguishing Characters	9
Chapter 2. **Review of literature**	12-35
2.1. Concept of stock and sustainable exploitation	12
2.2. Population genetics and conservation of species	14
2.2.1. Morphometrics and meristics	16
2.2.2. Molecular markers	20
2.2.2.1. Mitochondrial DNA analysis	24
2.2.2.1a. Concept of DNA barcoding	30
2.3. Indian scenario	33
Chapter 3. **Materials and methods**	36-56
3.1. Sampling	36
3.2. Morphometrics and meristics	36
3.2.1. Material	36
3.2.2. Morphometric characters	36
3.2.3. Meristic characters	39
3.2.4. Analysis of data	40
3.3. Mitochondrial DNA analysis	44
3.3.1. Genomic DNA isolation	44
3.3.2. Gene amplification using PCR	48
3.3.3. Gene sequencing	51
3.3.4. Analysis of data	53

3.3.4.1. Sequence alignment 53
3.3.4.2. Population genetic analysis 54
3.3.4.3. Phylogenetic and molecular evolutionary analysis 55
3.3.4.4. Inference on population demographic history 55

Chapter 4. **Results** 57-122

4.1. Ecological observations 57
4.2. Morphometric and meristic analysis 60
4.2.1. Morphometric traits 60
4.2.1.1. Descriptive statistics of morphometric traits 60
4.2.1.2. Principal Component Analysis (PCA) 61
4.2.1.2a. PCA between *P. denisonii* and *P. chalakkudiensis* 61
4.2.1.2b. PCA among populations of *P. denisonii* 62
4.2.1.2c. PCA between populations of *P. chalakkudiensis* 62
4.2.2. Meristic traits 76
4.2.2.1. Descriptive statistics of meristic traits 76
4.2.2.2. Principal Component Analysis (PCA) 77
4.3. Mitochondrial DNA analysis 82
4.3.1. ATPase 8/6 region 82
4.3.1.1. ATPase 8/6 sequence variations 82
4.3.1.2. Population variability 83
4.3.1.3. Phylogeographic relationships of populations 84
4.3.1.4. Phylogenetic analysis 84
4.3.1.5. Inference on past demography 85
4.3.2. COI region 99
4.3.2.1. COI sequence variations 99
4.3.2.2. Genetic divergence 100
4.3.2.2. Phylogenetic and geographical relationships among haplotypes 100
4.3.3. Cyt *b* region 106
4.3.3.1. Cyt *b* sequence variations 106
4.3.3.2. Genetic divergence 107
4.3.3.3. Phylogenetic and geographical relationships among haplotypes 107
4.3.4. 16S rDNA region 112
4.3.4.1. 16S rDNA sequence variations 112
4.3.4.2. Genetic divergence 112
4.3.4.3. Phylogenetic and geographical relationships among haplotypes 113
4.3.5. Analysis of combined data of 2477 bp mtDNA region 117
4.3.5.1. Sequence variations 117
4.3.5.2. Genetic divergence 117
4.3.5.3. Phylogenetic and geographical relationships among haplotypes 118

Chapter 5. **Discussion** 123-144

5.1. Species validity of *P. chalakkudiensis* 124
5.2. Morphologic differentiation between populations of *P. denisonii* and *P. chalakkudiensis* 126
5.2.1. Stock differentiation in *P. denisonii* 127
5.2.2. Stock differentiation in *P. chalakkudiensis* 129
5.3. Genetic variation and distribution of *P. denisonii* and *P. chalakkudiensis* 130
5.4. Population structure and divergence pattern in *P. denisonii* and *P. chalakkudiensis* 132
5.5. Phylogenetic relationships and demographic evolution of *P. denisonii* and *P. chalakkudiensis* populations 137
5.6. Implications for fisheries management and conservation 141

Chapter 6. **Summary and conclusion** 145-152

Bibliography 153-177

Appendices 178-194

1. Sequence alignments 178
2. Publications 190

List of Abbreviations

μg	:	Micro grams
μl	:	Micro litre
μM	:	Micro molar
AFLP	:	Amplified Fragment Length Polymorphism
AFR	:	branched rays of anal fin
AFS	:	soft rays of anal fin
AMOVA	:	analysis of molecular variance
ANOVA	:	Analysis of Variance
ATPase	:	ATP synthase
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pairs
CDR	:	Chandragiri population
CHD	:	Chalakkudy population
CLR	:	Chaliyar population
CMFRI	:	Central Marine Fisheries Research Institute
COI	:	cytochrome c oxidase sub-unit I
CPD	:	Depth of caudal peduncle
CV	:	coefficient of variation
Cyt *b*	:	cytochrome *b*
DFR	:	branched rays of dorsal fin
DFS	:	soft rays of dorsal fin
DNA	:	Deoxyribo Nucleic Acid
dNTPs	:	Deoxynucleoside tri phosphates
EDTA	:	Ethylene Diamine Tetra Acetic acid
FBAN	:	Anal fin base
FBDO	:	Dorsal fin base
F_{ST}	:	Co-efficient of genetic differentiation
GRC	:	gill raker count on the outer gill arch
HD	:	Head depth
HW	:	Width of head
IUCN	:	International Union for Conservation of Nature and Natural Resources
KGD	:	Kariangode population
L_{BC}	:	Length of body cavity
L_{CP}	:	Length of caudal peduncle
L_{H}	:	Head length
LLS	:	lateral line scale count
L_{MB}	:	Barbel length
L_{O}	:	Orbital length
L_{PD}	:	Post dorsal length
L_{PLFAF}	:	Pelvic to anal
L_{PO}	:	Post orbital length
L_{PRA}	:	Pre anal length
L_{PRD}	:	Pre dorsal length
L_{PRO}	:	Pre orbital length
L_{PRV}	:	Pre ventral length
L_{PTFPLF}	:	Pectoral to pelvic origin

L_S	:	Standard length
MBD	:	Maximum body depth
mg	:	milligram
ml	:	millilitre
mM	:	millimolar
mm	:	Millimeter
mtDNA	:	Mitochondrial DNA
MUs	:	Management Units
MW	:	Width of mouth
NBFGR	:	National Bureau of Fish Genetic Resources
NCBI	:	National Centre for Biotechnology Information
nDNA	:	Nuclear DNA
ng	:	nanogram
Nm	:	Rate of gene flow
nm	:	nanometer
OD	:	optical density
Pc	:	*Puntius chalakkudiensis*
PCA	:	principal component analysis
PCR	:	Polymerase Chain Reaction
Pd	:	*Puntius denisonii*
PER	:	Periyar population
PFR	:	pectoral fin rays
RAPD	:	Random Amplified Polymorphic DNA
rDNA	:	ribosomal DNA
RFLP	:	Restriction Fragment Length Polymorphism
RGCB	:	Rajiv Gandhi Centre for Biotechnology
RNA	:	Ribo Nucleic Acid
rpm	:	Revolutions per minute
SD	:	standard deviation
SDS	:	Sodium Dodecyl Sulphate
TL	:	Total Length
VFR	:	ventral fin rays
VLP	:	Valapattanam population

List of tables

		Page No.
Table 1.	Details of sampling of *P. denisonii* and *P. chalakkudiensis* for the present study	37
Table 2.	Description of morphometric characteristics of *P. denisonii* and *P. chalakkudiensis*	42
Table 3.	Details about the number of individuals sequenced for each gene from each location	53
Table 4.	Descriptive statistics of transformed morphometric variables, the coefficient of variation of each measurement, the multivariate coefficient of variation of each species and F-values of *P. denisonii* and *P. chalakkudiensis*	64
Table 5.	Descriptive statistics of transformed morphometric variables, the coefficient of variation of each measurement, the multivariate coefficient of variation of each species and F-values of four populations of *P. denisonii*	65
Table 6.	Descriptive statistics of transformed morphometric variables, the coefficient of variation of each measurement, the multivariate coefficient of variation of each species and F-values of two populations of *P. chalakkudiensis*	66
Table 7.	Summary of principal component analysis (PCA) for the morphometric variables of *P. denisonii* and *P. chalakkudiensis*	67
Table 8.	Factor loadings for the first three principal components formed from the morphometric variables of *P. denisonii* and *P. chalakkudiensis* specimens	68
Table 9.	Summary of principal component analysis (PCA) for the morphometric variables of *P. denisonii* populations	69
Table 10.	Factor loadings for the first three principal components formed from the morphometric variables of *P. denisonii* populations	70
Table 11.	Summary of principal component analysis (PCA) for the morphometric variables of *P. chalakkudiensis* populations	71
Table 12.	Factor loadings for the first three principal components formed from the morphometric variables of *P. chalakkudiensis* populations	72
Table 13.	Descriptive statistics of meristic variables, the coefficient of variation of each measurement, the multivariate coefficient of variation of each species and F-values of *P. denisonii* and *P. chalakkudiensis*	79
Table 14.	Summary of principal component analysis (PCA) for the meristic variables of *P. denisonii* and *P. chalakkudiensis* specimens	80

Table 15. Factor loadings for the first three principal components formed from the meristic variables of *P. denisonii* and *P. chalakkudiensis* specimens 80

Table 16. Nucleotide sequence characteristics of *ATPase 8* gene across six populations of *P. denisonii* 88

Table 17. Nucleotide sequence characteristics of *ATPase 6* gene across six populations of *P. denisonii* 88

Table 18. Molecular diversity indexes of 842 bp fragment of the *ATPase 8/6* gene across each population of *P. denisonii* 89

Table 19. Matrix showing *Nm* between populations of *P. denisonii* 89

Table 20. Distribution of haplotypes of 842 bp fragment of the *ATPase 8/6* gene among the populations of *P. denisonii* 90

Table 21. Relative haplotype frequencies of 842 bp fragment of the *ATPase 8/6* gene among the populations of *P. denisonii* 91

Table 22. Mean pairwise distances between the haplotypes of each population based on *ATPase 8/6* gene sequences 92

Table 23. Mean pairwise distances between populations of *P. denisonii* based on *ATPase 8/6* gene sequences 93

Table 24. Intrapopulation nucleotide (π) and haplotype (h) diversities of *P. denisonii* based on *ATPase 8/6* gene sequences 93

Table 25. Results Analysis of molecular variance (AMOVA), showing variation in mitochondrial *ATPase 8/6* region in *P. denisonii* 94

Table 26. Pairwise F_{ST} values for between populations and significance of *P. denisonii* and *P. chalakkudiensis* based on *ATPase 8/6* gene sequences 94

Table 27. Parameters of sudden expansion model and Goodness of fit test to the model for each population 98

Table 28. Nucleotide sequence characteristics of *COI* gene across six populations including *P. denisonii* and *P. chalakkudiensis* 102

Table 29. Distribution of haplotypes of 521 bp fragment of the *COI* gene among the populations of *P. denisonii* and *P. chalakkudiensis* 102

Table 30. Mean pairwise distances between the haplotypes of each population based on 521 bp region of mtDNA *COI* gene 103

Table 31. Mean pairwise distances between populations of *P. denisonii* and *P. chalakkudiensis* based on 521 bp region of mtDNA *COI* gene 103

Table 32. Nucleotide sequence characteristics of cyt *b* gene across six populations including *P. denisonii* and *P. chalakkudiensis* 109

Table 33. Distribution of haplotypes of 552 bp fragment of the cyt *b* gene among the populations of *P. denisonii* and *P. chalakkudiensis* 109

Table 34. Mean pairwise distances between populations of *P. denisonii* and *P. chalakkudiensis* based on 552 bp region of mtDNA cyt *b* gene 110

Table 35. Distribution of haplotypes of 16S rDNA among the populations of *P. denisonii* and *P. chalakkudiensis* 114

Table 36. Mean pairwise distances between populations of *P. denisonii* and *P. chalakkudiensis* based on partial sequence of 16S rDNA 115

Table 37. Molecular characterization and phylogenetic information content of the mitochondrial DNA regions in *P. denisonii* and *P. chalakkudiensis* 119

Table 38. Distribution of haplotypes of 2477 bp mtDNA region among the populations of *P. denisonii* and *P. chalakkudiensis* 120

Table 39. Mean pairwise distances between populations of *P. denisonii* and *P. chalakkudiensis* based on 2477 bp mtDNA region 121

List of Figures

		Page No.
Figure 1.	*Puntius denisonii* (Day, 1865)	10
Figure 2.	*Puntius chalakkudiensis* (Menon *et al*., 1999)	11
Figure 3.	Vertebrate mitochondrial genome	24
Figure 4.	Map showing the distribution of the sampling sites of *P. denisonii* and *P. chalakkudiensis*	38
Figure 5.	Schematic representation of *P. denisonii* / *P. chalakkudiensis* indicating the positions of the superficial points used to measure the morphometric data	42
Figure 6.	*P. chalakkudiensis* specimen collected from Periyar River	59
Figure 7.	Ripe ovary in *P. chalakkudiensis* observed in the month of December	59
Figure 8.	Juveniles of *P. denisonii* and *P. chalakkudiensis*	60
Figure 9.	Scatter diagram based on PCA of significant morphometric variables between *P. denisonii* and *P. chalakkudiensis* specimens	73
Figure 10.	Scatter diagram based on PCA of significant morphometric variables among populations of *P. denisonii*	74
Figure 11.	Scatter diagram based on PCA of significant morphometric variables between populations of *P. chalakkudiensis*	75
Figure 12.	Microscopic image of pharyngeal teeth arranged on one side of the pharyngeal bone of *P. denisonii*	77
Figure 13.	Microscopic image of gill rakers of outer gill arch of *P. denisonii*	78
Figure 14.	Microscopic image of gill rakers of outer gill arch of *P. chalakkudiensis*	78
Figure 15.	Scatter diagram based on PCA of significant meristic variables between *P. denisonii* and *P. chalakkudiensis*	81
Figure 16.	Amplified products of ATPase 8/6 genes separated on 1.5% agarose gel	86
Figure 17.	DNA sequence of ATPase 8/6 gene and translated protein sequences of a representative haplotype	87
Figure 18.	Mean pairwise distances between the haplotypes within the populations of *P. denisonii* based on *ATPase 8/6* gene sequences	92
Figure 19.	Neighbour-joining tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA ATPase 8/6 genes	95
Figure 20.	Maximum parsimony tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA ATPase 8/6 genes	96
Figure 21.	Pairwise mismatch distributions for each population of *P. denisonii*.	97

Figure 22.	Amplified products of COI gene separated on 1.5% agarose gel	101
Figure 23.	DNA sequence of COI gene and translated protein sequence of a representative haplotype	101
Figure 24.	Neighbour-joining tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA COI gene	104
Figure 25.	Maximum parsimony tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA COI gene	105
Figure 26.	Amplified products of cyt *b* gene separated on 1.5% agarose gel	108
Figure 27.	DNA sequence of cyt *b* gene and translated protein sequence of a representative haplotype	108
Figure 28.	Neighbour-joining tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA cyt *b* gene	110
Figure 29.	Maximum parsimony tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA cyt *b* gene	111
Figure 30.	Amplified products of 16S rDNA separated on 1.5% agarose gel	113
Figure 31.	16S rDNA - 562 bp partial sequence of a representative haplotype	114
Figure 32.	Neighbour-joining tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial 16S rDNA	115
Figure 33.	Maximum parsimony tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial 16S rDNA	116
Figure 34.	Neighbour-joining tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the 2477 bp mtDNA region	121
Figure 35.	Maximum parsimony tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the 2477 bp mtDNA region	122

Chapter **1**

INTRODUCTION

1.1. Background and scope of the study

The Western Ghats of peninsular India is one of the world's ten "hottest biodiversity hotspots" and has over 5000 species of flowering plants, 139 mammal species, 508 bird species, 179 amphibian species and 295 fish species (Shaji *et al*., 2000; Vijayan, 2005). At least 325 globally threatened species occur in the Western Ghats (Myers *et al*., 2000). This mountain range runs north to south along the west coast of the Deccan Plateau, and separates the plateau from a narrow coastal plain along the Arabian Sea. The range starts near the border of Gujarat and Maharashtra, south of the Tapti River, and runs approximately 1600 km through the states of Maharashtra, Goa, Karnataka, Tamil Nadu and Kerala ending at Kanyakumari, at the southern tip of the Indian peninsula. These hills cover almost 60,000 km² and form the catchment area of a complex of river systems that drain almost 40% of India (Vijayan, 2005) and the rivers originating from these mountain ranges have proved the most fertile fields for ichthyologic discoveries (Hora, 1941; Silas, 1951, 1954; Dahanukar *et al*., 2004).

The species *Puntius denisonii* is a brilliantly coloured ornamental teleost endemic to Kerala and found in selected west flowing rivers originating from the Western Ghats (AnnaMercy *et al*., 2007). It is popularly known as "red-line torpedo", "rose-line shark" or "chenkaniyan" in local parlance. Although the species was described from the Indian State of Kerala by the British ichthyologist Francis Day in 1865, it is of no interest to fisheries (Talwar and Jhingran, 1991) until 1996. Later, it has been identified as an ornamental fish and is being collected from the wild for aquarium trade. However, it exploded in popularity almost immediately, after it won an award in the 'new species' category at Singapore's Aquarama exhibition in 1997 (Anon. 2008). The species then

achieved high demand in domestic as well as in international aquarium trade and it is being exploited from the wild in large quantities (AnnaMercy *et al*., 2007).

Kottelat and Whitten (1996) identified the streams and rivers of Kerala (8^0 17'30"–12^0 27'40"N, 74^0 51'57"–77^0 24'47"E) as an exceptional hotspot of freshwater biodiversity and showing high degree of endemism. The National Bureau of Fish Genetic Resources (NBFGR) has enlisted 295 species of freshwater teleosts from the Western Ghats of which approximately 195 are endemic to the region (Shaji *et al.*, 2000). The highly endemic 'stream fishes' of the Southern region of the Western Ghats are now an important component of the global ornamental fish trade (Ponniah and Gopalakrishnan, 2000). Currently, 114 ornamental species from this region are being exported (Anon., 2005), having increased from just ten species at the beginning of the decade (Kurup *et al*., 2004). Presently *P. denisonii* is the most preferred ornamental fish from this region in the international trade (AnnaMercy *et al*., 2007) and fetches retail prices between US$ 20–35 (Anon., 2006; Anon., 2007).

Overexploitation from the wild is one of the major issues in the case of many indigenous ornamental fishes from the Western Ghat region including *P. denisonii*; and the species is categorized as "endangered" based on latest IUCN criteria, due to restricted distribution, loss of habitat, over exploitation, destructive fishing practices and trade (Anon., 1998). Till date, no hatchery rearing techniques were reported for this species, which in turn adding pressure on wild stocks. Over fishing, when particularly directed towards certain sizes or age classes, may reduce population sizes to levels at which inbreeding and loss of genetic diversity may become serious problems or result in extinction of local populations or population segments (Lakra *et al*., 2007). Habitat destruction is another reason that leads to the depletion of these natural resources. There are many factors, such as deforestation, watershed erosion, silting, agricultural runoffs, pesticides, fertilizers, sewage, and chemical pollutants which destroy the breeding habitats or added additional stresses to many aquatic species. Attempts to promote

aquaculture practices in the area using transplanted Indian major carps and other exotic species have led to further deterioration in the situation. Ponniah and Gopalakrishnan, (2000) reported an alarming rate of depletion of the fish diversity of the region due to over-exploitation and clandestine export. The fishery for ornamentals in the streams originating from the Western Ghats is an open-access one, devoid of any quotas or access restrictions and no regulation on either catch or export is in place, nor is there any policy directed towards native ornamental fisheries (AnnaMercy *et al*., 2007). There is an urgent need for the development of scientific management strategies for the sustainable utilization of these natural resources.

Assessment of the genetic make-up and variability of fish stocks is important for the scientific and judicious exploitation/management of fishery, conservation and rejuvenation of endangered species and improvement of stocks of cultivable species. Populations of most species are composed of subpopulations, also called genetic stocks, between which limited gene flow occurs. These subpopulations maintain their genetic makeup or characteristics distinct from other subpopulations of the same species due to genetic variation within the species. A strategy thus developed for the scientific management of these resources was to identify the natural units, the 'stocks' of the fishery resources under exploitation (Altukhov, 1981). This can be done by characterizing the genetic structure of the populations being harvested. A lack of knowledge about the genetic structure of these populations may result in the differential harvest of the populations that will ultimately have a drastic and long-term effect. To overcome this, there is always a need for investigations encompassing the genetic variations at the intra and inter-population levels as well as at the intra and inter- specific levels of the fish and shellfish resources of any nation (Allendorf and Utter, 1979).

There is no information on population/stock structure and basic genetic profile of the species *P. denisonii*, which is essential for the fishery management,

conservation and rehabilitation of the species. The species is also having taxonomic ambiguity with *Puntius chalakkudiensis*, another species described from Chalakkudy River by Menon *et al*. (1999).

Comparative examination of morphological characters was one of the traditional methods of distinguishing fish taxa and stocks (Hubbs and Lagler, 1947). The multivariate methods like principal component analysis of morphometric traits have been proposed as an efficient tool for stock identification in fishery management programmes (Winans, 1985). The development of DNA-based genetic markers has had a revolutionary impact on animal genetics. It is theoretically possible to observe and exploit genetic variation in the entire genome of organisms with DNA markers.

Allozymes, mitochondrial DNA, RFLP, RAPD, AFLP, microsatellite, SNP, and EST markers are the popular genetic markers employed in fisheries and aquaculture (Liu and Cordes, 2004). In recent years, mtDNA, because of its fast evolution - 5 to 10 times faster than single copy nuclear genes - (Avise 1994), has been widely applied in systematics, population genetics and conservation biology of animals (Wang *et al*., 2000). But the genetic markers in general are oversensitive to a low level of gene flow: a relatively low level of exchange between stocks, negligible from a management perspective, may be sufficient to ensure genetic homogeneity (Ward and Grewe, 1994; Carvalho and Hauser, 1994). Therefore, molecular markers alone may not be sufficient to detect existing genetic variation among populations, and also only a small proportion of DNA is analysed by molecular markers. However, phenotypic markers may detect morphological differentiation due to environmental differences in the habitats of partially-isolated stocks, which may be a practical level of partitioning among self-recruiting stocks. Such self-recruiting stocks may react independently to exploitation (Carvalho and Hauser, 1994), even without showing genetic differentiation (Turan, 1999). In this context the present work was undertaken to study the genetic diversity and population/stock structure in *P. denisonii* from

different geographically isolated environments and to resolve the taxonomic ambiguity between *P. denisonii* and *P. chalakkudiensis* using morphologic and molecular genetic tools.

1.2. Objectives of the study

For the judicious exploitation and conservation of fishery resources, it is apparent that understanding of correct taxonomic identity, the degree of population/stock structuring and evolutionary history is essential. In the present study, a holistic approach, combining phenotypic and genetic methods to analyze the taxonomic identity of *P. chalakkudiensis* and possible population/stock differentiation in the candidate species *P. denisonii* was performed. The major objectives of the present study are as follows:

- To resolve the taxonomic ambiguity of *P. denisonii* and *P. chalakkudiensis* using; (a) morphometric and meristic characters and (b) mitochondrial DNA sequence analysis.
- To understand the structuring of populations of the two species if any using morphologic (morphometric analysis) and genetic (mtDNA analysis) tools with an ultimate objective to devise stock-specific, propagation assisted rehabilitation programme.

1.3. Characterization of the study area

Rivers of the Southern region of the Western Ghats is selected as the study area based on the distribution of candidate species. *P. denisonii* is exhibiting a distribution of highly fragmented populations restricted to specific pockets of few west flowing rivers of Kerala (South India); whereas *P. chalakkudiensis* is restricted to the Chalakkudy River (Menon *et al*., 1999).

All the river basins in Kerala State can be divided into three natural zones based on elevation, consisting of low land or sea-board, midland and high land. The coast for a short distance along the borders of lakes is flat, retreating from it the surface roughens up into slopes which gradually combine and swell into mountains on the east. The low land area along sea coast is generally swampy and liable to be flooded during monsoon. The plains/midlands succeed low land in gentle ascents and valleys interspersed with isolated low hills. The high land on the eastern portion is broken by long spurs, dense forests, extensive ravines and tangled jungles. Towering above all their slopes are Western Ghats that form eastern boundary of the basins. The natural habitat of *P. denisonii* is mainly found in the midland regions of selected west flowing rivers of the southern region of the Western Ghats.

In the present study, river basin approach has been taken up for water shed basis study of racism and genetic diversity in *P. denisonii* and *P. chalakkudiensis*. Six geographically isolated river systems, which includes the habitats of *P. denisonii* and *P. chalakkudiensis* were short listed based on the available literature and the survey during the course of collection. Selected rivers from south to north are Periyar, Chalakkudy, Chaliyar, Valapattanam, Kariangode and Chandragiri (details of sampling and sampling sites are included in section 3.1., table 1 and fig. 4).

1.4. Description of the species

1.4.1. *Puntius denisonii* (Day)

Labeo denisonii, Day, *Proc. Zool. Soc*. 1865, p 299.
Puntius denisonii, Day, *Fish. Malabar*, p. 212, pl. XIV, f. 2.
Barbus denisonii, Day, *Fish. India*, p. 573, pl. CXLIII, f. 2.

Puntius denisonii (Fig. 1) was described by Francis Day (1865) in his work “On the fishes of Cochin on the Malabar cost of India”, as *Labeo denisonii*, obtained in the hill ranges of Travancore. Later, in his works on the “Fishes of

Malabar" and "The Fishes of India; Being a Natural History of the fishes known to inhabit the seas and fresh waters of India, Burma and Ceylon", he has again included about the species as *Puntius denisonii* and *Barbus denisonii* respectively.

P. denisonii is one of the most commercially important candidate species from the southern region of Western Ghats of Peninsular India. "Red-line torpedo fish", "Rose-line shark", "Denison's barb", "Chenkaniyan", "Chorakanni", and "Chorakaniyan", are the popular common names of this fish. This native barb has become one of India's biggest exports in recent times (Anon., 2006; Anon., 2008). Among the 106 species contributing the ornamental fish fauna of Kerala no single species has received attention as much as the red-lined torpedo fish, *P. denisonii* the endemic and endangered cyprinid (Sekharan and Ramachandran, 2006; AnnaMercy *et al*., 2007; Anon, 2008).

Taxonomic status

Phylum	:	Vertebrata
Subphylum	:	Craniata
Superclass	:	Gnathostomata
Series	:	Pisces
Class	:	Teleostei
Subclass	:	Actinopterygii
Superorder	:	Acanthopterygii
Order	:	Cypriniformes
Family	:	Cyprinidae
Genus	:	*Puntius*
Species	:	*denisonii*

Distinguishing Characters

Fin formula: D 2/8; P 15; V 9; A 2/5; C 19

Body elongated, compressed; profile from snout to dorsal slightly elevated, from whence to the caudal it gradually slopes. Its depth is about 3.57 times in standard length. Head about 4.5 times in standard length. Eye diameter about $^{1}/_{3}$ of length of head. Nostrils nearer orbit than end of snout, the posterior closed by a membranous valve, the margin of the anterior slightly elevated, so as to appear semi-tubular. Mouth small, directed forwards and slightly downwards, the upper jaw being the longest, and snout slightly prominent. Maxillary barbels one pair only, longer than orbit. Dorsal fin inserted nearer to tip of snout than to base of caudal fin; its last unbranched ray non-osseous, weak and articulated. Scales large; lateral line complete, curved slightly in its anterior portion, then passing direct to the caudal with about 28 scales; number of transverse scale rows 5/3; pre-dorsal scales 9.

Body silvery, with a line of black passing from the snout through the lower part of the eye to the centre of the base of the caudal fin; above it a wide line of bright scarlet, extending same distance. Back glossy metallic blue; abdomen silvery white. Caudal with an oblique black band crossing the posterior third of each lobe.

1.4.2. *Puntius chalakkudiensis* Menon, Remadevi and Thobias.

In 1972, two specimens of a colourful *Puntius* fish was collected by Dr. M.P. Thobias from the upper reaches of Chalakkudy River, Kerala which resembles *P. denisonii* (Day) at first glance were described as *Puntius chalakkudiensis* (Fig. 2) by Menon *et al*. (1999). The species can be distinguished from all other *Puntius* species by its inferior mouth (terminal or subterminal in all other species) and a distinct black blotch on its dorsal fin from *P. denisonii* (Menon *et al*., 1999). Taxonomic ambiguity exists between these species and the

taxonomic identity of specimens caught from Chalakkudy River needs to be confirmed.

Taxonomic status

Phylum	:	Vertebrata
Subphylum	:	Craniata
Superclass	:	Gnathostomata
Series	:	Pisces
Class	:	Teleostei
Subclass	:	Actinopterygii
Superorder	:	Acanthopterygii
Order	:	Cypriniformes
Family	:	Cyprinidae
Genus	:	*Puntius*
Species	:	*chalakkudiensis*

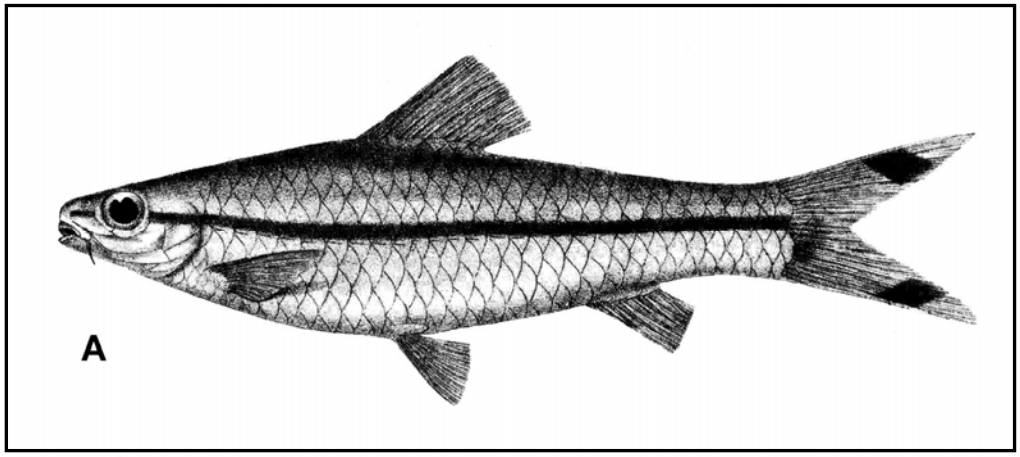
Distinguishing Characters

Fin formula: D 2/8; P 1,15; V 1,7; A 2/5; C 19

P. chalakkudiensis is distinguishable from all other *Puntius* species by its inferior mouth (terminal or subterminal in all other species) and from *P. denisonii* by the distinctive black blotch on its dorsal fin.

Body rather deep, its depth is about 3.45 times in standard length. Head 4.51, body depth 3.45, predorsal 2.16, postdorsal 1.87, preventral 1.95, preanal 1.35 times in standard length. Width of head 1.81, its depth 1.38, snout length 2.48, eye 4.17, interorbital width 2.76 times in head length. Mouth small, semicircular, inferior, width of mouth 2.36 in width of head. One pair of maxillary barbels, 1.15 in eye diameter, reaching to middle of eye when stretched. Lateral line scale count 28; number of transverse scale rows $5/2^{1}/_{2} - 3$; pre-dorsal scales 9 and 10 gill rakers on the first gill arch.

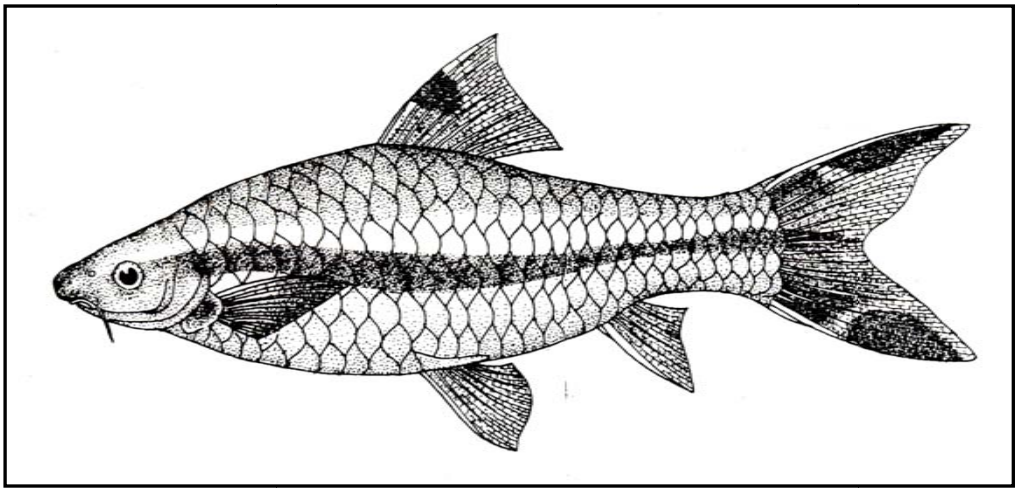
Body silvery with a black band from the snout to the base of caudal fin; a pale scarlet stripe above this; caudal fin with an oblique dark band crossing the posterior third of each lobe; dorsal with a dark mark in the middle of the outer surface extending up to the second branched ray.



A

B

Fig. 1. ***Puntius denisonii*** **(Day); A -** figure reproduced from Day, (1875) pl. CXLIII, figure 2.; **B** - specimen collected from Valapattanam River.



A

B

Fig. 2. ***Puntius chalakkudiensis*****; A -** figure reproduced from Menon *et al.*, (1999); **B -** specimen collected from Chalakkudy River.

Chapter 2

REVIEW OF LITERATURE

2.1. Concept of stock and sustainable exploitation

Since the early classical periods of Linnaeus, Lamark and Darvin, 'species' was the taxonomic unit of fishery resources. The species concept still remains as the corner stone of scientific research in various aspects of biology. Nevertheless, according to the Darwin's theory of origin of species and modern theories of evolution, the species cannot remain as a constant entity. In the course of time, each species may undergo micro-evolutionary processes leading to further speciation. The process and order of such speciation is primarily based on reproductive and geographic isolation and the transformation of its populations into different sub-populations or stocks and later into new species in an unspecified evolutionary time scale. 'Stocks' are said to be the natural units of a species. 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 term "stock" means different things to different people (Booke, 1981; Dizon *et al*., 1997). From a management perspective, stocks are understood to be groups of phenotypically similar individuals whose morphological and meristic homogeneity is due to environmental effects (Templeman, 1981). Implied is the idea that a stock could be manageable as a unit. An alternative approach to stock delimitation is genetic. From a population genetic perspective, stocks are equivalent to demes: closed populations that maintain themselves in a given area and are reproductively isolated from other such populations (Carr and Crutcher, 1998). Morphological differences among groups of genetically homogeneous fish from different areas may simply reflect different environmental conditions. For

example, vertebral counts are inversely correlated with water temperature at the time of spawning (Brander, 1979).

Genetics have contributed to the general globalisation of the term "stock" by creating concrete boundaries and offering a more detailed analysis of the history and evolution of populations. The stock concept implies that genetic diversity and population structure must be considered and preserved in order to optimise the resource use (Carvalho and Hauser, 1994).

Muzinic and Marr (1960) has stated the biological and fishery management principle that necessitates racial/stock/sub-species differentiation of fishery resources as "The logical and practical reasons for identifying population units are that such units may have their own characteristics of recruitment, growth, natural mortality, migration, behavior etc. more or less independent of the characteristics of other population units within the same species". Carvalho and Hauser (1994) has also supported this view by stating that, "The Fisheries management is mainly based on the assumption that a unit stock has definable patterns of recruitment and mortality and hence a sustainable yield". Current conservation plans mainly focuses to preserve the maximum amount of biodiversity, generally in terms of number of different species, with usually limited financial resource. In practice—when designing reserves for example—managers often resort to the principle of complementarity to make the most of the available resources and maximize the number of protected species (Bonin *et al*., 2007).

Grant *et al*., (1999) pointed out the importance of delineating the stocks and their boundaries which has become an essential part of fishery management/conservation. Lack of knowledge on the exact number of interbreeding populations of an exploited species will not help the management policies to achieve long-term conservation goals. So, it is required to generate

information on population/stock structure and basic genetic profile of the species for its effective and scientific management, conservation and rehabilitation.

2.2. Population genetics and conservation of species

Population genetics is concerned with the analysis of demographic and evolutionary factors affecting the genetic composition of a population (Hartl, 2000; Ewens, 2001). It is the application of Mendel's laws and other genetic principles to entire populations of organisms (Hartl and Clark, 1989). A population can be defined as a group of conspecific individuals forming a breeding unit sharing a particular habitat at a certain time. This is one of the widest concepts used as a sub-specific category (Slatkin, 1993). In the current context of biodiversity erosion, biologists face the urgent need for objective and unambiguous criteria to characterize conservation "units" that are the most worthwhile preserving. Among these criteria, the amount of intraspecific genetic variation is now widely accepted as a key parameter to determine populations to prioritize for protection purposes. Loss of genetic diversity within populations might be associated with inbreeding depression, which in turn results in reduced fitness and ultimately jeopardizes the population persistence (Bonin *et al*., 2007). Recent studies pointed out that intraspecific genetic diversity was also shown to favor species richness and to contribute to ecosystem functioning and recovery (Bonin *et al*., 2007; Dominguez *et al*., 2008).

By analysing genetic variation one can discriminate among fishes at the species, population and even individual levels; identify hybrids; establish species and population phylogeny and phylogeographic history; discriminate different stocks, analyse their migration patterns and estimate their effective size; assess individual stock contribution to mixed stock fisheries and evaluate the response of stocks to fishing exploitation (Wirgin and Waldman, 1994; Ferguson *et al.*, 1995; Huey *et al*., 2006; Pramuk *et al*., 2007; Ilves and Taylor, 2009). Maintenance of stock viability is greatly promoted by the conservation of their

gene pools, preserving genetic variability and hence, the possibility of future adaptation (Milligan *et al.*, 1994).

Genetic variation can be partitioned into two complementary but disconnected components that have to be assessed separately and differently (Bonin *et al*., 2007). The first is the selected (or functional) diversity arising directly from adaptive evolution due to natural selection and second is the neutral heritage of the population resulting from the effects of neutral evolutionary forces such as genetic drift, mutation, or migration. As a result of this, studies of genetic variability for population management are thus of two kinds. On the one hand quantitative traits that receive particular attention because they are the primary targets of natural selection and the variation at such traits is thus supposed to mirror the adaptive potential of the population or species (Bonin *et al*., 2007). On the other hand, some authors have underlined the importance of neutral genetic variation in conservation (Moritz, 1994).

The main evolutionary force, the natural selection, as introduced by Darwin is the capacity for genotypes to be selected by environmental factors and passed on to following generations. This is probably one of the main forces of evolution and is responsible for maintaining much of the phenotypic variation in nature. Natural selection can act in different directions and it can eliminate genetic variation or maintain it. In order to understand all the structuring of populations in nature we have to take in to consideration all these aspects and analyse them by looking at variation of distinct alleles at defined loci known as molecular or genetic markers (Allendorf *et al.*, 1987; Gavrilets, 2001).

Under the basic concept of evolution, every species is believed to be undergoing micro and macro evolutionary process resulting in the expression of significant genetic variations at levels of species specific chromosome morphology/structure, gene controlled protein structure and polygene controlled morphometrics and metrics (Ayala and Keiger, 1980). The classical

morphometrics / metrics and the modern molecular genetic tools have been employed in a number of teleosts to study intra-specific variations as well as inter generic / inter-specific differences.

2.2.1. Morphometrics and meristics

Meristic and morphometric characters have been commonly used in fisheries biology as powerful tools for measuring discreteness and relationships among various taxonomic categories (Ihssen *et al*., 1981; Melvin *et al*., 1992; Quilang *et al*., 2007; Nowak *et al*., 2008). Genetic markers are generally oversensitive to a low level of gene flow: a relatively low level of exchange between stocks, negligible from a management perspective, may be sufficient to ensure genetic homogeneity (Hubbs and Lagler, 1947; Ward and Grewe, 1994; Carvalho and Hauser, 1994). Therefore, molecular markers may not be sufficient to detect existing genetic variation among populations, and also only a small proportion of DNA is analysed by molecular markers. However, phenotypic markers may detect morphological differentiation due to environmental differences in the habitats of partially-isolated stocks, which may be a practical level of partitioning among self-recruiting stocks. Such self-recruiting stocks may react independently to exploitation (Carvalho and Hauser, 1994), even without showing genetic differentiation (Turan, 1999). Morphometric and meristic analysis can thus be a first step in investigating the stock structure of species with large population sizes (Turan, 1999). As a potential indicator of phenotypic stocks, analysis of morphometric landmarks is a valuable tool that compliments other stock identification methods. The identification, discrimination and delineation of phenotypic stocks are essential for population modeling, which generally assumes homogenous ontogenetic rates within a stock.

Heincke (1898) discovered significantly different vertebral counts among the European herring populations, which was the earliest report on study of morphometric variability among fish populations (Mohandas, 1997). Morphometric variability based on body parts such as length, depth, and width of

fish, body and that of head and tail regions were described by Hubbs and Lagler (1947). Meanwhile it was suspected that these measurements were related to the overall growth history of the fish because there appears to be distinct stanzas in the growth history of fishes, hence any comparison should be made within the same life history stanza (Martin, 1949).

There are many well documented studies on population/stock structure in fishes using morphological traits. Jayasankar *et al.* (2004) observed morphological homogeneity in Mackerals from Indian coasts. Significant morphologic heterogeneity was reported in silver perch (*Leiopotherapon plumbeus*) populations in the Philippines by Quilang *et al*. (2007). Morphometric analysis revealed a high degree of dissimilarity of the anchovy (*Engraulis encrasicolus*) in the Black, Aegean and Northeastern Mediterranean Seas (Turan *et al.*, 2004a). The studies of Uiblein (1995) in *Neobythites stefanovi*; Klingenberg and Ekau (1996) in Antarctic fishes (Perciformes:Nototheniidae); Elvira and Almodovar (2000) in *Acipenser sturio*; Smith *et al.* (2002) in black (*Allocytus niger*) and smooth oreos (*Pseudocyttus maculates*); Turan (2004) in Mediterranean horse mackerel (*Trachurus mediterraneus*); Turan *et al.* (2005) in African catfish (*Clarias gariepinus*); Katselis *et al.* (2006) in four Mediterranean grey mullet species; Pollar *et al*. (2007) in *Tor tambroides*; Mazlum *et al*. (2007) in eastern white river crayfish (*Procambarus acutus acutus*); Murphy *et al.* (2007) in river sturgeons (*Scaphirhynchus spp*.); Vasconcellos *et al.* (2008) in yellowtail snapper (*Ocyurus chrysurus*); Nowak *et al.* (2008) in common gudgeon (*Gobio gobio*) etc., are some other classical examples.

Genetic polymorphism or environmental factors may induce morphological variability among spatially separated fish populations (Carvalho, 1993), and phenotypic plasticity in fish morphology has been documented for various species (Wimberger, 1991, 1992; Gunawickrama, 2007). This phenotypic plasticity of fish allows them to respond adaptively to environmental change by modification in their physiology and behaviour which leads to changes in their

morphology, reproduction or survival that mitigate the effects of environmental variation (Stearns, 1983; Meyer, 1987). According to Sokal and Rinkel (1963) geographic variations is not likely due to the adaptation of a few characters to single environmental variable but a multidimensional process involving the adaptation of many characters to a myriad of interdependent environmental factors. Hence a better understanding of morphological variations may be achieved by thoroughly examining the patterns of variance and co-variance among as many characters as possible in a data set using multivariate statistical analysis (Gould and Johnston, 1972; Reyment *et al*., 1981; Thorpe, 1976, 1983, 1987). Early investigators used descriptive statistics and univariate analyses separately on each meristic and morphometric character, but these did not always yield efficient results (Surre *et al*., 1986). Thus, multivariate techniques such as principal component analysis, factor analysis, cluster analysis, and discriminant analysis, have been adopted in the study of population structure of fishes (Ihssen *et al*., 1981; Surre *et al*., 1986; Hedgecock *et al*., 1989; Melvin *et al*., 1992; Mamuris *et al*., 1998; Trapani, 2003; Quilang *et al*., 2007; Nowak *et al*., 2008).

The significant aspect of morphological characters is that they are polygenetically inherited but individually have low heritability and subjected to considerable environmental plasticity. Thus multivariate analysis of many sets of phenotypic characters are regarded as a more appropriate method than the use of single character for determining the extent to which populations or species may be genetically diverse (Smith and Chesser, 1981). The multivariate methods like principal component analysis have been proposed as an efficient tool for stock identification in fishery management programmes (Winans, 1985). The study of shape variation has advanced from measuring simple linear distances to deriving geometric variables. The shift from traditional morphometrics to more complex geometric functions was facilitated by the development of image processing tools. Recently developed geometric methods to morphometric analysis are generally categorized as either “landmark (truss network) methods or outline methods”. The landmark method analyses data derived from discrete

morphometric points, linear distances between point and geometric relationships among points, where as outline method quantify boundary shapes of structures such as otoliths or scales so that patterns of shape variation within and among groups can be evaluated (Cadrin and Friedland, 2005).

Meristic characters originally referred to characters that correspond to body segments (myomeres), such as number of vertebrae and fin rays. Now meristic is used for almost any countable structure, including numbers of scales, gill rakers, cephalic pores, and so on (Helfman *et al*., 1997). These characters are useful because they are clearly definable, and usually other investigators will produce the same counts. In most cases they are stable over a wide range of body size. Also, meristic characters are easier to treat statistically, so comparisons can be made between populations or species with a minimum of computational effort. Whereas, Morphometric studies are based on a set of measurements which represent size and shape variation and are continuous data, in contrast to meristic characters, which are discrete or noncontinous data. Therefore, the predictive abilities of morphometric and meristic characters differ statistically and are likely to be lower for the meristic characters (Ihssen *et al*., 1981). Thus, morphometric data should be analysed separately from meristic data in multivariate analyses (Ihssen *et al*., 1981).

Fishes are considered to be phenotypically more variable than most other vertebrates, having relatively higher within-population coefficients of variation of phenotypic characters (Carvalho, 1993). The differentiable variation in morphology among fish populations has been suggested as indicative of the presence of stock structuring and restricted movement among geographically isolated populations (Uiblein, 1995; Roby *et al*., 1991; Palumbi, 1994; Jerry and Cairns, 1998). Morphological characters for many fishes, including highly dispersible marine and estuarine species have revealed spatial separation of populations. Schaefer (1991) reported morphological heterogeneity among populations of yellowfin tuna (*Thunnus albacares*) from the Pacific Ocean based

on morphometric characters and gill raker counts; Elliott *et al*., (1995) observed spatial separation of populations in orange roughy (*Haplostethus atlanticus*) of southern Australia; Morphometric and meristic differences between shallow and deepwater populations of whitehake (*Urophycis tenuis*) was repoted by Hurlbut and Clay, (1998); Jerry and Cairns, (1998) observed morphological variation in the catadromous Australian bass, from seven geographically distinct riverine drainages. Morphological divergence has been reported in estuarine fish populations that are not completely geographically separated, suggesting that partial isolation may play a role in population subdivision. Roby *et al*. (1991) observed morphologic differentiation of capelin (*Mallotus villosus*) populations in the estuary and Gulf of St. Lawrence; and Suneetha and Naevdal, (2001) of the pearlside, *Maurolicus muelleri* (Pisces, Sternoptychidae) among Norwegian fjords and offshore area. Morphologic differentiation among stocks of Mediterranean horse mackerel, *Trachurus mediterraneus* (Turan, 2004); African catfish, *Clarias gariepinus* (Turan *et al*., 2005); silver perch, *Leiopotherapon plumbeus* (Quilang *et al*., 2007) were also reported. Even though, there are many well documented morphometric and meristic studies, which provide evidence for stock discrimination in fishes, the application of morphometrics in stock identification is complicated by the fact that phenotypic variations in these characters are often influenced by environmental factors and are not directly related to particular differences in the genome always (Clayton, 1981). Therefore, several new techniques using molecular markers came into practice to detect the stock structure and genetic variations of the organism.

2.2.2. Molecular markers

The development of DNA-based genetic markers has had a revolutionary impact on animal genetics. According to Liu and Cordes (2004), it is theoretically possible to observe and exploit genetic variation in the entire genome of organisms with DNA markers. Allozymes, mitochondrial DNA, RFLP, RAPD, AFLP, microsatellite, SNP, and EST markers are the popular genetic markers employed in fisheries and aquaculture.

Molecular markers are basically two types *viz*., protein and DNA markers. The DNA markers can be further classified into nuclear DNA and mitochondrial DNA (mtDNA) markers based on their transmission and evolutionary dynamics (Park and Moran, 1994). Popular nuclear DNA markers are Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Variable Number of Tandem Repeats loci (VNTRs: minisatellites, microsatellites) and Single Nucleotide Polymorphisms (SNPs) which are biparently inherited. Whereas, mitochondrial DNA markers are maternally inherited and non-recombining such that, they have one quarter the genetic effective population size (Ne) of nuclear markers (Ferguson and Danzmann, 1998). Studies of vertebrate species generally have shown that sequence divergence accumulates more rapidly in mitochondrial than in nuclear DNA (Brown, 1985).

One of the main questions at the beginning of any genome research is what type of marker is most suitable for the given project and to the species of interest. There is no simple answer to this question, and much depends on the specific objectives of the study. According to Carvalho and Hauser (1998) all approaches to describe population structure employing genetic markers are based on the principle that migration and mating patterns among populations determine the extent of a common gene pool and therefore their integrity; even though there are many types of genetic markers available for this (Park and Moran, 1994). Hillis *et al.* (1996) states that "no molecular marker is inherently superior to other and is worthwhile spending some time analysing which technique or techniques are best suited to approach a certain problem". Depending on the problem addressed, on the available resources, time and costing, some molecular markers can be more appropriate than others for studying a given problem. For example, DNA sequencing provides a resolution appropriate to phylogenetic and population-level studies but microsatellites are more appropriate to studies of parentage and mating systems and sometimes may not be sufficiently conserved for population-level comparisons (Creasey and Rogers, 1999). Sometimes only

by using a combination of different molecular markers can a problem be resolved.

From 1990's, an increasing number of studies have been published making use of random parts of a genome. One such approach involves PCR amplification of anonymous DNA fragments commonly known as Random Amplified Polymorphic DNA (RAPD) (Williams *et al.,* 1990; Welsh and Mc Clelland, 1990) to amplify stretches of DNA identified by random primers. A single short primer (10 base pairs) and low annealing temperature are combined to obtain specific amplification patterns from individual genomes. Priming sites are randomly distributed throughout a genome and polymorphisms in such sites result in differing amplification products, detected by the presence and absence of fragments. Such polymorphisms are generally inherited in a Mendelian fashion and can be used as genetic markers (Bardakci and Skibinski, 1994; Liu *et al*., 1999a; Appleyard and Mather, 2002). This technique is able to provide a convenient and rapid assessment of the differences in the genetic composition of related individuals (Kazan *et al*., 1993). RAPD fingerprinting has been used in teleosts in many studies *viz.,* for the identification of species and subspecies in tilapia (Bardakci and Skibinski, 1994; Sultmann *et al.,* 1995; Appleyard and Mather, 2002) and *Xiphophorus hellari* (Borowsky *et al.,* 1995); intraspecific genetic variation in red mullet (*Mullus barbatus*) (Mamuris *et al.,* 1998); monitoring of genetic polymorphism in sea bass after acclimation to freshwater (Allegrucci *et al.,* 1995) for discriminating the populations in hilsa shad, *Tenualosa ilisha* (Dahle *et al.,* 1997); for the study of heterosis in common carp, *Cyprinus carpio* (Dong and Zhou, 1998); for stock discrimination in *Hirundichthys affinis* (Gomes *et al.,* 1998); in yellow catfish, *Horabagrus brachysoma* (Muneer *et al.,* 2008) and for differentiating different species in grouper *Epinephelus* (Baker and Azizah, 2000; Govindaraju and Jayasankar, 2004; Christopher, 2004).

Variable Number of Tandem Repeats loci (VNTRs) are another type of genetic variation that of differences in the number of repeated copies of a segment of DNA. These sequences can be classified based on decreasing sizes into satellites, minisatellites and microsatellites (Tautz, 1993). Satellites consist of units of several thousand base pairs, repeated thousands or millions of times. Minisatellites consist of DNA sequences of some 9-100bp in length that is repeated from 2 to several 100 times at a locus. Microsatellites have a unique length of 1-6 bp repeated up to about 100 times at each locus (Litt and Luty, 1989). They are also called as 'simple sequence repeat' (SSR) by Tautz (1989) or 'short tandem repeat' (STR) DNA by Edwards *et al.* (1991). Jeffreys *et al.* (1988) and Weber (1990) opined that length variations in tandemly arrayed repetitive DNA in mini and microsatellites is usually due to increase or decrease of repeat unit copy numbers. These differences in repeat numbers represent the base for most DNA profiling techniques used today. The very high levels of variability associated with microsatellites, the speed of processing and the potential to isolate large number of loci provides a marker system capable of detecting differences among closely related populations. Microsatellites that have been largely utilized for population studies are single locus ones in which both the alleles in a heterozygote show co-dominant expression (Gopalakrishnan and Mohindra, 2001). Microsatellites are inherited in Mendelian fashion (O'Connell and Wright, 1997), give the information as distinguishable loci with codominant alleles and are more neutral and variable than allozymes (Queller *et al.*, 1993). Various authors have reported microsatellite polymorphism and sequences in several marine and freshwater fish species for the population/stock analysis (Estoup *et al*., 1993; Presa and Guyomand, 1996; Kirankumar *et al*., 2002; Buston *et al*., 2007; Muneer *et al*., 2008; Ghiasi *et al*., 2009).

Mitochondrial DNA (mtDNA), which generally exhibits a five to 10 times greater variability than single copy nuclear genes, has been used as a molecular marker and shown to be a powerful tool for elucidating population structures and estimating phylogenetic relationships in various groups of species (Howard and

Berlocher, 1998; Avise, 2000; Yamazaki, *et al*., 2003; Klinbunga *et al*., 2005; Doiuchi and Nakabo, 2006; Page and Hughes, 2007; Dominguez *et al*., 2008; Chan *et al*., 2009).

2.2.2.1. Mitochondrial DNA analysis

The mtDNA molecule exists in a high copy number in the mitochondria of cells and has a circular structure (Fig.3).

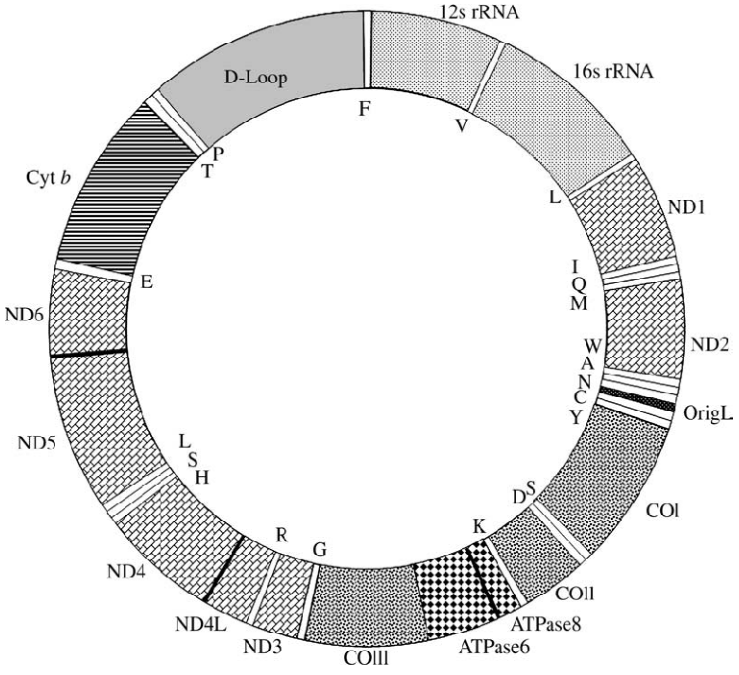


Fig.3. Vertebrate mitochondrial genome. Labels outside ring indicate the locations of the two rRNAs, the 13 mitochondrial-encoded polypeptides of OXPHOS and the light strand origin. Letters in the ring indicate locations of mtDNA produced tRNAs. Pi, inorganic phosphate. (Brown, 2008).

The vertebrate mitochondrial genome is composed of about 15 to 20 kb in different organisms, coding for 40 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) responsible 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 because the mitochondria do not have repair enzymes for errors in the replication, nor for the damages of the DNA (Clayton, 1982). In teleosts, mtDNA high mutation rate (average divergent rates of 2–4% per million years [Wilson *et al*., 1985] in vertebrates, is commonly accepted in fishes [Huey *et al*., 2006]) leads to a high degree of variability between individuals. It also has a large capacity to accumulate mutations that give it a faster rate of evolution than nuclear DNA in higher animals (Brown *et al.*, 1979). The whole molecule may be regarded as a single locus with multiple alleles (Park and Moran, 1995).

The technical advantages, such as requirement of only small amounts of fresh, frozen or alcohol-stored tissue, to amplify the genes has also made mtDNA a very practical genetic tool. Together with the fact that it is one of the most well studied parts of the animal genome, mtDNA sequencing has become the molecular marker of choice when studying cospecific populations (Gold *et al.*, 1993; Park and Moran, 1995; Stepien, 1999; Stammatis *et al.*, 2004; Dominguez *et al*., 2008). There are some disadvantages also with this marker like; maternal inheritance does not provide information about males in populations, which may display different dispersal behaviour to females and its haploid nature is also an issue as no inferences about the neutrality and equilibrium of populations, as well as other aspects based on allelic frequencies can be addressed.

In recent years, mtDNA, because of its fast evolution (Avise 1994), has been widely applied in systematics, population genetics, inference of migration routes and conservation biology of animals. In population studies, a hierarchical description of genetic diversity reflects phylogenetic relationships among populations distributed over different geographical regions, or may infer relationships among alleles based on the gene tree of organelle DNA (Avise 1994; Moritz and Faith 1998; Wang *et al*., 2000; Chen *et al*., 2007; Ilves and Taylor, 2009).

Due to its non-mendelian mode of inheritance, the mitochondrial DNA molecule is considered a single locus in genetic investigations (Avise, 1994). Uniparental inheritance also reduces the effective population size of mitochondrial genes, which means that variants are fixed more quickly between speciation events (Curole and Kocher, 1999). It is almost ten years since the polymerase chain reaction (PCR) has been used for amplifying and sequencing of microbial, plant and animal nuclear and mitochondrial genes (Kocher, 1989). Furthermore, mtDNA retains a history of past isolations for a longer period relative to nuclear DNA, but the maternal inheritance of mtDNA limits its ability to provide information on the male component of population.

From 1990's an increasing number of studies have been published making use of selected parts of mitochondrial and nuclear genome. 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 therefore is under fewer structural and functional constraints, leading to a high average substitution rate (Saccone *et al*., 1987). It is usually 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 (Caccone *et al*., 1996; Avise, 2000). Genetic variability is pivotal to maintaining the capability of restocked fish to adapt to a changing environment or demographic events (Avise, 1994; Nguyen *et al*., 2006). 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).

Several researchers from different parts of the world have used 16S rRNA gene sequences to study the phylogeny of different groups including fishes. Craig and Hastings (2007) have explained the molecular phylogeny of the groupers of the subfamily Epinephelinae (Serranidae) with a revised classification of the

Epinephelini using 12S and 16S rDNA sequences. The phylogenetic studies of Sparks and Smith (2004) in cichlid fishes; Vinson *et al*. (2004) in Sciaenid fishes; Lopez *et al*. (2000) in Esocoid fishes; Wiley *et al*. (1998) in Lampridiform fishes (Teleostei: Acanthomorpha) and Ilves and Taylor (2009) in Osmeridae are some other examples. Partial sequence of 16SrRNA gene has been used for resolving taxonomic ambiguities and analyzing phylogenetic relationship in bivalves (Bendezu *et al*., 2005; Klinbunga *et al*., 2005), crustaceans (Grandjean *et al*., 2002; Chan *et al*., 2009) and fishes (Doiuchi and Nakabo, 2006; Quenouille *et al*., 2004).

Cytochrome c Oxidase subunit I (COI / cox1) gene suppose to be evolving faster than 16S rDNA has been used widely in molecular taxonomy to resolve the phylogenetic relationships within the family and also to study intra-specific population genetic structure in several groups of eukaryotes including fishes. Klinbunga *et al*. (2005) utilized the COI sequences along with 16S and 18S rDNA to study the molecular taxonomy of Cupped Oysters (*Crassostrea, Saccostrea,* and *Striostrea*) in Thailand. Ecological speciation in a Caribbean marine sponge has been studied using mtDNA COI gene (Duran and Rutzler, 2006). Population structure and genetic divergences were examined between two cryptic species of Lethenteron by Yamazaki *et al*. (2003). Well documented studies on population genetic structure were reported in several species including fishes like, crayfishes of the genus *Euastacus* in Australia (Ponniah and Hughes, 2006); California red abalone (*Haliotis rufescens*) (Gruenthal *et al*., 2007); Antarctic coastal krill *Euphausia crystallorophias* (Jarman *et al*., 2002); and in atyid shrimp, *Paratya australiensis* (Baker *et al*., 2004a), utilizing COI sequences variations. Couceiro *et al*. (2007) described the population genetic structure and average dispersal distance of the netted dog whelk *Nassarius reticulatus* (Mollusca, Gastropoda, Prosobranchia) from 6 locations using sequence variations in a fragment of the mtDNA COI gene. Page and Hughes (2007) selected mtDNA COI region to study the phylogeographic structure of cryptic lineages within the freshwater shrimp *Caridina indistincta* in Australia, in an

attempt to unravel any potential genetic influences of Quaternary sea-level oscillations.

Partial sequence information of cytochrome *b* (Cyt *b*) gene is used to study species specific pattern in several animals. Several rationales exist for summarizing the Cyt *b* literature (Glen and Avis, 1998). This is perhaps the most extensively sequenced gene in the vertebrates (Irwin *et al*., 1991; Moore and De-Filippis, 1997). Primarily because (1) evolutionary dynamics of the Cyt *b* gene and the biochemistry of the proteins produced are better characterized than most other molecular systems (Esposti *et al.,* 1993) and (2) levels of genetic divergence typically associated with sister sps., congeners and co-familiar genera usually are in a range in which the Cyt *b* gene is phylogenetically informative and unlikely to be severely compromised by saturation effects involving superimposed nucleotide substitution (Mortiz *et al,* 1987; Meyer, 1994). Garcia *et al*. (2000) utilized 324 base pairs of the mitochondrial cytochrome b gene to examine the phylogenetic relationships in 14 species of the genus *Cynolebias* (Cyprinodontiformes, Rivulidae) while, Ghorashi *et al*. (2008) carried out the phylogenetic analysis of anemone fishes of the Persian Gulf. Phylogeography of *Glyptothorax fokiensis* and *Glyptothorax hainanensis* in Asia (Chen *et al*., 2007); Neotropical catfish *Pimelodus albicans* (Siluriformes: Pimelodidae) from South America (Vergara *et al*., 2008); and an endangered fish *Zoogoneticus quitzeoensis* (Cyprinodontiformes: Goodeidae) (Dominguez *et al*., 2008) are also well studied based on mtDNA cyt *b* sequence variations. Goswami *et al*. (2008) has also used mtDNA cyt *b* region to study the genetic stock structure of two seahorse species (*Hippocampus kuda* and *Hippocampus trimaculatus*) from the south east and south west coasts of India.

ATPase gene of mtDNA is comparatively faster evolving and is extremely useful in assessing population structure, levels of connectivity and influence of historical processes in fish species (Mc Glashan and Hughes, 2001a; Ovenden and Street, 2003). Mc Glashan and Hughes (2001a) reported significant

levels of genetic subdivision among populations from 8 sites of the Australian freshwater fish, *Craterocephalus stercusmuscarum* using 7 polymorphic allozyme loci and sequence information on the ATPase gene of mictochondrial DNA. Hughes and Hillyer (2006) used allozymes and ATPase gene of mtDNA and found significant levels of differentiation between drainages of two fish species, *Nematolosa erebi* and *Retropinna semoni*. Sequence information on the mtDNA control region and ATPase6 gene were utilized in the genetic population structure analysis of 8 populations of mangrove jack, *Lutjanus argentimaculatus* as reported by Ovenden and Street (2003). Direct sequencing of mtDNA D-loop (745 bp) and mtATPase6/8 (857 bp) regions was used to investigate genetic variation within common carp (*Cyprinus carpio*) and develop a global genealogy of common carp strains (Thai *et al*., 2004). Machordom and Doadrio (2001) studied the phylogenetic relationships and evolutionary patterns in the cyprinid genus *Barbus* through the analysis of the complete sequences of mitochondrial ATPase and cytochrome *b* genes. Perdices *et al*. (2002) studied the evolutionary history of the genus *Rhamdia* (Teleostei: Pimelodidae) in Central America; whereas, Perdices *et al*. (2005) inferred the phylogenetic relationships of *Synbranchus marmoratus* and *Ophisternon aenigmaticum* from 45 drainages throughout South, Central America and Cuba, based on mitochondrial genes cytochrome *b* and ATPase 8/6.

In recent years, several authors have reported studies on phylogenetic relationships, evolutionary pattern and genetic variability among species, sub-species and populations in the genus of family cyprinidae using mitochondrial DNA sequences. Alves *et al*. (2001) observed significant mtDNA variation in the highly endangered cyprinid fish *Anaecypris hispanica* and identified four Management Units (MUs) among specimens representative of nine tributaries in the Iberian Peninsula. Nucleotide sequences of cyt *b*, tRNA genes, 12S rDNA and control region were used to assess the genetic and phylogeographic structure of *Acrossocheilus paradoxus* populations in 12 major streams from 3 geographical regions in Taiwan by Wang *et al*. (2000). The genealogical

relationships and diversity within common carp (*Cyprinus carpio*) were well studied using allozyme, microsatellites and mitochondrial DNA markers (Kohlmann *et al*., 2003; Thai *et al*., 2004). Durand *et al*., (2002) had utilized mitochondrial DNA cytochrome *b* region to study the phylogeny and biogeography of the family cyprinidae in the Middle East. Phylogeographic studies on the cyprinid *Squalius aradensis* were carried out based on the sequence information of cyt *b* and microsatellites (Mesquita *et al*., 2005). Li *et al*., (2008) utilized the 16S rDNA sequences from 93 cyprinid fishes to reconstruct the phylogenetic relationships within the diverse and economically important subfamily Cyprininae. The phylogeny and speciation modes in the European cyprinids (Briolay *et al*., 1998; Zardoya and Doadrio, 1999); cyprinid genus *Barbus* (Machordom and Doadrio, 2001); Eurasian and American cyprinids (Cunha *et al*., 2002); East Asian cyprinids (Liu and Chen, 2003); finescale shiners of the genus *Lythrurus* (Cypriniformes: Cyprinidae), a group of 11 species of freshwater minnows widely distributed in eastern North America (Pramuk *et al*., 2007); East Asian cyprinid genus *Sarcocheilichthys* (Zhang *et al*., 2008) were studied by analyzing different regions of mitochondrial genome are well documented.

2.2.2.1a. Concept of DNA barcoding

The use of short DNA sequences for the standardized identification of organisms has recently gained attention under the terms DNA barcoding or DNA taxonomy (Floyd *et al*., 2002; Hebert *et al*., 2003a,b and Tautz *et al*., 2003): analogy to 'UPC (Universal Product Code) system' used to identify manufactured goods. DNA barcoding clearly has enormous potential to relieve taxonomists of routine identifications, providing more time to focus on new taxonomic hypotheses and to concentrate on rare, poorly characterized, and new species. The ability to quickly put a name to an unknown specimen benefits not only conservationists, but is also a tremendous tool for ecologists as well (Newmaster *et al*., 2006). In addition to enabling species identification, DNA barcoding will aid phylogenetic analysis and help reveal the evolutionary history

of life on earth. It could also be applied where traditional methods are unrevealing, for instance identification of eggs and immature forms, and analysis of stomach contents or excreta to determine food webs. Selection of an appropriate gene is a critical strategic and practical decision, with significant consequences for the overall success of barcoding. An appropriate target gene for DNA barcoding is conserved enough to be amplified with broad-range primers and divergent enough to allow species discrimination.

Hebert *et al*. (2003a,b) has established that the mitochondrial gene cytochrome *c* oxidase I (COI) can serve as the core of a global bio-identification system for animals. The selection of COI as a target gene for DNA barcoding is supported by published and ongoing work, which demonstrates that barcoding via COI, will meet the goals for a wide diversity of animal taxa (http: //www.barcodinglife.com//). A model COI profile, based upon the analysis of a single individual from each of 200 closely allied species of lepidopterans, was 100% successful in correctly identifying subsequent specimens (Hebert *et al*., 2003a). Two hundred and seven species of fish, mostly Australian marine fish, were sequenced (barcoded) for a 655 bp region of the mitochondrial cytochrome oxidase subunit I gene (cox1) as reported by Ward *et al*. (2005). Spies *et al*. (2006) examined the variation at the mtDNA COI gene in 15 species of North Pacific skates and indicated that, a DNA-based barcoding approach may be useful for species identification. DNA barcoding reveals a likely second species of Asian sea bass (barramundi) (*Lates calcarifer*); Ward *et al*., (2008a) strongly suggest that barramundi from Australia and from Myanmar are different species based on the sequencing of 650 base pair region of the mitochondrial COI gene. Out of fifteen fish species barcoded from Northern (Atlantic and Mediterranean) and Southern (Australasian) Hemisphere waters using COI sequences, Ward *et al*. (2008b) observed significant evidence of spatial genetic differentiation for this gene in two fishes; the silver scabbardfish (*Lepidopus caudatus*) and John dory (*Zeus faber*). These observations further supported the scope of barcoding in identifying species and also the geographical variations expected within species.

When fully developed, a COI identification system will provide a reliable, cost-effective and accessible solution to the current problem of species identification as stated by Hebert *et al*. (2003a).

Even though the efficiency of COI gene as DNA barcodes (Hebert *et al*., 2003a,b) has been demonstrated in a wide variety of organisms (Hebert *et al*., 2004a,b; Saunders, 2005; Ward *et al*., 2005); concerns have been raised (Vences *et al*., 2005a,b; Lockridge Mueller, 2006) regarding the efficacy of the CO1 approach to DNA barcoding. It has also been suggested that, it is undesirable to rely on a single sequence for taxonomic identification (Sites and Crandall, 1997; Mallet and Willmortt, 2003; Matz and Nielsen, 2005). Thus the feasibility of using additional genes, particularly ribosomal RNA genes, as DNA barcodes has also been explored. For instance, different rRNA genes have been proposed to be good DNA barcodes in nemotades (Floyd *et al*., 2002; Power, 2004), tardigrades (Blaxter *et al*., 2004) and amphibians (Vences *et al*., 2005b). Vences *et al*. (2005b) have compared the performance of 16S and COI genes in representatives of different frog, salamander and caecilian genera and showed the superiority of 16S fragment over COI based on universality of priming sites and identification of major vertebrate clades that studied. They have strongly advocated the use of 16S rRNA as standard DNA barcoding marker for vertebrates to complement COI, especially if samples a priori could belong to various phylogenetically distant taxa and false negatives would constitute a major problem. However, Smith *et al*. (2008) have concluded that there is greater potential for a COI barcode's use with amphibians than has been reported to date, based on their work carried out with a group of Holarctic amphibians.

An intrinsic problem of using rRNA as barcodes resides in sequence alignment (Lutzoni *et al*., 2000; Noe and Kucherov, 2004). The use of mitochondrial genes encoding ribosomal (12S and 16S) DNA in broad taxonomic analyses is constrained by the prevalence of base insertions and deletions (indels) that complicate sequence alignments (Doyle and Gaut, 2000; Hebert *et al*.,

2003a). Since indels are common in rRNA sequences, every sequence with indels has to be assigned gaps for alignment with the others. The DNA sequences for barcoding purpose should be intact, i.e. not incorporated with any artifacts, including gaps. Otherwise, the same sequence may be referred to as different barcodes by different laboratories because of differences in alignment. Thus, sequence alignment is a major obstacle that limits the effective use of rRNAs for barcode purposes. An attempt has been done by Chu *et al*. (2006) to analyze rRNA sequences without alignment, using a simple correlation analysis based on composition vectors (CVs) derived from sequence data (Yu and Jiang, 2001; Qi *et al*., 2004a,b), with a view to test the feasibility of using rRNAs as molecular barcodes. They have considered assembling large datasets of rDNA sequences from GenBank database for their feasibility study in line with the studies which demonstrate the use of COI as DNA barcodes by Hebert *et al*. (2003a, b). By analyzing a total of seven rRNA datasets from a wide variety of organisms and taxonomic levels, from archaea to tetrapods, from class to species they have demonstrated that unaligned rRNA gene sequences could be used as convenient and reliable DNA barcodes.

However, DNA barcoding has evolved as a modern tool of taxonomy; conflict still remains in the selection of a standard marker for this even with in vertebrates. Both COI and 16S (unaligned) rRNA genes could be used as DNA barcodes for effective and more accurate identification of species can be suggested as a solution.

2.3. Indian scenario

Genetics has become an important tool in species conservation and several studies of this matter have been conducted on both marine and freshwater fish species, especially on those subject to exploitation. Molecular techniques can provide valuable data about phylogeny, structure and endemicity of target species. Together with morphometric and hydrographical data can be used to

infer population structure, gene flow, recruitment, reproductive strategies and behavior of fish species.

Over the last quarter of century numerous genetic studies have been undertaken to determine variation and population structure of fresh water fishes using a variety of molecular techniques. Allozyme electrophoresis was the technique used by many authors to study this in the early days. With progress, molecular techniques like RFLP's, RAPD's and mtDNA sequencing were quickly adopted. Jayasankar *et al*. (2004) has reported a holistic approach, combining morphometric and genotypic methods to analyze possible population differences in Indian mackerel (*Rastrelliger kanagurta*).

Several attempts have been made by many authors to study the genetic differentiation and population structure of some fresh water fish species from India using genetic markers. Sarangi and Mandal (1996) reported isozyme polymorphism in diploid and tetraploid Indian major carps, *Labeo rohita*. Gopalakrishnan *et al.* (1997) identified species-specific esterase markers in rohu and mrigal, while Singh *et al.* (2004) identified allozyme markers helpful in population genetic analysis of *Cirrhinus mrigala*. Mohindra *et al*. (2001 a, b; 2002 a, b, c, d) have carried out cross-species amplification of *C. catla* G1 primer in *Catla catla* from Gobindsagar; *L. rohita*, *Labeo dero*, *L. dyocheilus* and *Morulius calbasu*, and sequenced the loci in these species. Das and Barat (2002a, b, c) also carried out charecterisation of dinucleotide microsatellite repeats in *Labeo rohita*. Genetic variation among three riverine and one hatchery populations of this species has also been studied by Islam and Alam (2004) using RAPD. Silas *et al*. (2005) utilized RAPD marker to reveal the genetic identity of an endemic cyprinid of the Western Ghats, *Tor malabaricus*. In another endemic cyprinid of the Western Ghats (*Labeo dussumieri*), Gopalakrishnan *et al*. (2002) sequenced microsatellite loci by cross-species amplification of *C. catla* G1 primer. Kirankumar *et al*. (2002) reported the complete sequence of a repeat like region in Indian rosy barb (*Puntius conchonius*). Seven polymorphic

microsatellite DNA markers were developed by cross-species amplification of primers from other cyprinid species in *Labeo dyocheilus* for population-genetic studies by Mohindra *et al*. (2005). Gopalakrishnan *et al*. (2006) identified polymorphic microsatellite loci in sun-catfish, *Horabagrus brachysoma* by cross-species amplification of primers from the orders Osteoglossiform and Siluriform. High level of genetic variation between 3 populations of *H. brachysoma* using 14 polymorphic allozyme loci (Muneer *et al*., 2007) and significant levels of genetic differentiation has also reported in the same species using RAPD and microsatellite markers (Muneer *et al*., 2008). Gopalakrishnan *et al*. (2007) has reported low genetic differentiation in the populations of the Malabar carp *Labeo dussumieri* using allozymes, RAPD and microsatellites while, Johnson *et al*. (2007) has reported some degree of genetic structuring among the populations of the freshwater cyprinid *Puntius filamentosus* using Restriction Fragment Length Polymorphism (RFLP) analysis from the Western Ghats.

In *Puntius denisonii*, 6 polymorphic RAPD markers and 11 microsatellite markers developed by cross-species amplification was reported by Lijo (2004). However, no attempts have been made to study the stock structure and basic genetic profile of the species that are essential for good fishery management, conservation and rehabilitation of this species. This prompted me to take up the present work with a view to study the genetic diversity and population/stock structure in *P. denisonii* from different geographically isolated environments and to delineate the taxonomic ambiguity existing with *P. chalakkudiensis* using morphologic and molecular genetic tools. In the present study the mtDNA regions such as 16S rRNA, Cyt *b*, COI and ATPase 8/6 genes were used to study the taxonomic status and genetic heterogeneity in different riverine populations of *P. denisonii* and *P. chalakkudiensis*.

Chapter 3

MATERIALS AND METHODS

3.1. Sampling

Specimens of *Puntius denisonii* and *Puntius chalakkudiensis* were collected from selected six geographically isolated locations comprising the rivers Chandragiri, Kariangode, Valapattanam, Chaliyar, Chalakkudy, and Periyar (Fig. 4) throughout the species distribution (Menon *et al*., 1999). 30–50 specimens including juveniles (TL ranges 1.5 – 4.0 cm), fingerlings (TL ranges 4.0 – 10.0 cm) and adults (TL ranges 10.0 – 15.0 cm) were collected from each location using eco-friendly methods (Table 1). Specimens of *P. denisonii* and *P. chalakkudiensis* were identified up to species level in the field following Talwar and Jhingran, (1991), Jayaram (1999) and Menon *et al*. (1999). For DNA extraction, a piece of tissue (fin clips of approximately 5x5mm size from pectoral and pelvic fins of right side of the specimens) was excised and placed in 95% alcohol. Further the specimens were fixed in 30% formalin, and preserved in 10% formalin for further morphometric measurements.

3.2. Morphometrics and meristics

3.2.1. Material

A total of 180 specimens were used for morphometric study. 30 specimens of *P. denisonii* and *P. chalakkudiensis* each collected from each location as mentioned in Section 3.1. and Table 1.

3.2.2. Morphometric characters

Twenty one reliably measurable morphometric characters were selected for the study (Table 2). The distance between the vertical or horizontal lines across the identified superficial landmark points were measured using digital calipers (Fig. 5). All body measurements were taken point to point to the nearest of 0.1 mm following Kottelat (1990) and routinely taken from the left side.

Table 1. Details of sampling of *P. denisonii* and *P. chalakkudiensis* for the present study.

River basin	Site code	Geographic location	Date of capture	n_1	n_t
Chandragiri	CDR	12^0 34' N 75^0 23' E	Apr. 2007 Jun. 2007 May 2008	04 19 27	50
Kariangode	KGD	12^0 17' N 75^0 23' E	Jun. 2007 May 2008	15 17	32
Valapattanam	VLP	12^0 04' N 75^0 43' E	Feb. 2007 Mar. 2007 Jun. 2007 May 2008	04 16 17 15	52
Chaliyar	CLR	11^0 23' N 76^0 01' E	Mar. 2007 Jun. 2007	19 17	36
Chalakkudy	CHD	10^0 17' N 76^0 32' E	Jul. 2007 Dec. 2007 Jul. 2008	16 12 07	35
Periyar	PER	10^0 08' N 76^0 47' E	Dec. 2007 Feb. 2008 May 2008	17 15 20	52

n_1 – no. of specimens captured in each collection, n_t – total no. of specimens collected from each location.

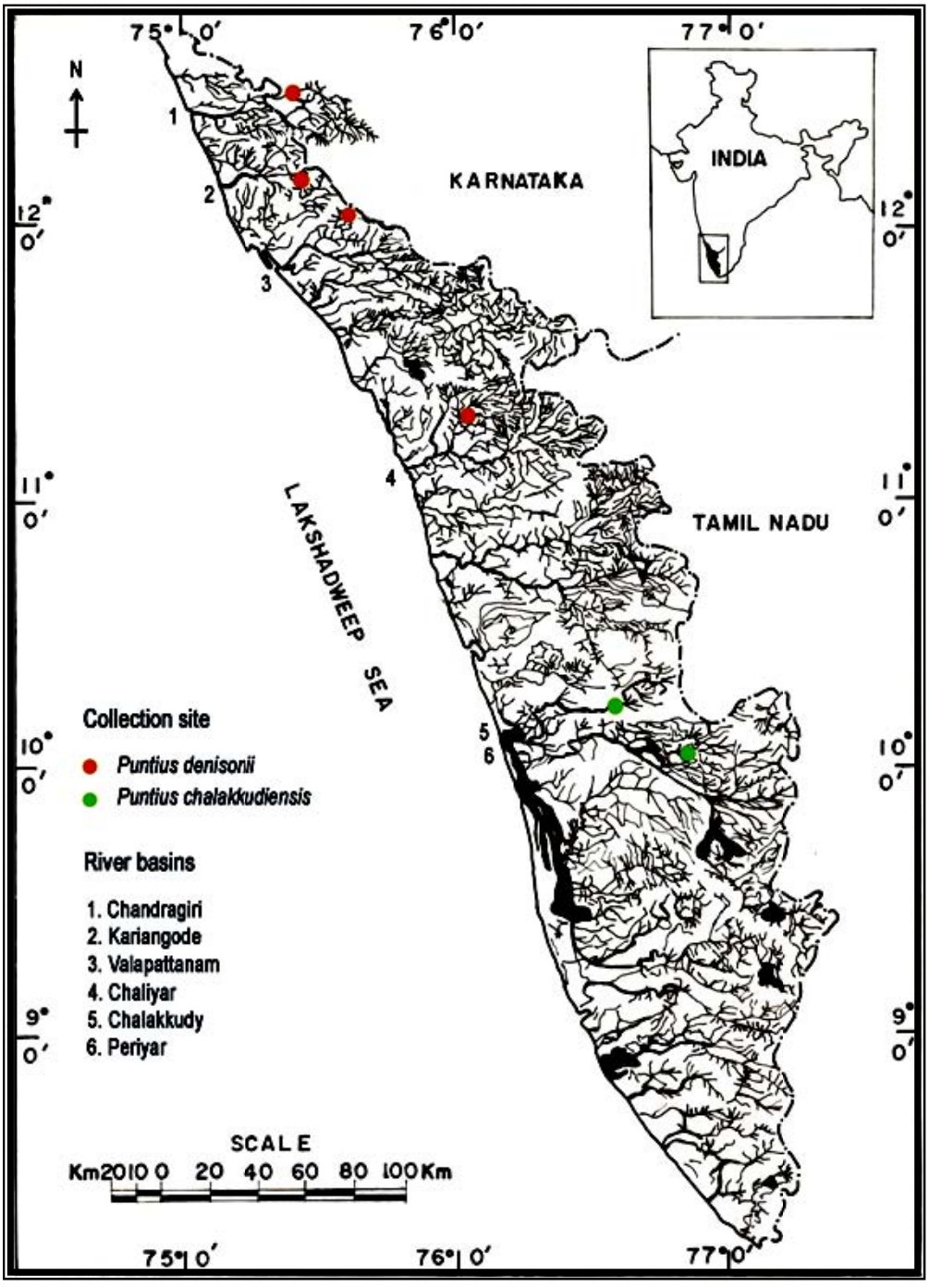


Fig. 4. Map of Kerala showing the distribution of the sampling sites of *P. denisonii* and *P. chalakkudiensis*

All length measurements (*L*) were taken parallel to the antero-posterior body axis. Head depth (HD) was taken perpendicular to the body axis between dorsal and ventral margins of the head, starting from the point where the ventral edge of the operculum intersects the ventral body margin. Maximum body depth (MBD) was measured between the two visually detectable widest points of the trunk, perpendicular to the body axis. Width of mouth (MW) was measured between the points of lateral edges of jaws of the mouth. The length of the fin bases was measured between the verticals made across the externally visible origin of the first spine and the last ray of the fin. Measurements of parts of the head are given as percentages of head length (L_H). The head length and measurements of other parts of the body are represented as percentages of standard length (L_S).

3.2.3. Meristic characters

Meristic characters studied include fin ray and scale count. The fins of bony fishes are supported by hard spines and segmented soft rays. Soft rays are either simple or branched. One of the most reliable ways of identifying fish species is by the fin ray formula. Spines (whether true spines or modified soft rays) are designated by means of roman capital letters (I, II, III etc.). Simple or unbranched segmented rays are designated by roman lower case letters (i,ii,iii etc.) are always at the beginning. Branched rays are represented by arabic numerals (1, 2, 3 etc.) and almost without exception are in the hinder part. The dorsal fin is prefixed D and the anal fin A (eg. D.II, 6; A.iii, 11). The pectoral fin is prefixed P and the ventral or pelvic fin V (eg. P.14;V.6).

In the present study, eight meristic characters and pharyngeal teeth were counted to find out any variation between the two species *viz.*, *P. denisonii* and *P. chalakkudiensis*. The meristic characters included the number of soft rays of dorsal fin (*DFS*); number of branched rays of dorsal fin (*DFR*); number of pectoral fin rays (*PFR*); number of ventral fin rays (*VFR*); number of soft rays of anal fin (*AFS*); number of branched rays of anal fin (*AFR*); number of gill rakers

on the outer gill arch (*GRC*); and lateral line scale count (*LLS*). The counts were routinely taken from the left side where as gill rakers were counted on the right side. The pharyngeal teeth were counted and represented by a formula adopting the method of Hubbs and Lagler (1947), e.g. the formula 2,3,5-5,3,2 indicates that the pharyngeal bones of both left and right sides bears three rows, with two teeth in the outer row, three in the middle and five in the inner. The structure and arrangement of pharyngeal teeth and gill rakers were observed under a binocular microscope (Nikon DS-L2) and images were digitally captured.

3.2.4. Analysis of data

In morphometric analyses, allometric growth *i.e*., heterogeneity in body size among samples can result in heterogeneity of shape without providing information on differences in body proportions among populations (Reist, 1985). The morphometric variables were transformed with natural logarithms and rectified as allometric variations for principal component analysis. Size-dependent variation for morphometric characters was excluded using the formula by Elliott *et al*. (1995):

$$M_{adj} = M(L_s/L_o)^b$$

where, M is the original morphometric measurement, M_{adj} the size adjusted measurement, L_o the standard length of fish, and L_s the overall mean of standard length for all fish from all samples for each variable. The parameter b was estimated for each character from the observed data as the slope of the regression of log M on log Lo, using all specimens. The efficiency of size adjustment transformations was assessed by testing the significance of correlations between transformed variables and standard length. Both univariate and multivariate analysis of variance were carried out to test the significance of morphometric differences among populations. The descriptive statistics *viz.*, minimum, maximum, mean and standard deviation for morphometric traits were estimated using SPSS (ver. 10.0).

The coefficient of variation (CV) was computed for each character using the formula (Van Valen, 1978):

$CV = (100\times SD)/X_m$,

where *SD* is the standard deviation and X_m is the mean of the transformed measurements of characters in each species. In each species' sample group, morphological variability was estimated by the multivariate generalization of the coefficient of variation (CVp) using the formula (Van Valen, 1978):

$CV_p = 100 \times \sqrt{\sum S_x / \sum M_x}$,

where S_x is the variance of each morphometric variable and M_x is the mean squared.

To identify whether there are any statistically significant differences between the species/population for each character, one-way analysis of variance (ANOVA) was performed (Snedecor and Cochran, 1980; Zar, 1984; Katselis, *et al*., 2006) using SPSS software (ver. 10.0). In addition, size-adjusted data were standardized and submitted to principal component analysis (PCA) and the scatter plots were generated using the software PAST (ver. 1.89).

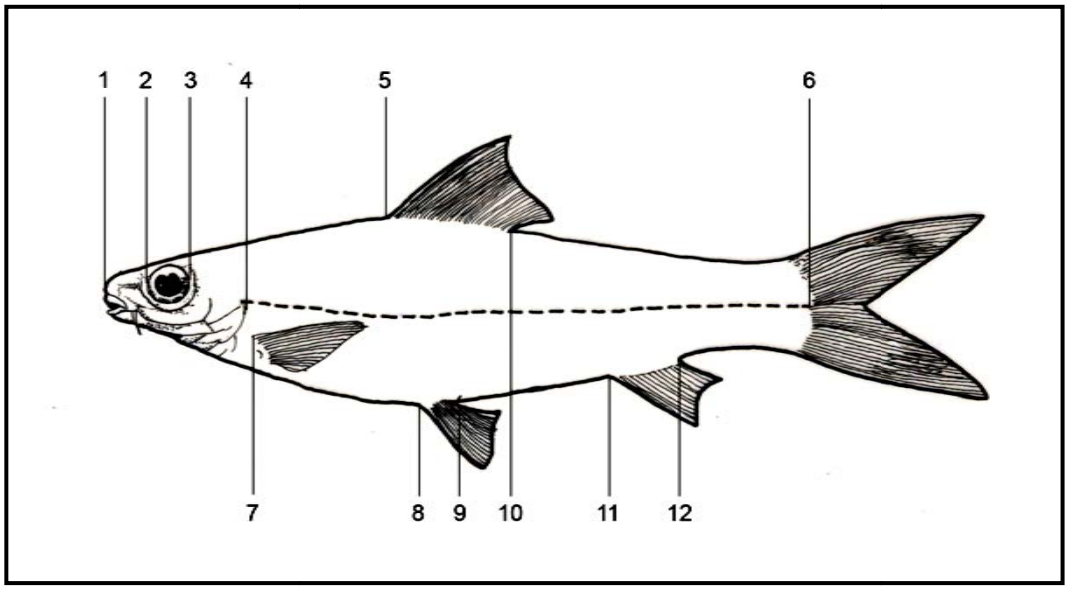


Fig. 5. Schematic representation of *P. denisonii* / *P. chalakkudiensis* indicating the positions of the superficial points used to measure the morphometric data (Table 2 for description).

Table 2. Description of morphometric characteristics of *P. denisonii* and *P. chalakkudiensis*.

Character	Code	Description	Number code (Fig. 5)
Standard length	L_S	snout tip to the midpoint of caudal fin origin	1 – 6
Head length	L_H	snout tip to the posterior edge of operculum	1 – 4
Maximum body depth	MBD	distance between points at deepest part of body (measured vertically)	not shown
Pre dorsal length	L_{PRD}	snout tip to the origin of dorsal fin	1 – 5
Post dorsal length	L_{PD}	length from the last ray of the dorsal fin to origin of caudal fin	10 – 6

Table 2 - continued.

Pre ventral length	L_{PRV}	snout tip to the origin of ventral fin	1 – 8
Pre anal length	L_{PRA}	snout tip to the origin of anal fin	1 – 11
Pectoral to pelvic origin	L_{PTFPLF}	length from the origin of pectoral fin to that of pelvic fin	7 – 8
Pelvic to anal	L_{PLFAF}	length from the origin of pelvic fin to that of anal fin	8 – 11
Length of body cavity	L_{BC}	length from the first ray of pectoral fin to vent	not shown
Dorsal fin base	FBDO	length between the visible origins of the first spine and the last ray of the dorsal fin	5 – 10
Anal fin base	FBAN	length between the visible origins of the first spine and the last ray of the anal fin	11 – 12
Length of caudal peduncle	L_{CP}	length from the anal fin insert to the midpoint of the caudal peduncle	12 – 6
Depth of caudal peduncle	CPD	least depth measured vertically	not shown
Head depth	HD	depth at the nape measured vertically	not shown
Width of head	HW	Width behind orbit perpendicular to the longitudinal axis	not shown
Width of mouth	MW	length between the points of lateral edges of jaws of the mouth	not shown
Orbital length	L_{O}	length (along axis) of the orbit	2 – 3
Pre orbital length	L_{PRO}	mouth tip to anterior edge of orbit	1 – 2
Post orbital length	L_{PO}	posterior edge of orbit to posterior edge of operculum	3 – 4
Barbel length	L_{MB}	Length of maxillary barbel on left side	not shown

3.3. Mitochondrial DNA analysis

3.3.1 Genomic DNA isolation

Total DNA was extracted from the tissue (fin clips) samples following the procedure of Miller *et al.* (1988) with minor modifications. Fifteen samples each from each location (included representative sample from each collection) were selected for isolating the total DNA.

Reagents required:

Stock solutions:

1. 0.5M Tris Cl (pH-8.0)

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

2. 0.5M EDTA (pH-8.0)

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

3. 10mM Tris Cl (pH-7.5)

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

4. RNAase buffer

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

Working Solutions

1. Solution 1:

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

2. Solution 2:

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

3. Proteinase K

Proteinase K - 20mg/ml

Prepared in autoclave double distilled water and store at -20°C.

4. TE buffer

Tris Cl (pH-8.0) - 10mM

EDTA (pH-8.0) - 1mM

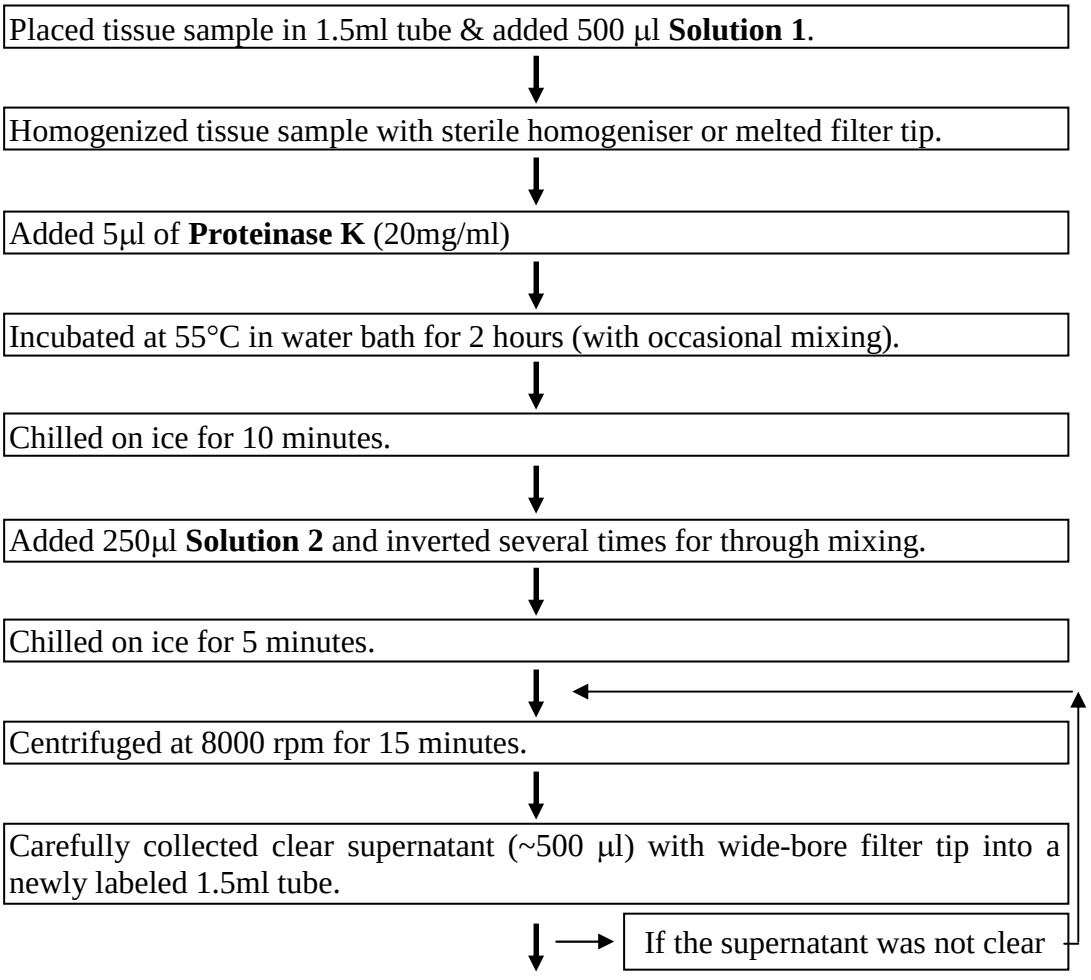
Prepared in double distilled water. Autoclave and store at 4°C

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.



Added 1.5µl RNase (final conc.20µg/ml) and incubated at 37°C on heating block for 15 minutes.

↓

Added twice the volume (~1ml) of ice cold 100% molecular biology grade Ethanol to precipitate the DNA.

↓

Incubated overnight at -20°C.

↓

Next day, centrifuged at 11000rpm for 15 minutes and removed supernatant.

↓

Rinsed DNA pellet in 250 µl of ice-cold 70% ethanol.

↓

Centrifuged at 11000rpm for 5 minutes.

↓

Carefully removed supernatant and partially dry with lid off at room temperature.

↓

Resuspended partially dried DNA in 50-200µl (depending on size of pellet) of TE buffer (pH-8) by gently pipetting sample with wide-bore filter tip until dissolved.

The DNA samples were stored at -20^0C for further use.

Agarose electrophoresis and visualization of bands

The extracted DNA was checked through 0.7% agarose gel (10x4cm) electrophoresis with ethidium bromide incorporated in 1X TBE buffer.

Reagents required:

1. TBE buffer 10X (pH-8.0)

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

2. Gel loading buffer

Bromophenol blue - 0.5%
Glycerol (mol. grade) - 30%
Prepared in 1XTBE
Store at 4°C.

3. Agarose solution (0.7%)

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

3. Ethidium bromide solution

Ethidium bromide	- 10mg
Distilled water	- 2 ml

4. 1X TBE buffer

10X TBE	- 10 ml
Distilled Water	- 90 ml

Protocol:

Arranged the gel casting unit according to instructions of manufacturer.

Boiled the Agarose solution to dissolve agarose.

↓

Cooled it down to approximately 50°C.

↓

Added 2.5µl 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 2µl of DNA solution and 2µl of sample loading buffer in each wells

↓

Electrophoresis was done at constant voltage (80V) for 1hr.

↓

After electrophoresis the gel was observed in ultraviolet light and documented using the gel documentation system Image Master VDS (Pharmacia Biotech).

DNA Quantification using spectrophotometer

The quality and quantity of the extracted DNA was checked in UV spectrophotometer (Beckman, USA) 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.

3.3.2. 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). PCR reactions were carried out in PTC 200 gradient thermal cycler (M.J. research, Inc., Watertown, Massachusetts, USA) employing specific universal primers for amplifications of partial sequences of mitochondrial DNA regions such as 16S rRNA, Cyt *b*, COI and complete sequence of ATPase 8/6 genes.

Amplification of ATPase 8/6 region

The entire ATP synthase 6 and 8 (ATPase 8/6) 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 (100mM Tris, 500mM KCl, 0.1% gelatin, pH 9.0) with 1.5mM $MgCl_2$ (Genei, Bangalore, India), 5pmoles of each primer, 200μM of each dNTP (Genei, Bangalore, India), 1.5U *Taq* DNA polymerase and 20ng 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 min, denaturation 94°C for 30 s, annealing 55^{o} C for

30 s, extension 72°C for 45 s, repeated for 29 cycles, followed by a final extension for 5 min at 72°C.

Amplification of COI region

The partial sequence of cytochrome oxidase sub-unit I genes was PCR amplified using primers Fish F1 (5' – TCA ACC AAC CAC AAA GAC ATT GGC AC - 3') and Fish R1 (5' – TAG ACT TCT GGG TGG CCA AAG AAT CA - 3') (Ward *et al*., 2005). The amplifications were performed in 25 µl reactions containing 1x assay buffer (100mM Tris, 500mM KCl, 0.1% gelatin, pH 9.0) with 1.5mM $MgCl_2$ (Genei, Bangalore, India), 5pmoles of each primer, 200µM of each dNTP (Genei, Bangalore, India), 1.5U *Taq* DNA polymerase and 20ng 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 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.

Amplification of Cyt *b* region

The partial sequence of cytochrome *b* genes was PCR amplified using primers Cyt*b*A (5' – CCA TGA GGA CAA ATA TCA TTY TGR GG - 3') and Cyt*b*C (5' – CTA CTG GTT GTC CTC CGA TTC ATG T - 3') (Bossuyt and Milinkovitch, 2000). The amplifications were performed in 25 µl reactions containing 1x assay buffer (100mM Tris, 500mM KCl, 0.1% gelatin, pH 9.0) with 1.5mM $MgCl_2$ (Genei, Bangalore, India), 5pmoles of each primer, 200µM of each dNTP (Genei, Bangalore, India), 1.5U *Taq* DNA polymerase and 20ng 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 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.

Amplification of 16S rDNA region

The partial sequence of 16S ribosomal DNA was PCR amplified using primers L2510 (5' – CGC CTG TTT ATC AAA AAC AT - 3') and H3080 (5' – CCG GTC TGA ACT CAG ATC ACG T - 3') (Palumbi *et al*., 1991). The amplifications were performed in 25 µl reactions containing 1x assay buffer (100mM Tris, 500mM KCl, 0.1% gelatin, pH 9.0) with 1.5mM $MgCl_2$ (Genei, Bangalore, India), 5pmoles of each primer, 200µM of each dNTP (Genei, Bangalore, India), 1.5U *Taq* DNA polymerase and 20ng 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 min, denaturation 94°C for 30 s, annealing 58^{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.

Test Run

3µl PCR products along with marker (100bp DNA ladder; Genei, Bangalore, India) were electrophoresed in 1.5% agarose gel (10x4cm) using 1X TBE buffer for 90 minutes at constant voltage (80V) and stained with ethidium bromide. The gel was visualized under UV transilluminator and documented using Image Master VDS (Pharmacia Biotech, USA).

Purification of PCR product

The remaining PCR product was purified using GeNei^{TM} Quick PCR purification kit (Genei, Bangalore, India) following the instructions given by the manufacturer.

3.3.3. Gene sequencing

The cleaned up PCR products were used as the template for sequencing PCR. Nucleotide sequencing was performed 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 dideoxynucleotides 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 the two amplification primers.

Composition of reaction mix:

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

Cycle sequencing conditions were as follows:

ATPase 8/6 region: 95^{o} C for 30 s, 55^{o} C for 5 s and 60^{o} C for 4 min repeated for 25 cycles and finally stored at 4^{o} C.

COI region: 95^{o} C for 30 s, 50^{o} C for 5 s and 60^{o} C for 4 min repeated for 25 cycles and finally stored at 4^{o} C.

Cyt *b* region: 95^{o} C for 30 s, 50^{o} C for 5 s and 60^{o} C for 4 min repeated for 25 cycles and finally stored at 4^{o} C.

16S rDNA region: 95^{o} C for 30 s, 58^{o} C for 5 s and 60^{o} C for 4 min repeated for 25 cycles and finally stored at 4^{o} C.

A total of 75 sequences information were generated in the present study. Table 3 give details about the number of individuals sequenced from each location for each gene.

Clean up for Sequencing:

The resulting cycle sequencing fragments were cleaned up by the following procedure.

2µl of 125mM EDTA was added to each tube (PCR tube containing the PCR product) and mix.

↓

73µl H_2O was added to this tube (final volume was adjusted to 100µl)

↓

It was transferred to a 1.5ml tube and 10µl 3M sodium acetate (pH-4.6) was added.

↓

250µl of 100% ethanol was added and mixed gently.

↓

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 250µl of 70% ethanol was added.

↓

Tubes were again spun for 10min and the ethanol was decanted.

↓

The above step (ethanol wash) was repeated again.

↓

The supernatant was decanted and the pellet was air dried for 25-30min.

Table 3. Details about the number of individuals sequenced for each gene from each location.

Sampling site	Number of specimens sequenced for each gene				Total
	ATPase 8/6	COI	Cyt *b*	16S rRNA	
Chandragiri	6	2	2	2	12
Kariangode	5	2	2	2	11
Valapattanam	8	2	2	2	14
Chaliyar	6	2	2	2	12
Chalakkudy	9	2	2	2	15
Periyar	5	2	2	2	11
Total	39	12	12	12	75

Electrophoresis and visualization 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).

3.3.4. Analysis of Data

3.3.4.1. Sequence alignment

The raw DNA sequences were edited using BioEdit sequence alignment editor version 7.0.5.2 (Hall, 1999). One example each of 16S, cyt *b*, COI and ATPase 6 & 8 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/). The most similar sequences obtained

from BLAST searches were added as outgroups for phylogenetic analysis to the data sets obtained for *P. denisonii* and *P. chalakkudiensis* in the present study. Multiple alignment of sequences was performed using CLUSTAL X version 2 (Larkin *et al*., 2007) alignment editor. Alignment was then manually checked and corrected. Nucleotide sequence characteristics after alignment were analysed using the program DnaSP version 4.10 (Rozas *et al*., 2003). The sequences (different haplotypes only) after their confirmation were submitted in GenBank (List of NCBI accessions generated were given as Appendix II) using a standalone multiplatform submission programme called "sequin" (www.ncbi.nlm.nih.gov/Sequin/index.html).

3.3.4.2. Population genetic analysis

All the six populations were grouped into two for further analysis by taxonomic position based on published accounts of their distribution (Menon *et al*., 1999). In group 1 (*Pd*) included the populations from the distribution sites of *P. denisonii* and in group 2 (*Pc*) that of *P. chalakkudiensis*. The samples collected from Periyar River appeared morphologically similar to *P. chalakkudiensis* and has been included in group 2.

Populations analyzed	
Group 1 (*Pd*)	**Group 2 (*Pc*)**
Chandragiri (CDR)	Chalakkudy (CHD)
Kariangode (KGD)	Periyar (PER)
Valapattanam (VLP)	
Chaliyar (CLR)	

The sequence information generated that of ATPase 6 & 8 genes were used for performing the population genetic analysis. Intrapopulation diversity was analysed by estimating gene diversity (*h*) - the probability that 2 randomly chosen haplotypes are different (Nei, 1987), and nucleotide diversity (π) – the probability that 2 randomly chosen homologous nucleotides are different

(Tajima, 1983; Nei, 1987). Gene flow (*Nm*) between populations was also estimated. 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 same site 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 species. Finally, variation among *P. denisonii* and *P. chalakkudiensis* 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).

3.3.4.3. Phylogenetic and molecular evolutionary analysis

Phylogenetic and molecular evolutionary analysis was conducted using MEGA version 4 (Tamura *et al.,* 2007). Sequence data was subsequently analysed using distance (Neighbour-Joining) and Maximum Parsimony methods. The sampling error of Neighbour-Joining and Maximum Parsimony Trees was analysed using bootstraps of 10,000 replicates where possible followed by the construction of Majority Rule Trees. 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.

3.3.4.4. 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 analysing 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 more ragged distributions indicate the population is in stable equilibrium. Agreement between the observed distributions and expected distributions under a sudden-expansion model was tested following Schneider and Excoffier (1999). In addition, the raggedness index (Harpending, 1994) was calculated, which has larger values for stable populations versus expanding populations. Assuming neutrality, evidence of a population expansion was also tested using Tajima's (1989a, b) 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.

Chapter 4
RESULTS

4.1. Ecological observations

Size, body shape and colour differences were readily noticeable between the samples obtained from Chalakkudy / Periyar rivers (*P. chalakkudiensis*) and Chandragiri / Kariangode / Valapattanam / Chaliyar rivers (*P. denisonii*). Body of *P. chalakkudiensis* specimens was moderately deeper (especially in larger specimens) with slightly rounded snout and comparatively more greenish dorsum. A prominent black bloch on the dorsal fin were also present in *P. chalakkudiensis*. In *P. denisonii*, body was comparatively slender and streamlined with a pointed snout. The body length of *P. denisonii* was relatively shorter (max. total length observed 13.5cm) than that of *P. chalakkudiensis* (max. total length observed 17.6cm). In addition, *P. chalakkudiensis* (Fig. 2) has a black longitudinal stripe from the snout extending along the lateral line more clearly defined from post orbit to the caudal peduncle, while a clearly defined black longitudinal stripe starts from the side of the snout passing through mid orbit and abruptly ending at caudal peduncle is distinct in *P. denisonii* (Fig. 1).

The species, *P. chalakkudiensis* was described based on two specimens collected from the upper reaches of Chalakkudy River of the Western Ghats and its distribution is confined only to this river (Menon *et al*., 1999). In the present study, specimens collected from Periyar River resembled *P. chalakkudiensis* in their colour characteristics (Fig. 6).

These species inhabits the rocky and sandy bottom and mid-waters in the upper middle reaches of the rivers with fast running and clean water. It was supposed to be an omnivore, apparently feeds on insects and some algal matters attached to the rocks. No sexual dimorphism could be observed. Sexually

matured females appeared to be more robust and round bellied than the comparatively slimmer males. Mature males oozes out milt when pressed at the abdomen towards vent region. Specimens with fully matured gonads (ripe ovary [Fig. 7] and milt oozing males) were observed in the month of December. Early juveniles were found near the sides of the river in calm waters with sandy and rocky bottom during January onwards. Juveniles of different size ranges (10mm to 40mm) were seen during the months of May - June at the commencement of monsoon.

Juveniles of both the species exhibited different colour pattern from that observed in adult specimens (Fig. 8). Shape and colour pattern of early juveniles of *P. denisonii* and *P. chalakkudiensis* were also distinguishable between each other. The prominent red or scarlet horizontal band observed in adult specimens was not prominent in early juveniles, whereas three prominent black vertical bands were observed. The early juveniles of *P. denisonii* (Fig. 8-A) possess comparatively narrow and diffuse vertical bands on sides of body, whereas that of *P. chalakkudiensis* (Fig. 8-B) found to be broader and more prominent. However, this pattern disappears in sub-adults and adults in both species.

Interaction with the local fishermen and collectors revealed that the abundance of both the species in the streams of Kerala, especially in Chalakkudy and Valapattanam river systems has been reduced drastically due to over fishing in recent years to meet the high demand for “Redline torpedo fish” in the aquarium trade. Personal observations during the course of sampling suggests that *P. chalakkudiensis* census size is usually lower than *P. denisonii*, as catch per hour for the former was found to be considerably lesser, especially in river Chalakkudy.

Fig. 6. *P. chalakkudiensis* specimen collected from Periyar River.

Fig. 7. Ripe ovary in *P. chalakkudiensis* (specimen collected from Periyar River) observed in the month of December.

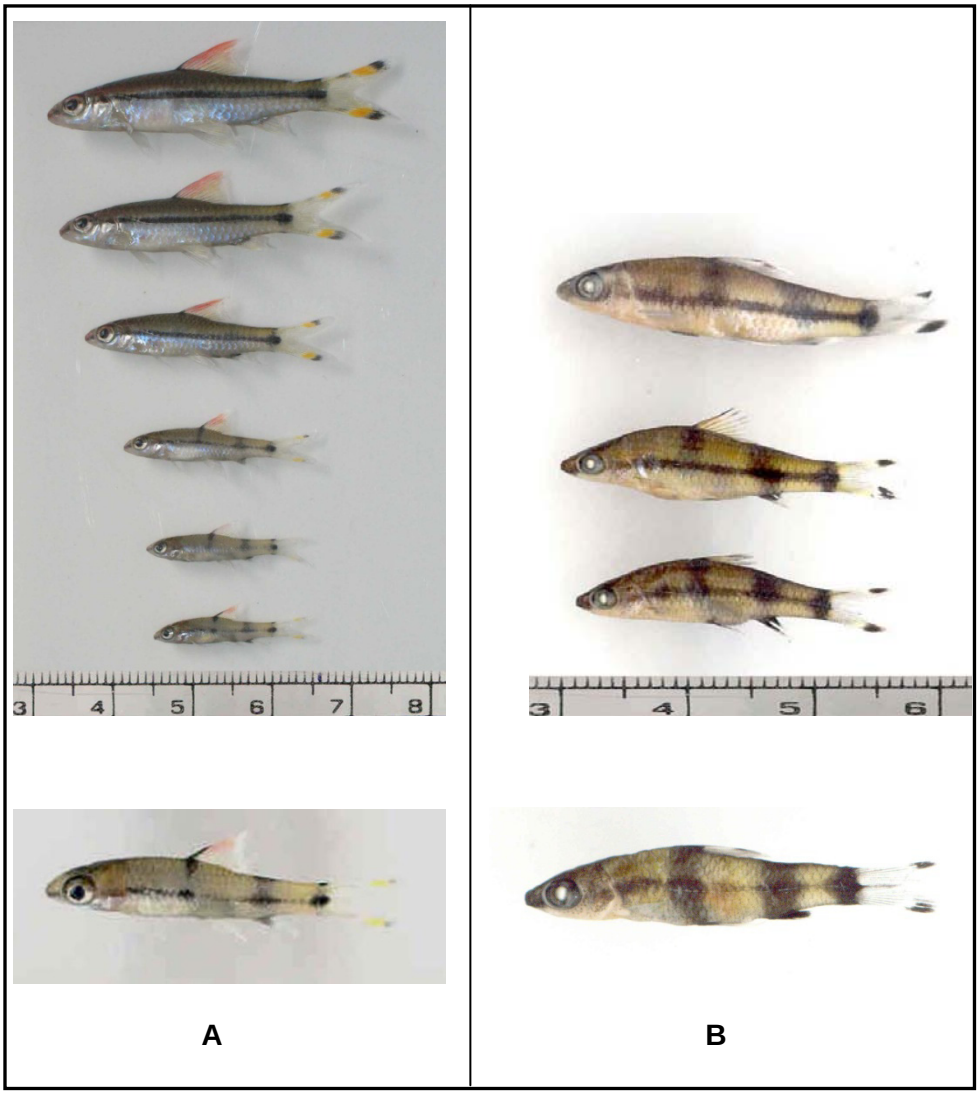


Fig. 8. A – Juveniles of *P. denisonii*. B – Juveniles of *P. chalakkudiensis*.

4.2. Morphometric and meristic analysis

4.2.1. Morphometric traits

The species wise and population wise descriptive statistics *viz.*, minimum, maximum, mean, standard deviation; the coefficient of variation (CV) of all morphometric traits, the multivariate coefficient of variation (CVp) and the Principle Component Analysis were carried out. The results are as follows.

4.2.1.1. Descriptive statistics of morphometric traits

Descriptive statistics for each of the morphometric variables of *P. denisonii* and *P. chalakkudiensis* are denoted in Table 4. The population wise descriptive statistics for the populations of *P. denisonii* and *P. chalakkudiensis* are represented in Table 5 and 6 respectively. Generally low coefficients of variation were obtained for the morphometric characters of *P. denisonii* (2.19 – 10.73%) and *P. chalakkudiensis* (2.32 – 9.16%). The CVs were still lower within each population of *P. denisonii* (1.30 – 9.49%) and *P. chalakkudiensis* (1.87 – 8.75%). The multivariate generalized coefficient of variation (CVp) in each species and population was also relatively low. *P. chalakkudiensis* showed higher CVp (5.85%) than *P. denisonii* (5.79%). Specimens from Periyar showed the highest CVp (5.51%), followed by Valapattanam (5.38%), Chalakkudy (5.36%), Kariangode (5.32%), Chaliyar (4.81%) and Chandragiri (4.80%), with relatively low values; indicates minimal or very low intra-population variation.

When the two species *P. denisonii* and *P. chalakkudiensis* were compared (specimens from different sites combined together for each species) the univariate analysis of variance (ANOVA) showed significant differences at the $p<0.05$ and $p<0.01$ levels of significance in 18 out of 20 morphometric characters (only L_{BC} and L_O were not significantly different, $P>0.05$) excluding standard length (Table 4). Univariate analysis of variance also showed that, fish samples from different sites differed significantly (at $p<0.05$ and $p<0.01$ levels of significance) in 18 and 15 out of the 20 morphometric characters examined in *P. denisonii* (Table 5) and *P. chalakkudiensis* (Table. 6) respectively, leading to the rejection of the null

hypothesis of 'no heterogeneity in fish morphology among riverine populations' of these species. There were no significant differences (P>0.05) in FBDO & L_O among samples of *P. denisonii* and in MBD, L_{PRD}, L_{BC}, FBDO & FBAN between samples of *P. chalakkudiensis*.

P. denisonii samples from four sites shared several (but not uniform) of the morphometric characters that are significantly different from those in *P. chalakkudiensis* with high F values. In this respect, they have shorter HD, MBD, FBAN and L_{PD}. Moreover, larger mean L_{PRV}, L_{PRA}, L_{PTFPLF}, MW, L_{PO}, L_{MB} and L_H identified *P. denisonii* specimens. Whereas, *P. chalakkudiensis* specimens could be identified by larger mean L_{PLFAF}, L_{CP}, CPD, HW and L_{PRO} (Table 4).

4.2.1.2. Principal Component Analysis (PCA).

Principal component analysis was carried out factoring the correlation matrix of the morphometric data, between the two species and also among and between populations of *P. denisonii* and *P. chalakkudiensis* respectively.

4.2.1.2a. PCA between *P. denisonii* and *P. chalakkudiensis*.

PCA of the 18 significant variables (Table 4) between *P. chalakkudiensis* and *P. denisonii* yielded 8 principal components accounting for 82.98% of the total variation in the original variables (Table 7). The variance explained by the first three components was 58.83%, whose factor loadings are shown in Table 8. The first factor was mainly defined by measurements of post dorsal length (L_{PD}), head depth (HD), maximum body depth (MBD), depth of caudal peduncle (CPD), pre ventral length (L_{PRV}) and length of caudal peduncle (L_{CP}). The second component was mainly correlated with length of pectoral to pelvic origin (L_{PTFPLF}), width of mouth (MW), width of head (HW), and pre anal length (L_{PRA}). These observations indicated that the above morphometric characters which contributed the maximum to differentiate *P. denisonii* and *P. chalakkudiensis*. The scatter diagram based on PCA clearly distinguishes the two major groups which are evidently distinct as separate and two species (Fig. 9).

4.2.1.2b. PCA among populations of *P. denisonii*.

Eighteen significant morphometric variables (Table 5) among populations of *P. denisonii* were used for PCA and yielded 6 principal components accounted to describe 83.14% of the total variation in the original variables (Table 9). The factor loadings of the first three components are shown in Table 10. The first two components explained 57.82% variance. The first component was mainly defined by measurements of head depth (HD), width of head (HW), width of mouth (MW), post orbital length (L_{PO}), pre anal length (L_{PRA}) and length of pectoral to pelvic origin (L_{PTFPLF}). Whereas, the second component was mainly correlated with, length of maxillary barbal (L_{MB}), width of mouth (MW), measurements of head (HD & HW), length of caudal peduncle (L_{CP}) and pre ventral length (L_{PRV}). These indicated that the above morphometric characters contributed the maximum to differentiate *P. denisonii* populations. The bivariate scatter plot of component 1 and 2 was found to be sufficient to outline the morphological heterogeneity existing among *P. denisonii* populations (Fig. 10). The samples collected from Kariangode and Valapattanam Rivers showed similarity, is depicted in the form of overlapping clusters in the scatter plot.

4.2.1.2c. PCA between populations of *P. chalakkudiensis*.

Out of twenty morphometric measurements (excluding L_S) taken, fifteen were found to be significant between *P. chalakkudiensis* (Table 6) populations, which were used for PCA. Six principal components accounted for 81.40% of the total variation in the original variables (Table 11). The factor loadings of the first three components are shown in Table 12. The first two components explained 52.79% variance. The first component was mainly defined by pre ventral length (L_{PRV}), post dorsal length (L_{PD}), pre orbital length (L_{PRO}), length of orbit (L_O) and width of head (HW); and the second component was mainly correlated with, length of pelvic to anal (L_{PLFAF}), head length (L_H), post orbital length (L_{PO}), length of pectoral to pelvic origin (L_{PTFPLF}) and post dorsal length (L_{PD}). These indicated that the above morphometric characters contributed the maximum to differentiate *P. chalakkudiensis* populations. The bivariate scatter plot of component 1 and 2 was found to be sufficient to outline the morphological heterogeneity existing between populations of *P. chalakkudiensis* (Fig.11).

Table 4. Descriptive statistics of transformed morphometric variables, the coefficient of variation (*CV*) of each measurement, the multivariate coefficient of variation of each species (*CVp*) and F-values (derived from the analysis of variance) of *P. denisonii* and *P. chalakkudiensis*.

	***P. denisonii* (n = 120)**		***P. chalakkudiensis* (n = 60)**		
	Mean ± SD (min. - max.)	**CV**	**Mean ± SD (min. - max.)**	**CV**	**F value**
L_S	81.08 ± 8.43 (64.55-101.30)	-	89.52 ± 18.69 (64.75-129.75)	-	-
L_H	23.96 ± 0.93 (22.01-25.93)	3.88	22.75 ± 1.51 (19.99-24.88)	6.65	43.43**
MBD	24.42 ± 1.23 (21.96-26.41)	5.04	26.95 ± 0.86 (25.48-28.86)	3.18	202.65**
L_{PRD}	47.12 ± 1.03 (44.96-49.51)	2.19	46.34 ± 1.17 (44.51-48.55)	2.54	11.54**
L_{PD}	37.22 ± 1.09 (35.15-39.96)	2.93	40.49 ± 1.80 (37.51-43.96)	4.46	227.87**
L_{PRV}	52.99 ± 2.01 (49.19-58.95)	3.79	49.75 ± 1.38 (47.01-52.26)	2.78	125.72**
L_{PRA}	75.55 ± 1.69 (72.31-78.88)	2.24	73.90 ± 1.71 (70.17-77.25)	2.32	38.14**
L_{PTFPLF}	28.10 ± 1.38 (25.13-30.77)	4.91	26.71 ± 1.51 (24.12-31.03)	5.65	37.98**
L_{PLFAF}	24.59 ± 1.14 (21.89-26.62)	4.64	25.05 ± 1.10 (22.80-29.00)	4.40	6.59*
L_{BC}	48.98 ± 2.34 (44.72-53.25)	4.78	48.87 ± 2.02 (44.72-53.23)	4.14	0.10 NS
FBDO	16.41 ± 0.63 (15.18-18.45)	3.84	16.01 ± 0.82 (14.75-17.54)	5.15	12.86**
FBAN	08.03 ± 0.51 (06.83-08.88)	6.35	09.50 ± 0.75 (08.37-11.72)	7.88	238.16**
L_{CP}	15.95 ± 1.43 (13.13-18.67)	8.97	18.36 ± 1.22 (16.30-20.65)	6.63	124.13**
CPD	10.99 ± 0.51 (10.27-12.65)	4.64	12.07 ± 0.52 (10.47-13.14)	4.35	172.94**
HD	61.31 ± 2.74 (55.55-67.85)	4.47	68.34 ± 3.12 (62.25-75.67)	4.56	239.87**
HW	55.80 ± 2.45 (50.77-61.95)	4.39	57.09 ± 1.98 (53.49-60.73)	3.47	12.57**
MW	26.22 ± 2.01 (22.60-31.23)	7.67	25.17 ± 1.06 (21.44-26.74)	4.22	14.23**
L_O	31.45 ± 1.60 (28.56-34.22)	5.09	31.95 ± 2.15 (27.34-35.20)	6.73	3.13 NS
L_{PRO}	32.59 ± 1.67 (28.97-35.88)	5.12	34.82 ± 3.18 (28.10-40.46)	9.12	38.37**
L_{PO}	38.39 ± 1.82 (34.28-41.94)	4.74	35.56 ± 2.35 (32.19-42.37)	6.62	79.27**
L_{MB}	32.06 ± 3.44 (22.56-38.31)	10.73	28.26 ± 2.59 (22.94-33.09)	9.16	56.88**
CVp		**5.79**		**5.85**	

See Table 2 for explanations of acronyms. *Significant at the 5% level; **Significant at the 1% level; NS, not significant at the 5% level; *SD* is standard deviation.

Table 5. Descriptive statistics of transformed morphometric variables, the coefficient of variation (*CV*) of each measurement, the multivariate coefficient of variation of each species (*CVp*) and F-values (derived from the analysis of variance) of four populations of *P. denisonii*.

	Chandragiri (n = 30)		Kariangode (n = 30)		Valapattanam (n = 30)		Chaliyar (n = 30)		
	Mean ± SD (min. - max.)	CV	Mean ± SD (min. - max.)	CV	Mean ± SD (min. - max.)	CV	Mean ± SD (min. - max.)	CV	F value
L_S	74.08 ± 4.58 (67.34-85.70)	-	85.47 ± 7.20 (70.64-98.52)	-	87.11 ± 8.18 (70.66-101.30)	-	77.67 ± 5.63 (64.55-85.20)	-	-
L_H	24.68^{a} ± 0.48 (23.85-25.93)	1.94	24.26^{ab} ± 0.91(22.80-25.63)	3.75	23.85^{b} ± 0.76 (22.16-25.11)	3.21	23.06^{c} ± 0.66 (22.01-24.06)	2.86	27.74**
MBD	23.86^{a} ± 0.75 (22.82-25.13)	3.14	23.93^{ab} ± 1.28(21.96-25.71)	5.36	24.69^{bc} ± 1.33(22.32-26.22)	5.37	25.21^{c} ± 0.99 (23.42-26.41)	3.93	10.06**
L_{PRD}	47.64^{a} ± 0.62 (46.49-49.51)	1.30	46.39^{b} ± 0.81 (45.21-47.64)	1.74	46.35^{b} ± 0.69 (44.96-47.53)	1.50	48.11^{a} ± 0.63 (47.07-49.37)	1.31	49.51**
L_{PD}	37.32^{a} ± 1.14 (35.49-39.96)	3.05	36.88^{ab} ± 1.02(35.51-38.74)	2.78	36.97^{a} ± 1.17 (35.15-39.06)	3.18	37.72^{ac} ± 0.83 (36.10-38.90)	2.20	3.95*
L_{PRV}	52.52^{a} ± 1.12 (50.59-54.33)	2.13	54.06^{b} ± 1.62(51.60-56.45)	2.99	52.09^{a} ± 3.18(49.19-58.95)	6.11	53.29^{ab} ± 0.54(51.12-53.99)	1.01	6.31**
L_{PRA}	75.75^{a} ± 1.34 (72.86-77.51)	1.77	75.14^{ab} ± 1.52(72.45-77.49)	2.03	74.22^{b} ± 1.54 (72.31-77.55)	2.08	77.11^{c} ± 0.80 (75.67-78.88)	1.04	24.71**
L_{PTFPLF}	27.73^{a} ± 1.16 (25.82-29.51)	4.18	27.82^{a} ± 1.01 (26.02-29.38)	3.62	27.20^{a} ± 1.16 (25.13-29.49)	4.25	29.63^{b} ± 0.81 (28.60-30.77)	2.73	31.04**
L_{PLFAF}	23.80^{a} ± 0.87 (21.89-25.04)	3.66	24.71^{bc} ± 1.15(22.93-26.50)	4.66	24.52^{ab} ± 1.25(23.24-26.62)	5.09	25.34^{bc} ± 0.68(23.46-26.56)	2.68	11.79**
L_{BC}	49.39^{a} ± 2.25 (45.56-53.25)	4.56	48.17^{ab} ± 2.09(44.72-51.31)	4.34	47.02^{b} ± 1.37 (45.51-50.62)	2.91	51.35^{c} ± 0.78 (49.96-52.59)	1.52	34.59**
FBDO	16.35^{a} ± 0.39 (15.76-17.01)	2.39	16.47^{a} ± 0.57 (15.63-17.21)	3.47	16.45^{a} ± 0.55 (15.53-17.22)	3.32	16.36^{a} ± 0.92 (15.18-18.45)	5.62	0.26 NS
FBAN	08.43^{a} ± 0.21 (08.09-08.88)	2.49	08.05^{b} ± 0.39 (07.25-08.74)	4.87	08.20^{ab} ± 0.46(07.12-08.76)	5.55	07.45^{c} ± 0.37 (06.83-08.02)	4.97	38.13**
L_{CP}	14.31^{a} ± 0.73 (13.13-15.65)	5.10	16.27^{bd} ± 1.03(14.61-17.92)	6.32	17.38^{c} ± 0.95 (15.35-18.67)	5.47	15.85^{d} ± 0.94 (14.42-17.39)	5.93	57.30**
CPD	10.74^{a} ± 0.35 (10.31-11.67)	3.26	11.09^{b} ± 0.51(10.41-11.98)	4.59	11.33^{c} ± 0.62 (10.48-12.65)	5.49	10.82^{ab} ± 0.26(10.27-11.23)	2.40	10.76**
HD	60.70^{a} ± 1.70 (55.55-63.44)	2.80	59.70^{a} ± 1.85 (57.01-62.84)	3.10	60.24^{a} ± 1.94 (56.71-62.92)	3.22	64.61^{b} ± 2.30 (61.35-67.85)	3.56	38.93**
HW	57.51^{a} ± 1.54 (52.98-59.96)	2.68	54.11^{b} ± 1.73 (50.77-56.70)	3.21	54.24^{b} ± 1.47 (52.43-56.51)	2.71	57.33^{a} ± 2.50 (54.07-61.95)	4.36	30.66**
MW	27.85^{a} ± 1.47 (24.71-31.23)	5.28	24.81^{b} ± 0.79 (23.44-26.15)	3.18	24.68^{b} ± 0.83 (22.60-25.84)	3.36	27.53^{a} ± 2.01 (23.05-29.80)	7.30	46.64**
L_O	31.32^{a} ± 1.72 (28.56-33.99)	5.49	32.09^{a} ± 1.84 (28.71-34.22)	5.75	31.17^{a} ± 1.19 (28.83-33.43)	3.83	31.21^{a} ± 1.48 (28.74-32.92)	4.74	2.28 NS
L_{PRO}	32.73^{a} ± 1.10 (29.80-34.02)	3.36	32.28^{ba} ± 1.64(29.29-34.50)	5.08	31.58^{b} ± 1.85(28.97-34.53)	5.86	33.75^{ca} ± 1.23 (31.88-35.88)	3.64	11.31**
L_{PO}	38.29^{a} ± 1.14 (34.98-39.82)	2.98	39.53^{b} ± 1.21 (37.53-41.46)	3.07	39.64^{b} ± 1.03 (38.15-41.94)	2.61	36.10^{c} ± 1.16 (34.28-38.53)	3.21	62.50**
L_{MB}	28.55^{a} ± 2.07 (22.56-30.56)	7.25	32.32^{bc} ± 3.07(27.92-36.84)	9.49	32.87^{cd} ± 2.94(28.84-36.99)	8.94	34.48^{d} ± 2.58 (27.73-38.31)	7.48	26.10**
CVp	**4.80**		**5.32**		**5.38**		**4.81**		

For each morphometric variable, means with the same letter superscript are not significantly different. See Table 2 for explanations of acronyms. *Significant at the 5% level; **Significant at the 1% level; NS, not significant at the 5% level; *SD* is standard deviation.

Table 6. Descriptive statistics of transformed morphometric variables, the coefficient of variation (*CV*) of each measurement, the multivariate coefficient of variation of each species (*CVp*) and F-values (derived from the analysis of variance) of two populations of *P. chalakkudiensis*.

	Chalakkudy (n = 30)		Periyar (n = 30)		
	Mean ± SD (min. - max.)	**CV**	**Mean ± SD (min. - max.)**	**CV**	**F value**
L_S	100.73 ± 7.94 (85.32-112.44)	-	78.31 ± 19.69 (64.75-129.75)	-	-
L_H	22.21 ± 1.27 (20.30-24.47)	5.72	23.30 ± 1.56 (19.99-24.88)	6.70	8.68**
MBD	26.92 ± 0.91 (25.48-28.63)	3.40	26.98 ± 0.81 (25.87-28.86)	3.00	0.06 NS
L_{PRD}	46.11 ± 1.33 (44.51-48.45)	2.88	46.57 ± 0.97 (44.93-48.55)	2.08	2.32 NS
L_{PD}	41.48 ± 1.19 (39.66-43.30)	2.86	39.49 ± 1.78 (37.51-43.96)	4.51	25.83**
L_{PRV}	48.89 ± 1.07 (47.01-50.52)	2.20	50.61 ± 1.10 (47.67-52.26)	2.17	37.54**
L_{PRA}	73.04 ± 1.58 (70.17-75.48)	2.16	74.75 ± 1.39 (72.41-77.25)	1.87	19.77**
L_{PTFPLF}	26.11 ± 1.57 (24.12-28.71)	6.00	27.31 ± 1.20 (25.96-31.03)	4.38	11.19**
L_{PLFAF}	24.77 ± 0.82 (23.32-25.87)	3.31	25.33 ± 1.28 (22.80-29.00)	5.06	4.12*
L_{BC}	49.14 ± 2.18 (45.43-53.10)	4.44	48.60 ± 1.84 (44.72-53.23)	3.80	1.06 NS
FBDO	15.97 ± 0.83 (14.75-17.54)	5.20	16.05 ± 0.83 (14.83-17.50)	5.17	0.15 NS
FBAN	09.41 ± 0.67 (08.37-10.48)	7.17	09.60 ± 0.82 (08.59-11.72)	8.51	0.96 NS
L_{CP}	19.02 ± 1.01 (17.03-20.65)	5.33	17.69 ± 1.03 (16.30-20.28)	5.82	25.52**
CPD	12.22 ± 0.48 (11.45-13.14)	3.89	11.91 ± 0.53 (10.47-13.04)	4.49	5.53*
HD	69.68 ± 2.73 (65.63-74.34)	3.92	67.00 ± 2.93 (62.25-75.67)	4.37	13.44**
HW	58.36 ± 1.82 (55.54-60.73)	3.12	55.82 ± 1.15 (53.49-58.79)	2.06	41.97**
MW	25.48 ± 0.69 (24.01-26.48)	2.69	24.86 ± 1.27 (21.44-26.74)	5.12	5.68*
L_O	30.68 ± 1.98 (27.34-33.60)	6.45	33.22 ± 1.47 (29.14-35.20)	4.42	31.83**
L_{PRO}	37.38 ± 1.99 (34.18-40.46)	5.33	32.26 ± 1.74 (28.10-36.18)	5.38	112.34**
L_{PO}	34.33 ± 1.00 (32.19-35.69)	2.92	36.79 ± 2.67 (33.91-42.37)	7.27	22.27**
L_{MB}	26.68 ± 2.33 (22.94-32.38)	8.75	29.84 ± 1.74 (27.25-33.09)	5.81	35.57**
CVp		**5.36**		**5.51**	

See Table 2 for explanations of acronyms. *Significant at the 5% level; **Significant at the 1% level; NS, not significant at the 5% level; *SD* is standard deviation.

Table 7. Summary of principal component analysis (PCA) for the morphometric variables of *P. denisonii* and *P. chalakkudiensis*.

Component	Eigenvalue	% variance	Cumulative %
1	6.2768	34.87	34.87
2	2.4246	13.47	48.34
3	1.8879	10.49	58.83
4	1.1930	6.63	65.46
5	1.0811	6.01	71.47
6	0.7775	4.32	75.79
7	0.7362	4.09	79.88
8	0.5576	3.10	82.98
9	0.4814	2.67	85.65
10	0.4292	2.38	88.03
11	0.3840	2.13	90.16
12	0.3448	1.92	92.08
13	0.3018	1.68	93.76
14	0.2735	1.52	95.28
15	0.2511	1.39	96.67
16	0.2394	1.33	98.00
17	0.2150	1.19	99.19
18	0.1454	0.81	100.0

Table 8. Factor loadings for the first three principal components formed from the morphometric variables of *P. denisonii* and *P. chalakkudiensis* specimens.

Morphometric variables	PC1	PC2	PC3
L_{H}	-0.2265	0.2242	-0.2935
MBD	0.3032	-0.1210	0.1712
L_{PRD}	-0.1233	-0.0947	0.0006
L_{PD}	0.3329	-0.0748	0.0726
L_{PRV}	-0.2970	-0.1035	0.0613
L_{PRA}	-0.1945	-0.3802	0.0996
L_{PTFPLF}	-0.1938	-0.4101	0.2162
L_{PLFAF}	0.0564	-0.2155	0.5160
FBDO	-0.1085	0.0477	0.1901
FBAN	0.2741	0.1673	-0.0719
L_{CP}	0.2859	0.1553	0.2709
CPD	0.3024	0.1375	0.0986
HD	0.3253	-0.2378	0.1098
HW	0.1489	-0.4058	-0.2572
MW	-0.0734	-0.4096	-0.3520
L_{PRO}	0.2272	-0.1646	-0.2026
L_{PO}	-0.2559	0.2440	0.1797
L_{MB}	-0.2310	-0.0415	0.3908
Explained variance (%)	**34.87**	**13.47**	**10.49**

Table 9. Summary of principal component analysis (PCA) for the morphometric variables of *P. denisonii* populations.

Component	Eigenvalue	% variance	Cumulative %
1	16.9659	31.87	31.87
2	13.8130	25.95	57.82
3	5.4256	10.19	68.01
4	3.3171	6.23	74.24
5	2.4654	4.63	78.87
6	2.2736	4.27	83.14
7	1.7150	3.22	86.36
8	1.2798	2.40	88.76
9	1.2545	2.36	91.12
10	1.0367	1.95	93.07
11	0.9200	1.73	94.80
12	0.7903	1.48	96.28
13	0.6106	1.15	97.43
14	0.5800	1.09	98.52
15	0.2919	0.55	99.07
16	0.2230	0.42	99.49
17	0.1834	0.34	99.83
18	0.0935	0.18	100.0

Table 10. Factor loadings for the first three principal components formed from the morphometric variables of *P. denisonii* populations.

Morphometric variables	PC1	PC2	PC3
L_{H}	-0.0968	0.1106	-0.0709
MBD	0.1192	-0.0458	0.1662
L_{PRD}	0.1729	0.0252	0.0433
L_{PD}	0.0640	0.0104	0.0265
L_{PRV}	0.0511	-0.1326	-0.7420
L_{PRA}	0.2717	-0.0678	-0.2674
L_{PTFPLF}	0.2248	-0.1091	-0.1564
L_{PLFAF}	0.0618	-0.1146	-0.0674
FBDO	-0.0199	-0.0095	-0.0269
FBAN	-0.0449	0.0569	-0.0215
L_{CP}	-0.1398	-0.1441	0.2212
CPD	-0.0303	-0.0095	-0.0097
HD	0.5572	-0.1767	0.3601
HW	0.4629	0.1566	-0.1193
MW	0.3424	0.1956	0.1108
L_{PRO}	0.1913	-0.0480	-0.3165
L_{PO}	-0.3310	0.0176	-0.0658
L_{MB}	-0.0264	-0.9039	0.0529
Explained variance (%)	**31.87**	**25.95**	**10.19**

Table 11. Summary of principal component analysis (PCA) for the morphometric variables of *P. chalakkudiensis* populations.

Component	Eigenvalue	% variance	Cumulative %
1	5.1534	34.36	34.36
2	2.7640	18.43	52.79
3	1.5166	10.11	62.90
4	1.1314	7.54	70.44
5	0.9244	6.16	76.60
6	0.7205	4.80	81.40
7	0.6295	4.20	85.60
8	0.5124	3.42	89.02
9	0.3687	2.46	91.48
10	0.3281	2.19	93.67
11	0.2833	1.89	95.56
12	0.2403	1.60	97.16
13	0.2031	1.35	98.51
14	0.1458	0.97	99.48
15	0.0784	0.52	100.0

Table 12. Factor loadings for the first three principal components formed from the morphometric variables of *P. chalakkudiensis* populations.

Morphometric variables	PC1	PC2	PC3
L_H	0.2372	-0.3852	0.1664
L_{PD}	-0.3270	0.2745	0.0524
L_{PRV}	0.3564	-0.1567	0.1646
L_{PRA}	0.2927	0.0695	0.3651
L_{PTFPLF}	0.1648	0.3254	0.0508
L_{PLFAF}	0.1149	0.4782	-0.0190
L_{CP}	-0.2932	0.1565	0.2288
CPD	-0.1369	-0.2435	0.5009
HD	-0.2220	0.2562	0.4223
HW	-0.3116	-0.1054	0.2211
MW	-0.1554	-0.0510	-0.4215
L_O	0.3169	-0.1554	-0.0371
L_{PRO}	-0.3228	-0.2129	0.0540
L_{PO}	0.1556	0.3665	-0.0929
L_{MB}	0.2823	0.2133	0.2917
Explained variance (%)	**34.36**	**18.43**	**10.11**

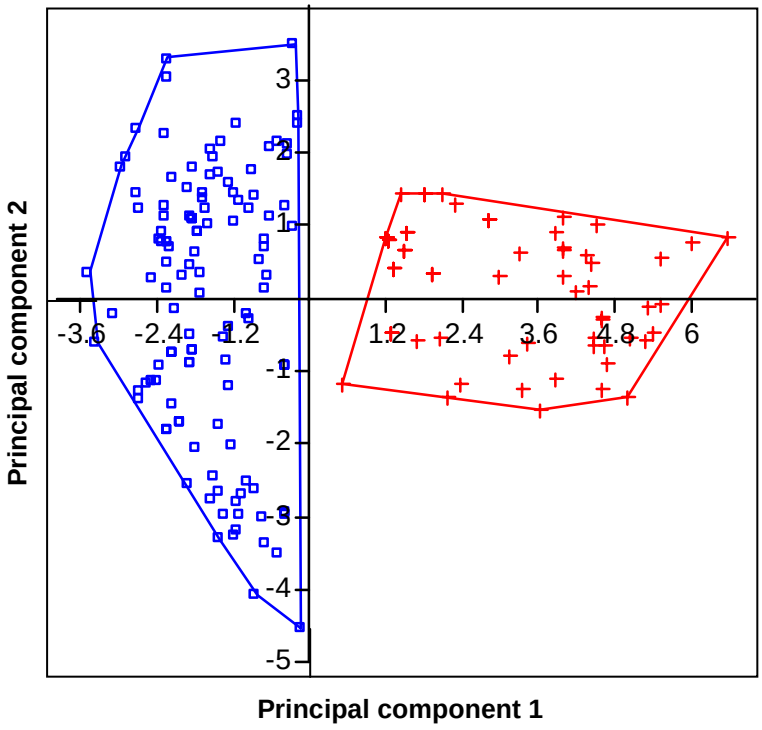


Fig. 9. Scatter diagram based on PCA of significant morphometric variables between *P. denisonii* (blue squares) and *P. chalakkudiensis* (red crosses) specimens.

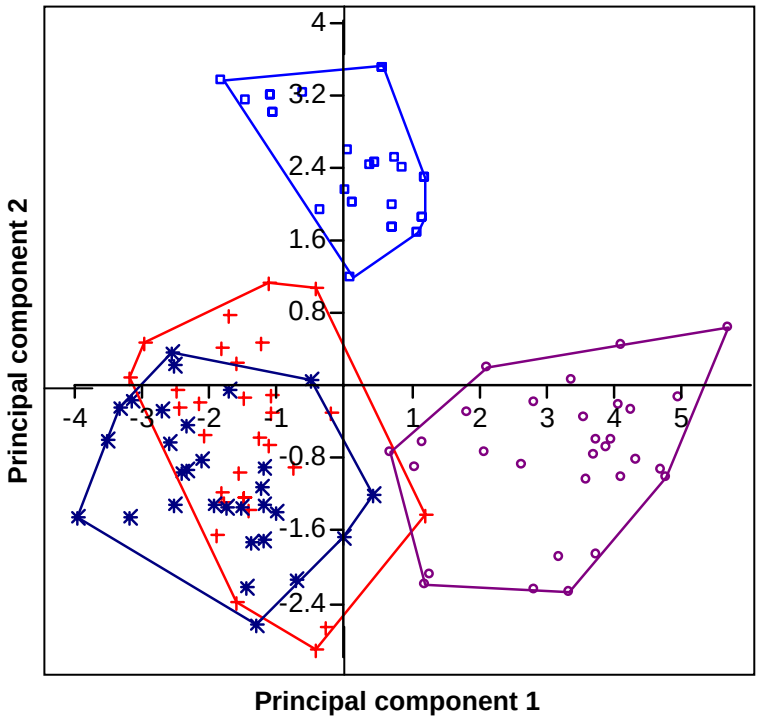


Fig. 10. Scatter diagram based on PCA of significant morphometric variables among populations of *P. denisonii* (Chandragiri – blue squares; Kariangode – red crosses; Valapattanam – dark blue stars; Chaliyar - purple circles).

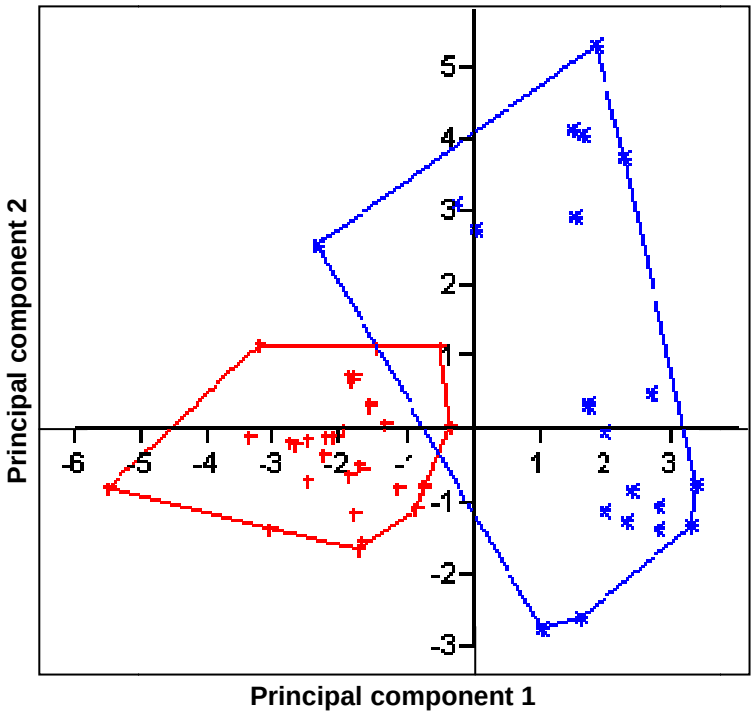


Fig. 11. Scatter diagram based on PCA of significant morphometric variables between populations of *P. chalakkudiensis* (Chalakkudy – red crosses; Periyar – blue stars).

4.2.2. Meristic traits

Eight meristic characters were counted; structure and arrangement of pharyngeal teeth and gill rakers were examined to find out any variation between the two species *viz.*, *P. denisonii* and *P. chalakkudiensis*.

Microscopic examination of pharyngeal teeth revealed the presence of an additional pair of teeth in the inner most rows on pharyngeal bones in *P. denisonii* (pharyngeal teeth formula given by Day (1865) for this species was 4,3,2-2,3,4) and *P. chalakkudiensis* (no earlier information available). It was found to be arranged as 5,3,2-2,3,5 in both species. The pharyngeal bones of both left and right sides possess three rows, with two teeth in the outer row, three in the middle and five in the inner. Out of the 5 teeth in the inner row, four were of similar size and the fifth was rudimentary (Fig. 12). Gill rakers of *P. denisonii* were slender and villiform in structure (Fig. 13) and that of *P. chalakkudiensis* were comparatively stout with blunt tip (Fig. 14).

4.2.2.1. Descriptive statistics of meristic traits

Descriptive statistics for each of the meristic variables of *P. denisonii* and *P. chalakkudiensis* are denoted in Table 13. Generally low coefficients of variation (CVs) were obtained for the meristic characters of *P. denisonii* (0.0 – 8.73%) and *P. chalakkudiensis* (0.0 – 5.96%). The multivariate generalized coefficients of variation (CVp) were slightly high. *P. denisonii* showed higher CVp (14.87%) than *P. chalakkudiensis* (12.28%).

The univariate analysis of variance (ANOVA) showed significant differences at $p<0.05$ and $p<0.01$ levels of significance in 6 out of 8 meristic characters (Table 13). There were no significant differences observed in *DFS* and *AFS* counts between samples of *P. denisonii* and *P. chalakkudiensis*.

4.2.2.2. Principal Component Analysis (PCA).

Principal component analysis was carried out factoring the correlation matrix of the meristic data, between the two species *P. denisonii* and *P. chalakkudiensis*.

PCA of the 6 significant variables between *P. chalakkudiensis* and *P. denisonii* yielded 4 principal components accounting for 87.46% of the total variation in the original variables (Table 14). The variance explained by the first two components was 63.97%, and the factor loadings for the first three components are shown in Table 15. The first component was mainly defined by counts of gill rakers of outer gill arch (*GRC*), branched rays of dorsal fin (*DFR*), pectoral fin rays (*PFR*) and anal fin rays (*AFR*). The second component was mainly correlated with counts of lateral line scales (*LLS*), ventral fin rays (*VFR*) and anal fin rays (*AFR*). These indicated that the above meristic characters contributed the maximum to differentiate *P. denisonii* and *P. chalakkudiensis*. Bivariate scatter plot of the first and second components easily distinguished two major groups (Fig. 15) resulting in an evident separation of the two species.

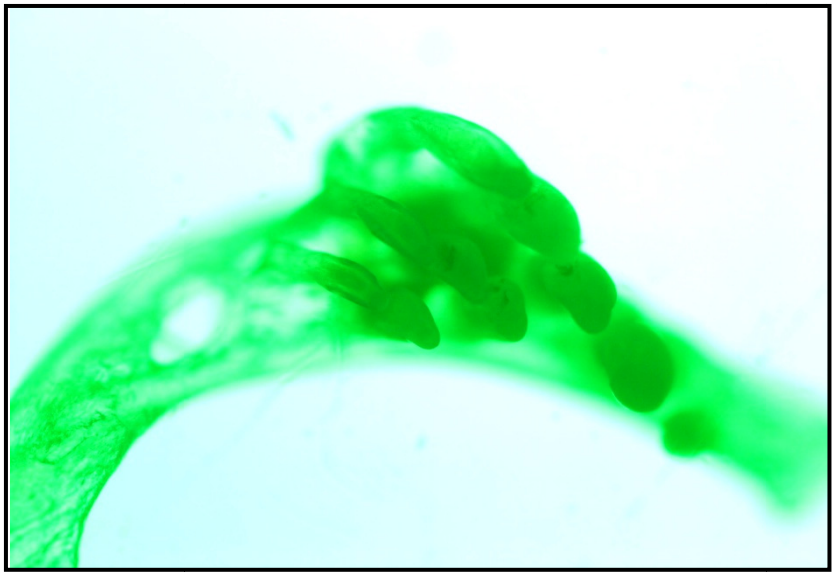


Fig. 12. Microscopic image of pharyngeal teeth arranged on one side of the pharyngeal bone of *P. denisonii*.

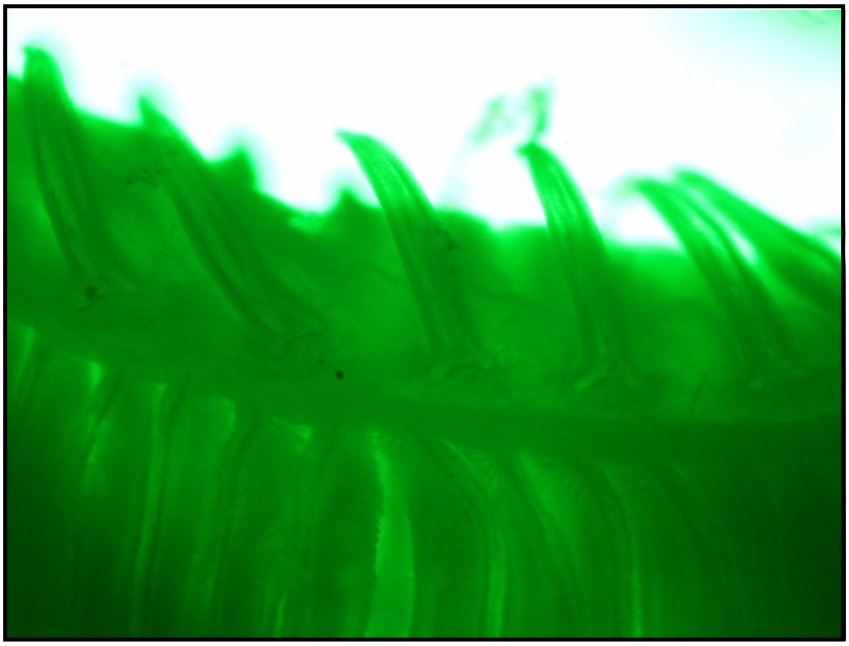


Fig. 13. Microscopic image of gill rakers of outer gill arch of *P. denisonii*

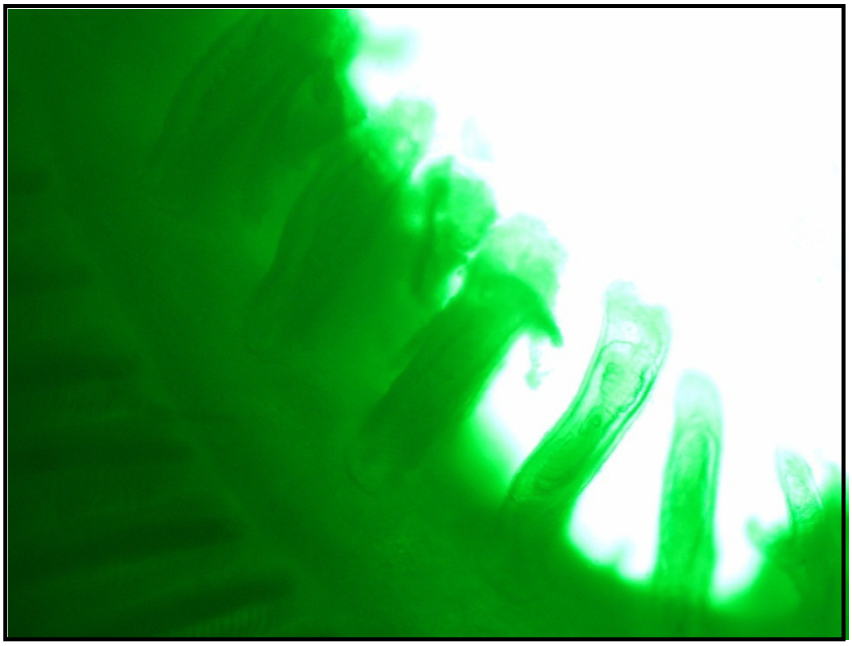


Fig. 14. Microscopic image of gill rakers of outer gill arch of *P. chalakkudiensis*

Table 13. Descriptive statistics of meristic variables, the coefficient of variation (*CV*) of each measurement, the multivariate coefficient of variation of each species (*CVp*) and F-values (derived from the analysis of variance) of *P. denisonii* and *P. chalakkudiensis*.

	***P. denisonii* (n = 120)**		***P. chalakkudiensis* (n = 60)**		
	Mean ± SD (min. - max.)	**CV**	**Mean ± SD (min. - max.)**	**CV**	**F value**
DFS	02.00 ± 0.00 (02.0-02.0)	0.00	02.00 ± 0.00 (02.0-02.0)	0.00	NS
DFR	08.20 ± 0.41 (08.0-09.0)	5.00	09.00 ± 0.00 (09.0-09.0)	0.00	56.00**
PFR	13.80 ± 0.56 (13.0-15.0)	4.06	14.60 ± 0.51 (14.0-15.0)	3.49	16.80**
VFR	08.87 ± 0.35 (08.0-09.0)	3.95	09.13 ± 0.35 (09.0-10.0)	3.83	4.31*
AFS	02.00 ± 0.00 (02.0-02.0)	0.00	02.00 ± 0.00 (02.0-02.0)	0.00	NS
AFR	05.27 ± 0.46 (05.0-06.0)	8.73	05.87 ± 0.35 (05.0-.6.0)	5.96	16.20**
GRC	12.27 ± 0.59 (11.0-13.0)	4.81	10.60 ± 0.51 (10.0-11.0)	4.81	68.36**
LLS	27.60 ± 0.51 (27.0-28.0)	1.85	28.13 ± 0.52 (27.0-29.0)	1.85	8.15**
CVp		**14.87**		**12.28**	

*Significant at the 5% level; **Significant at the 1% level; NS, not significant at the 5% level; *SD* is standard deviation.

Table 14. Summary of principal component analysis (PCA) for the meristic variables of *P. denisonii* and *P. chalakkudiensis* specimens.

Component	Eigenvalue	%variance	Cumulative
1	2.7687	46.15	46.15
2	1.0690	17.82	63.97
3	0.8290	13.82	77.79
4	0.5799	9.67	87.46
5	0.4462	7.44	94.90
6	0.3073	5.12	100.0

Table 15. Factor loadings for the first three principal components formed from the meristic variables of *P. denisonii* and *P. chalakkudiensis* specimens.

Meristic variables	PC1	PC2	PC3
DFR	-0.4961	0.1465	-0.0409
PFR	-0.3989	0.0918	-0.6948
VFR	-0.2855	0.6118	0.5850
AFR	-0.3913	-0.3541	0.3167
GRC	0.5209	-0.1084	0.1248
LLS	-0.2980	-0.6773	0.2399
Explained variance (%)	**46.15**	**17.82**	**13.82**

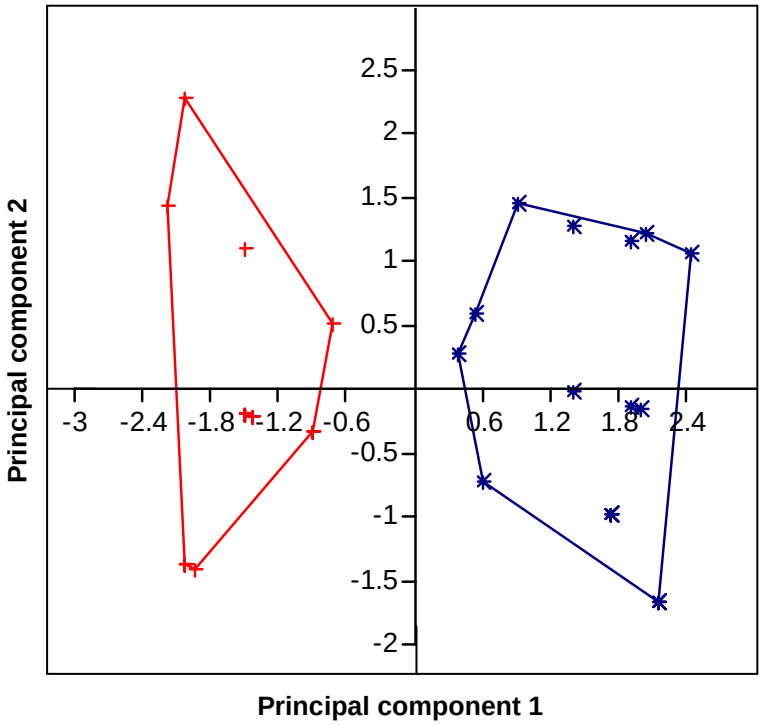


Fig. 15. Scatter diagram based on PCA of significant meristic variables between *P. denisonii* (blue stars) and *P. chalakkudiensis* (red crosses) specimens.

4.3. Mitochondrial DNA Analysis

4.3.1. ATPase 8/6 region

The complete ATPase 8/6 genes were sequenced from 39 samples including *P. denisonii* and *P. chalakkudiensis* from six different geographic locations. The size of amplified products was approximately 950 bp (Fig. 16). The ATPase 8/6 genes were slightly overlapping with 842 bp. The stop codon for ATPase 8 was at position 163-165. At position 159-164 two successive methionines (ATGATA) are coded. Thus the start codon for ATPase 6 might be at position 159-161. DNA sequence of a representative haplotype and its translated protein sequences are given in Fig. 17.

4.3.1.1. ATPase 8/6 sequence variations

Out of the 842 characters obtained, 719 (85.39%) were constant and 123 (14.61%) were variable, in which 118 (14.01%) were informative for parsimony. According to codon position, the most informative was the third position (78 informative characters) followed by the first position (28 informative characters). The empirical percentages of the different nucleotides were A = 32.8%, C = 25.7%, G = 12.2% and T = 29.3%. Most nucleotide variation resulted from transitions (88%) followed by transversions (12%). The transition-to-transversion rate (Ts/Tv) estimate for the ingroup was 7.3. This parameter was taken into account in the maximum-parsimony analysis. The nucleotide sequence characteristics of ATPase 8 and 6 genes across six populations including *P. denisonii* and *P. chalakkudiensis* were given separately in Table 16 and 17 respectively and the molecular diversity indexes were given in Table 18. All together 15 different haplotype were identified out of 39 sequences based on the nucleotide variations. The multiple alignment showing nucleotide variations among these haplotypes were presented in Appendix 1 - Table 1.

Amino Acid Translations - Nucleotide base pairs of the different haplotypes from all populations were translated into amino acid residues. Out of 281 total

residues (54 of ATPase 8 and 227 of ATPase 6), 259 (92.17%) were constant and of the variable characters only 1 (0.36%) was parsimony uninformative and 21 (7.47%) were parsimony informative across the groups. Not many variations can be detected in amino acid sequences with in populations. The multiple alignment showing amino acid variations among the haplotypes were presented in Appendix 1 - Table 2.

4.3.1.2. Population Variability

Comparison of the sequences revealed 15 different haplotypes out of 39 individuals from six different geographic locations, defined by 123 divergent nucleotide sites. All the 14 samples of group 2 (*Pc*) populations possessed the same haplotype; where as the Valapattanam population of group 1 (*Pd*) possessed the maximum number, six haplotypes out of eight samples (Table 20). No haplotypes were found to be sharing between any populations of group 1 (*Pd*), which indicated significant genetic separation between these populations. The *Nm* values estimated were significantly lower (0.006 – 0.223) suggesting no effective gene flow between populations of *P. denisonii* (Table 19). Relative frequencies of haplotypes among populations were presented in Table 21.

The mean pairwise distances between the haplotype of each population calculated by the Kimura 2-parameter method ranged from 0.0 (Chalakkudy and Periyar populations) to 0.0029 (Valapattanam population) (Fig. 18, Table 22). The mean pairwise distances among populations of group 1 (*Pd*) ranged from 0.0056 (between Kariangode and Valapattanam) to 0.0622 (between Kariangode and Chaliyar) indicated population structuring within the group (Table 23). The mean pairwise distances between populations of the two groups - group 1 (*Pd*) and group 2 (*Pc*) - was found to be significantly higher (0.1116), indicated apparent genetic divergence among these two groups.

Haplotype diversity (*h*), within the geographic populations was high in the case of group 1, ranging from 0.8667 in the Chandragiri population to 0.3333 in

Chaliyar and the nucleotide diversity (π) was generally low ranging from 0.0005 in the Kariangode population to 0.0024 in Valapattanam population (Table 24). Both haplotype and nucleotide diversities were found to be 0.0 in group 2.

4.3.1.3. Phylogeographic relationships of populations

The analysis of molecular variance (Table 25) for the two species indicated that a high proportion of the total variance (71.50%) was attributed to differences between them (Φ_{CT}=0.715), which was approaching significance (*P* = 0.068±0.006). The marginally significant *P*-value might be due to small sample size in some of the populations studied. The analysis also showed differences between the geographically isolated populations (Φ_{SC}=0.966), which was significant (*P* <0.001).

Estimates of genetic differentiation between all the six populations, using F-statistics, are given in Table 26. The group 1 (*Pd*) populations showed high levels of genetic differentiation among populations and also from other populations of group 2 (*Pc*). F_{ST} values were extremely high for all comparisons with in the group 1 (*Pd*) populations, (ranging from 0.6919 to 0.9889) denoting high genetic differentiation among these populations. No genetic differentiation was detected between populations of group 2 (*Pc*) (F_{ST} = 0.0). F_{ST} between the populations of group 1 and 2 were extremely higher (ranging from 0.9875 to 0.9973) indicated significant genetic divergence between *P. denisonii* and *P. chalakkudiensis*. The *p*-values associated with these comparisons were all significant (*P*<0.005).

4.3.1.4. Phylogenetic analysis

Phylogenies were constructed using the Neighbour joining (NJ) (Fig. 19) and Maximum parsimony (MP) (Fig. 20) methods. Both trees showed almost the same topology. They consistently indicated that two major groups (Group 1 and Group 2) could be recognized in both analyses. Group 1 formed a monophyletic

group included all the four sub populations of *P. denisonii* with high bootstrap values in both analyses. The group 1 (Pd) was divided into three major clades - clade 1, clade 2 and clade 3 - all with high support values (bootstrap value 97 – 100%) indicated clear population structuring. No subgroups or clades were recognized in group 2 (*Pc*). The clade 1 consisted of 4 haplotypes (6 samples) of Chandragiri population and clade 3 of 2 haplotypes (6 samples) of Chaliyar population. Both showed 100% bootstrap values in NJ and 99% in MP analyses. Valapattanam (6 haplotypes of 8 samples) and Kariangode (2 haplotypes of 5 samples) populations showed not much differentiation between them and they together formed clade 2 with high bootstrap support (100% in NJ and 99% in MP analyses).

4.3.1.5. Inference on past demography

Mismatch distribution analysis was performed separately for each population of group1 (*Pd*) because evidence of genetic population structure was found. In the case of Chalakkudy and Periyar populations of group 2 (*Pc*), the variance of the mismatch distribution was little and no demographic parameters could be estimated. Pairwise mismatch distributions and results of Tajima's D-test performed on each population of *P. denisonii* – Chandragiri, Kariangode, Valapattanam and Chaliyar – are given in Fig. 21. The parameters of the model of sudden expansion and the goodness of fit test to the model are also represented in Table 27.

The mismatch distributions for the Chandragiri and Valapattanam populations were appeared close to an estimated Poisson-shaped curve, expected for a population that has undergone a sudden expansion in effective population size and Tajima's D values were negative, but not significant. Together it suggested a history of genetic bottleneck events, with subsequent population expansion. A significant negative value for Tajima's D implies that there were more rare nucleotide site variants than would be expected under a neutral model of evolution (Rand, 1996). For Chaliyar and Kariangode populations Tajima's D-

values were negative but not significant, and the mismatch distributions were not fit to an expansion model curve suggested a history of long-term population stasis and a lack of clear expansion. The raggedness index (R) was the highest (0.667) for Chaliyar population (Table 27) signifies the stability of the population. The Chalakkudy and Periyar populations appear to have undergone either long-term small population size or a severe and recent genetic bottleneck, as evidenced by extremely low levels of genetic diversity (low *h* and π values, Table 24).

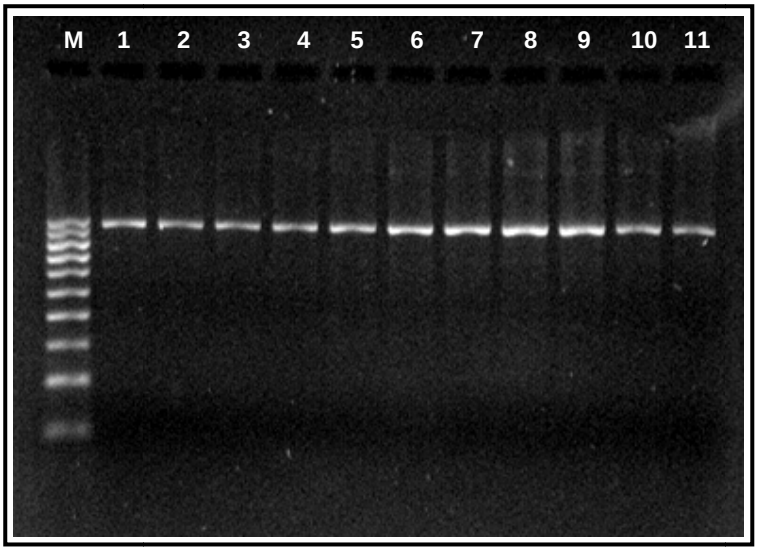


Fig. 16. Amplified products of ATPase 8/6 genes separated on 1.5% agarose gel. Lane 1 to 11 – eleven samples and M – 100 bp DNA ladder.

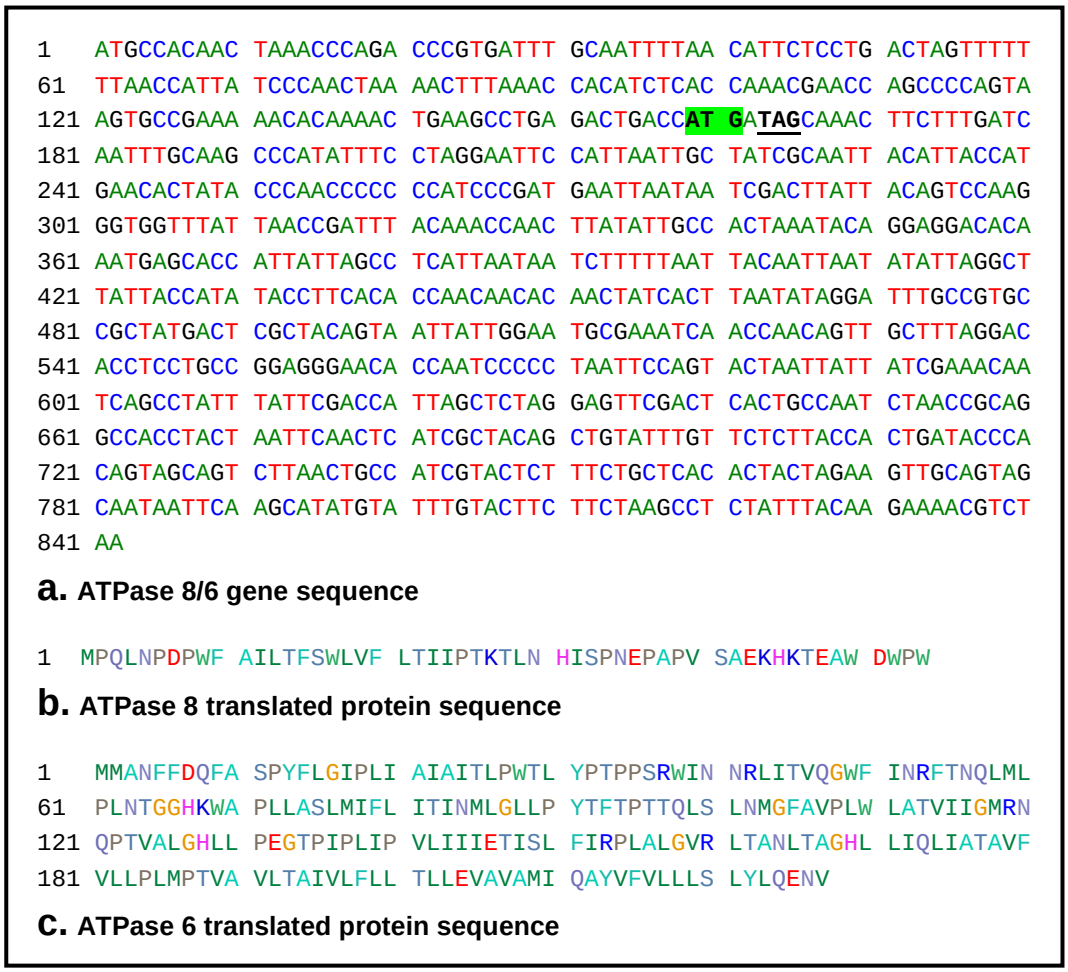


Fig. 17. DNA sequence of ATPase 8/6 gene and translated protein sequences of a representative haplotype. Underlined region is the stop codon of ATPase 8 gene and green highlighted region is the start codon of ATPase 6 gene.

Table 16. Nucleotide sequence characteristics of *ATPase 8* gene across six populations including *P. denisonii* and *P. chalakkudiensis*.

	1st Codon	2nd Codon	3rd Codon	Totals
Characters / sites	55	55	55	165
Invariable (monomorphic) sites	50	50	44	144 (87.27%)
Variable (polymorphic) sites	5	5	11	21 (12.73%)
Singleton variable sites	-	-	2	2 (1.21%)
Parsimony informative sites	5	5	9	19 (11.52%)

Table 17. Nucleotide sequence characteristics of *ATPase 6* gene across six populations including *P. denisonii* and *P. chalakkudiensis*.

	1st Codon	2nd Codon	3rd Codon	Totals
Characters / sites	228	228	228	684
Invariable (monomorphic) sites	205	220	157	582 (85.09%)
Variable (polymorphic) sites	23	8	71	102 (14.91%)
Singleton variable sites	-	1	2	3 (0.44%)
Parsimony informative sites	23	7	69	99 (14.47%)

Table 18. Molecular diversity indexes of 842 bp fragment of the *ATPase 8/6* gene across each population of *P. denisonii* and *P. chalakkudiensis*.

Statistics	*Pd*				*Pc*	
	CDR	**KGD**	**VLP**	**CLR**	**CHD**	**PER**
No. of transitions	4	1	3	2	0	0
No. of transversions	0	0	3	0	0	0
No. of substitutions	4	1	6	2	0	0
No. of indels	0	0	0	0	0	0
No. of transition sites	4	1	3	2	0	0
No. of transversion sites	4	1	3	2	0	0
No. of substitution sites	4	1	6	2	0	0
No. private substitution sites	4	1	3	2	0	0
No. of indel sites	0	0	0	0	0	0

Table 19. Matrix showing *Nm* between populations of *P. denisonii*

	CDR	**KGD**	**VLP**	**CLR**
CDR	****			
KGD	0.021	****		
VLP	0.040	0.223	****	
CLR	0.014	0.006	0.016	****

Table 20. Distribution of haplotypes of 842 bp fragment of the *ATPase 8/6* gene among the populations of *P. denisonii* and *P. chalakkudiensis*.

Site	Haplotype (Representative Sample Name)															n_t	n_h
	A (CDR-p01)	**B** (CDR-p02)	**C** (CDR-p05)	**D** (CDR-p06)	**E** (KGD-p01)	**F** (KGD-p02)	**G** (VLP-p01)	**H** (VLP-p04)	**I** (VLP-p05)	**J** (VLP-p06)	**K** (VLP-p07)	**L** (VLP-p08)	**M** (CLR-p01)	**N** (CLR-p04)	**O** (CHD-p01)		
Pd																	
CDR	2	1	2	1	0	0	0	0	0	0	0	0	0	0	0	6	4
KGD	0	0	0	0	4	1	0	0	0	0	0	0	0	0	0	5	2
VLP	0	0	0	0	0	0	1	3	1	1	1	1	0	0	0	8	6
CLR	0	0	0	0	0	0	0	0	0	0	0	0	5	1	0	6	2
Pc																	
CHD	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	9	1*
PER	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	5	1*
Total	2	1	2	1	4	1	1	3	1	1	1	1	5	1	14	39	15

n_t - number of individuals analyzed per site; n_h - number of haplotypes per site.

*CHD and PER populations are having same haplotype.

Table 21. Relative haplotype frequencies of 842 bp fragment of the *ATPase 8/6* gene among the populations of *P. denisonii* and *P. chalakkudiensis*.

Haplotype	Populations					
	Pd				*Pc*	
	CDR (n=6)	**KGD** (n=5)	**VLP** (n=8)	**CLR** (n=6)	**CHD** (n=9)	**PER** (n=5)
A	0.333	0	0	0	0	0
B	0.167	0	0	0	0	0
C	0.333	0	0	0	0	0
D	0.167	0	0	0	0	0
E	0	0.800	0	0	0	0
F	0	0.200	0	0	0	0
G	0	0	0.125	0	0	0
H	0	0	0.375	0	0	0
I	0	0	0.125	0	0	0
J	0	0	0.125	0	0	0
K	0	0	0.125	0	0	0
L	0	0	0.125	0	0	0
M	0	0	0	0.833	0	0
N	0	0	0	0.167	0	0
O	0	0	0	0	1.000	1.000

Table 22. Mean pairwise distances between the haplotypes of each population based on *ATPase 8/6* gene sequences.

Population Names	n_t	n_h	Mean pairwise distances
Pd			
Chandragiri (CDR)	6	4	0.0024 ± 0.0012
Kariangode (KGD)	5	2	0.0012 ± 0.0
Valapattanam (VLP)	8	6	0.0029 ± 0.0024
Chaliyar (CLR)	6	2	0.0024 ± 0.0
Pc			
Chalakkudy (CHD)	9	1	0.0000
Periyar (PER)	5	1	0.0000

n_t - number of individuals analyzed per site; n_h - number of haplotypes per site.

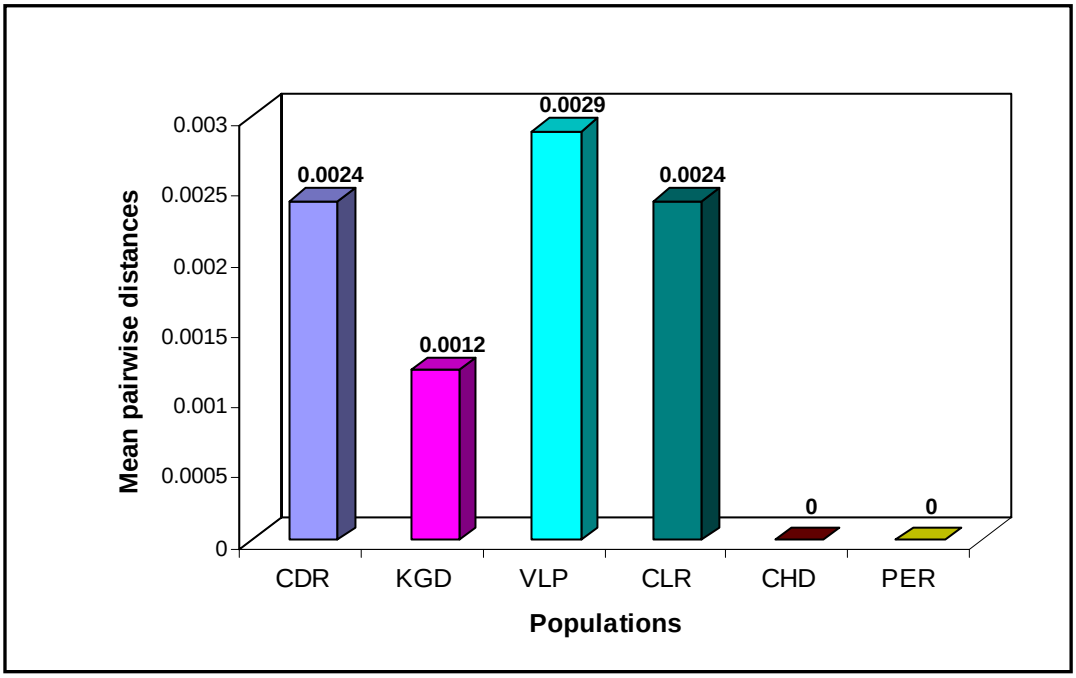


Fig. 18. Mean pairwise distances between the haplotypes within the populations of *P. denisonii* and *P. chalakkudiensis* based on *ATPase 8/6* gene sequences.

Table 23. Mean pairwise distances between populations of *P. denisonii and P. chalakkudiensis* based on *ATPase 8/6* gene sequences.

	Pd				***Pc***	
	CDR	**KGD**	**VLP**	**CLR**	**CHD**	**PER**
Pd						
CDR						
KGD	0.0363					
VLP	0.0322	0.0058				
CLR	0.0537	0.0622	0.0589			
Pc			***0.1116***			
CHD	0.1139	0.1122	0.1133	0.1071		
PER	0.1139	0.1122	0.1133	0.1071	0.0000	

Table 24. Intrapopulation nucleotide (π) and haplotype (h) diversities of *P. denisonii* and *P. chalakkudiensis* based on *ATPase 8/6* gene sequences.

Population Names	**Nucleotide diversity (π)**	**Haplotype diversity (h)**
Pd		
Chandragiri (CDR)	0.0021	0.8667
Kariangode (KGD)	0.0005	0.4000
Valapattanam (VLP)	0.0024	0.8929
Chaliyar (CLR)	0.0008	0.3333
Pc		
Chalakkudy (CHD)	0.0000	0.0000
Periyar (PER)	0.0000	0.0000

Table 25. Results of the hierarchical analysis of molecular variance (AMOVA) of populations of *P. denisonii* and *P. chalakkudiensis* based on mitochondrial ATPase 8/6 region.

Source of variation	*df*	% total variance	Φ	*P*-value
Among species	1	71.50	$\Phi_{CT} = 0.715$	0.068±0.006
Among populations within species	4	27.52	$\Phi_{SC} = 0.966$	<0.001
Within populations	33	0.98	$\Phi_{ST} = 0.990$	<0.001

Table 26. Pairwise F_{ST} values for between populations (above horizontal) and significance (below horizontal) of *P. denisonii* and *P. chalakkudiensis* based on *ATPase 8/6* gene sequences.

	Pd				***Pc***	
	CDR	**KGD**	**VLP**	**CLR**	**CHD**	**PER**
Pd						
CDR		0.9605	0.9265	0.9723	0.9923	0.9875
KGD	+		0.6919	0.9889	0.9984	0.9973
VLP	+	+		0.9693	0.9891	0.9837
CLR	+	+	+		0.9969	0.9949
Pc						
CHD	+	+	+	+		0.0000
PER	+	+	+	+	−	

+, significant pairwise F_{ST} at $p<0.005$ after sequencial Bonferroni agjustment; −, not significant at this level

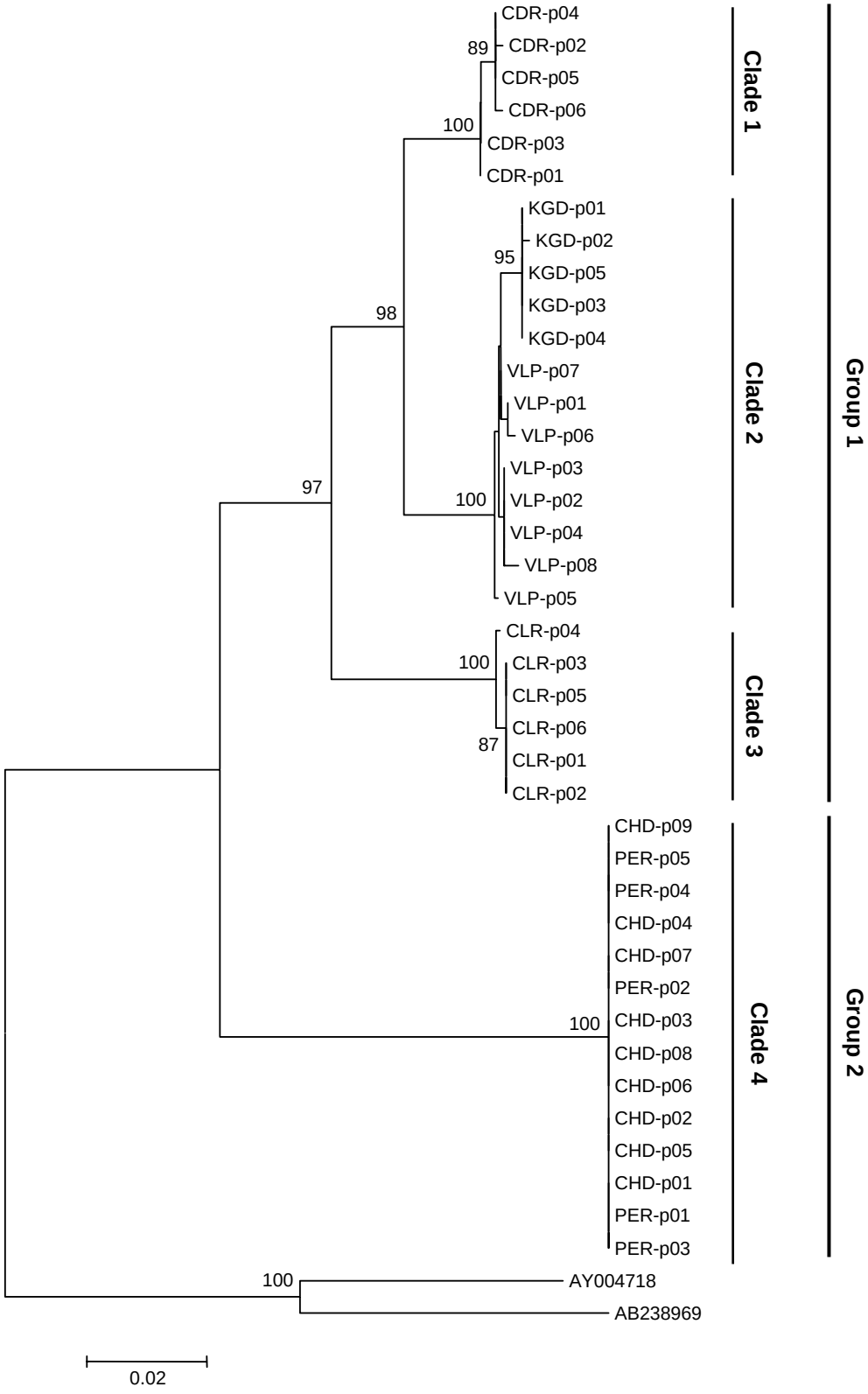


Fig. 19. Neighbour-joining tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA ATPase 8/6 genes. Numbers at nodes indicate the bootstrap values. AY004718 (*Puntius conchonius*) and AB238969 (*Puntius ticto*) are from genbank and are included as outgroup species.

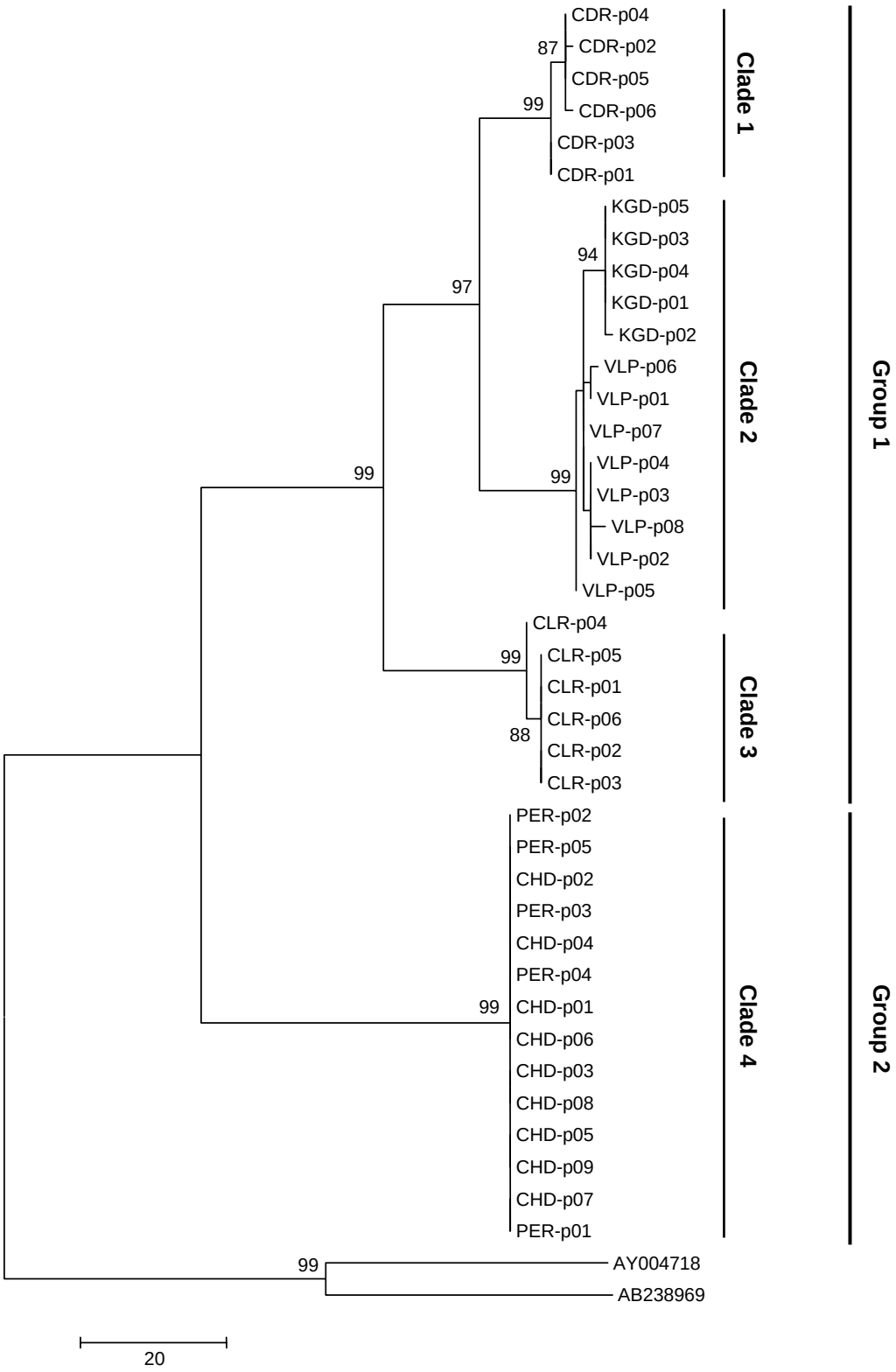


Fig. 20. Maximum parsimony tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA ATPase 8/6 genes. Numbers at nodes indicate the bootstrap values. AY004718 (*Puntius conchonius*) and AB238969 (*Puntius ticto*) are from genbank and are included as outgroup species.

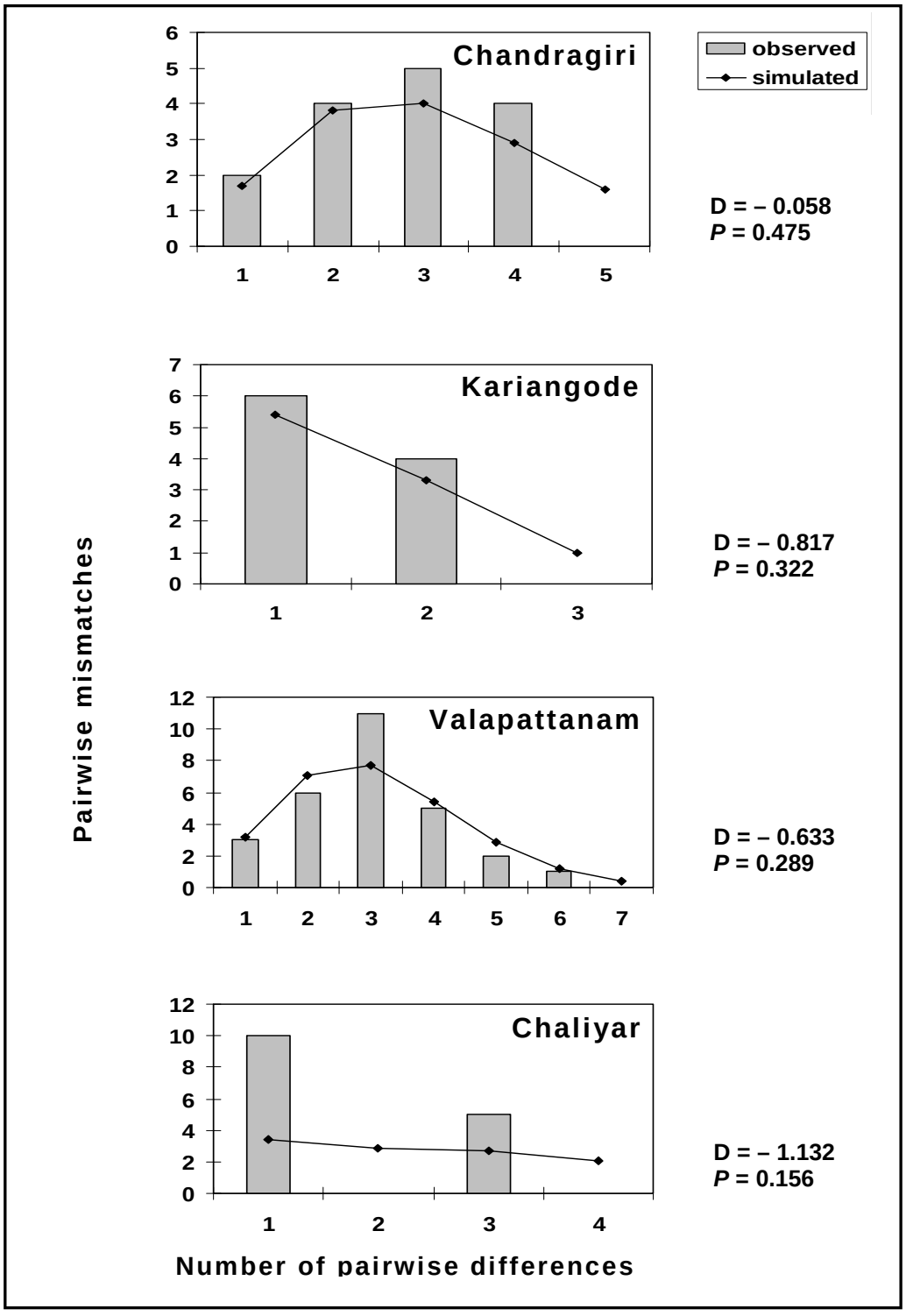


Fig. 21. Pairwise mismatch distributions (Rogers & Harpending 1992), simulated model of sudden expansion (Rogers 1995) and results of Tajima's D-test with associated probability for each population of *P. denisonii*.

Table 27. Parameters of sudden expansion model and Goodness of fit test to the model for each population.

Statistics	Populations					
	Pd				***Pc***	
	CDR	**KGD**	**VLP**	**CLR**	**CHD**	**PER**
Demographic expansion						
S	4	1	6	2	0	0
θ_0	0.004	0.005	0.000	0.900	–	–
θ_1	99999.0	99999.0	99999.0	3.600	–	–
τ	2.152	0.562	2.137	2.982	–	–
Goodness of fit test						
SSD	0.010	0.007	0.018	0.260	–	–
P	0.733	0.771	0.516	0.091	–	–
R	0.098	0.200	0.103	0.667	–	–
P	0.756	0.925	0.481	0.208	–	–

S-number of polymorphic sites, θ_0 – pre-expansion population size, θ_1 – post-expansion population size, τ - time in number of generations, **SSD** – sum of squared deviations, **R**- raggedness index, ***P*** = p-values.

4.3.2. COI region

The partial sequence of COI gene was generated from 12 samples including *P. denisonii* and *P. chalakkudiensis* from six different geographic locations. The size of amplified products was approximately 700 bp (Fig. 22). A 521 bp region was finally obtained for analysis after sequencing and sequence editing. DNA sequence of a representative haplotype and the translated protein are given in Fig. 23.

4.3.2.1. COI sequence variations

The alignment of the sequences revealed 7 different haplotypes defined by 68 (13.05%) divergent nucleotide sites out of 521 characters obtained. There were 67 (12.86%) sites which are parsimony informative. According to codon position, the most informative was the third position (79.41%) followed by the first position (16.17%). Most nucleotide variation resulted from transitions followed by transversions with a ratio (Transition/Transversion) of 9.0 and no indels were observed. The nucleotide frequencies were T = 29.0, C = 27.8, A = 26.3, G = 16.8.

The nucleotide sequence characteristics of 521bp partial sequence of COI gene across six populations including *P. denisonii* and *P. chalakkudiensis* were given in Table 28 and the multiple alignment showing nucleotide variations among these haplotypes were presented in Appendix 1 - Table 3.

Amino Acid Translations - Nucleotide base pairs of the different haplotypes from all populations were translated into amino acid residues. Out of 173 total residues, 5 (2.9%) characters were variable and parsimony informative. The multiple alignment showing amino acid variations among the haplotypes were presented in Appendix 1 - Table 4.

4.3.2.2. Genetic divergence

A total of 7 different haplotypes were identified out of 12 samples sequenced from six different geographic locations. As observed in ATPase 8/6, all the samples of group 2 (*Pc*) populations possessed the same haplotype; whereas, six different haplotypes were observed out of eight samples from four populations of group 1 (*Pd*) (Table-29). For Chandragiri and Chaliyar, specific haplotypes were observed within populations and at same time a haplotype was found to be sharing between Kariangode and Valapattanam populations.

The mean pairwise distances between the haplotype of each population calculated by the Kimura 2-parameter method ranged from 0.0 (Kariangode, Chalakkudy and Periyar populations) to 0.0019 (Chandragiri, Valapattanam and Chaliyar populations) (Table 30). The mean pairwise distances between each population of group 1 (*Pd*) ranged from 0.0019 (between Kariangode and Valapattanam) to 0.0821 (between Kariangode and Chaliyar). The mean pairwise distances between populations of the two groups - group 1 (*Pd*) and group 2 (*Pc*) - was found to be significantly higher (0.0903) (Table 31). These results were found to be in concordance with that observed in ATPase 8/6 genes analyses indicated clear population structuring within group 1 and significant genetic divergence between the two groups.

4.3.2.3. Phylogenetic and geographical relationships among haplotypes

Phylogenetic trees constructed using the Neighbour joining (NJ) (Fig. 24) and Maximum parsimony (MP) (Fig. 25) methods show the same topology. They consistently indicated that two major groups (Group 1 and Group 2) could be recognized in both analyses with high bootstrap support, as the same observed in the case of ATPase analyses. The group 1 (*Pd*) was divided into three well separated clades - clade 1, clade 2 and clade 3 - all with high support values (bootstrap value 99 - 100) indicated clear population structuring. No subgroups or clades were recognized in group 2 (*Pc*). The Chandragiri (clade 1) and Chaliyar (clade 3) populations were found to be separated with high bootstrap support of

97 – 100% in both analyses; where as the haplotypes of Kariangode and Valapattanam populations together formed clade 2 with high bootstrap support of 99% in NJ and MP analyses.

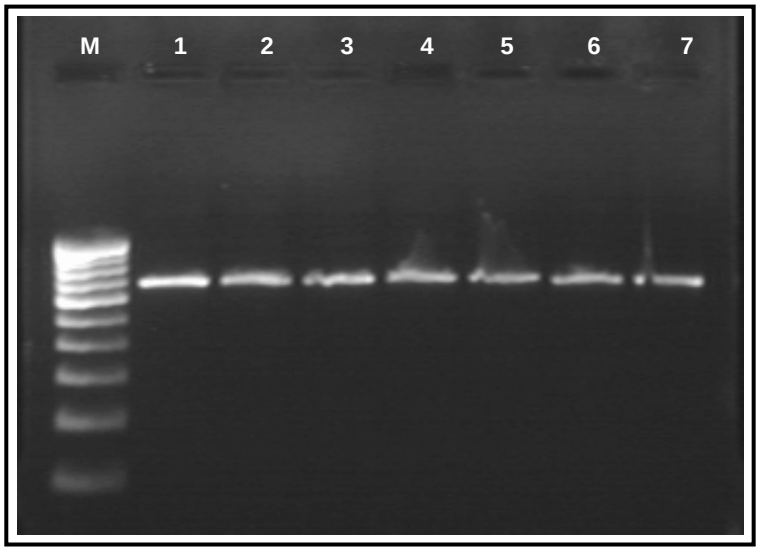


Fig. 22. Amplified products of COI gene separated on 1.5% agarose gel. Lane 1 to 7 – seven samples and M – 100 bp DNA ladder.

```
1   CCGCCCTAAG CCTCCTCATC CGAGCTGAGC TAAGCCAGCC AGGATCACTC TTAGGTGACG
61  ACCAAATTTA TAATGTCATC GTTACTGCTC ACGCCTTCGT AATAATTTTC TTTATAGTAA
121 TGCCTGTCCT TATTGGGGGG TTTGGAAACT GACTAGTACC ACTAATAATT GGAGCCCCCG
181 ACATAGCATT CCCACGAATA AACAATATAA GCTTCTGGCT ACTACCACCA TCGTTCCTAC
241 TTCTATTAGC CTCCTCCGGC GTTGAAGCTG GAGCGGGAAC GGGGTGAACA GTGTACCCGC
301 CACTTGCAGG AAATCTAGCT CACGCCGGAG CATCCGTTGA CCTAACAATT TTCTCACTGC
361 ACTTAGCAGG TGTCTCATCA ATCCTCGGAG CAATCAATTT TATCACTACA ACCATCAACA
421 TGAAACCCCC TACTACTTCA CAATATCAAA CACCTCTATT TGTTTGATCT GTGCTTGTAA
481 CTGCTGTATT ATTACTACTC TCACTTCCAG TCTTAGCTGC C
```

a. COI - 521bp partial sequence

```
1   ALSLLIRAEL SQPGSLLGDD QIYNVIVTAH AFVMIFFMVM PVLIGGFGNW LVPLMIGAPD
61  MAFPRMNNMS FWLLPPSFLL LLASSGVEAG AGTGWTVYPP LAGNLAHAGA SVDLTIFSLH
121 LAGVSSILGA INFITTTINM KPPTTSQYQT PLFVWSVLVT AVLLLLSLPV LAA
```

b. COI - translated protein sequence

Fig. 23. DNA sequence of COI gene and translated protein sequence of a representative haplotype.

Table 28. Nucleotide sequence characteristics of *COI* gene across six populations including *P. denisonii* and *P. chalakkudiensis*.

	1st Codon	2nd Codon	3rd Codon	Totals
Characters / sites	173	174	174	521
Invariable (monomorphic) sites	162	171	120	453 (86.95%)
Variable (polymorphic) sites	11	03	54	68 (13.05%)
Singleton variable sites	-	01	-	01 (0.19%)
Parsimony informative sites	11	02	54	67 (12.86%)

Table 29. Distribution of haplotypes of 521 bp fragment of the *COI* gene among the populations of *P. denisonii* and *P. chalakkudiensis*.

Site	Haplotype (Representative Sample Name)							n_t	n_h
	A (CDR-c01)	**B** (CDR-c02)	**C** (KGD-c01)	**D** (VLP-c01)	**E** (CLR-c01)	**F** (CLR-c02)	**G** (CHD-c01)		
Pd									
CDR	1	1	0	0	0	0	0	2	2
KGD	0	0	2	0	0	0	0	2	1
VLP	0	0	1	1	0	0	0	2	2*
CLR	0	0	0	0	1	1	0	2	2
Pc									
CHD	0	0	0	0	0	0	2	2	1*
PER	0	0	0	0	0	0	2	2	1*
Total	1	1	3	1	1	1	4	12	7

n_t - number of individuals analyzed per site; n_h - number of haplotypes per site.

*CHD and PER populations are having same haplotype; a haplotype shared between KGD and VLP.

Table 30. Mean pairwise distances between the haplotypes of each population based on 521 bp region of mtDNA *COI* gene.

Population Names	n_t	n_h	Mean pairwise distances
Pd			
Chandragiri (CDR)	2	2	0.0019
Kariangode (KGD)	2	1	0.0000
Valapattanam (VLP)	2	2	0.0019
Chaliyar (CLR)	2	2	0.0019
Pc			
Chalakkudy (CHD)	2	1	0.0000
Periyar (PER)	2	1	0.0000

n_t - number of individuals analyzed per site; n_h - number of haplotypes per site.

Table 31. Mean pairwise distances between populations of *P. denisonii* and *P. chalakkudiensis* based on 521 bp region of mtDNA *COI* gene.

	Pd				***Pc***	
	CDR	**KGD**	**VLP**	**CLR**	**CHD**	**PER**
Pd						
CDR						
KGD	0.0245					
VLP	0.0255	0.0019				
CLR	0.0763	0.0821	0.0809			
Pc			***0.0903***			
CHD	0.0928	0.0872	0.0884	0.0928		
PER	0.0928	0.0872	0.0884	0.0928	0.0000	

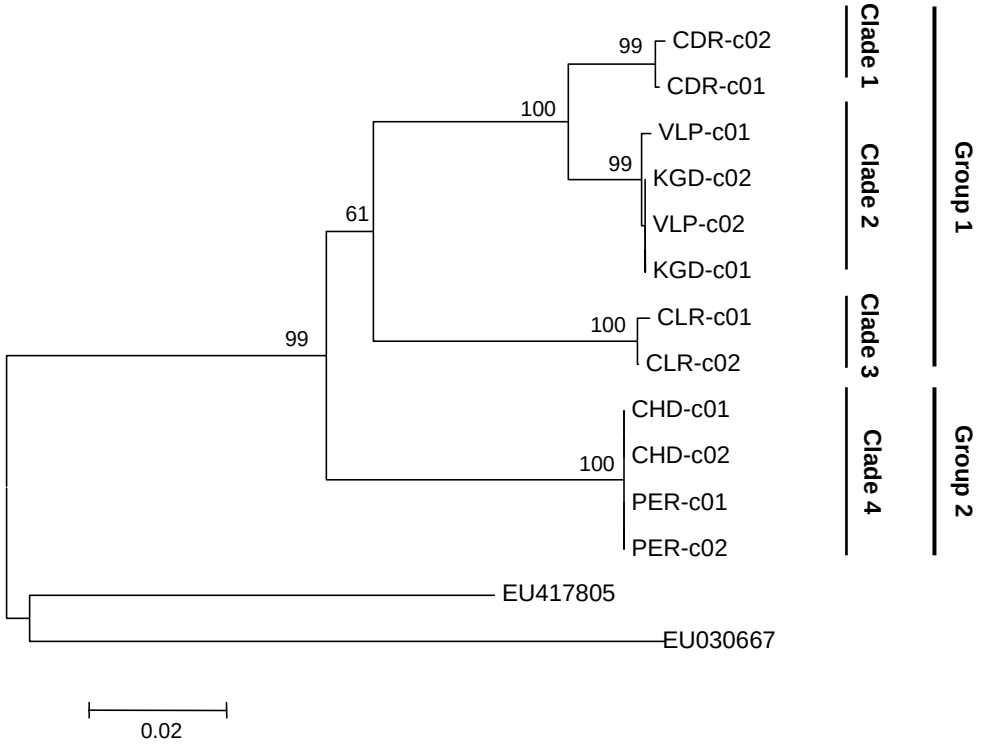


Fig. 24. Neighbour-joining tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA COI gene. Numbers at nodes indicate the bootstrap values. EU417805 (*Puntius sarana*) and EU030667 (*Labeo bata*) are from genbank and are included as outgroup species with original sequences for easy comparison.

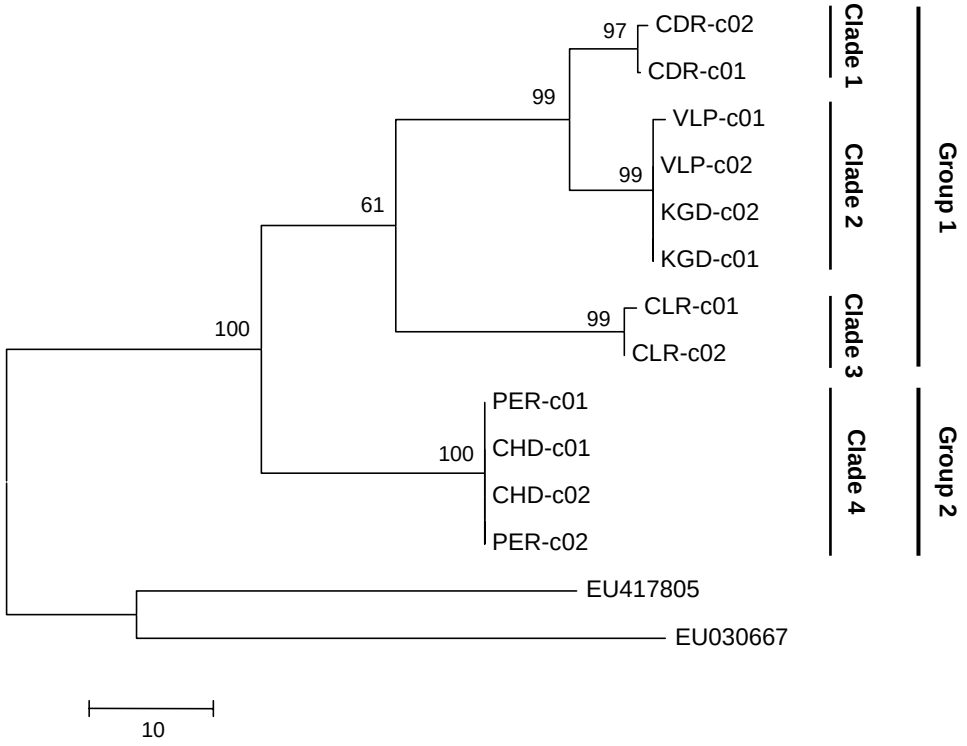


Fig. 25. Maximum parsimony tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA COI gene. Numbers at nodes indicate the bootstrap values. EU417805 (*Puntius sarana*) and EU030667 (*Labeo bata*) are from genbank and are included as outgroup species with original sequences for easy comparison.

4.3.3. Cyt *b* region

A 552 bp sequence of mtDNA cyt *b* region was sequenced from 12 individuals including *P. denisonii* and *P. chalakkudiensis* from six different geographic locations. The size of amplified products was approximately 650 bp (Fig. 26). DNA sequence of a representative haplotype and the translated protein are given in Fig. 27.

4.3.3.1. Cyt *b* sequence variations

When all the sequences were compared, 6 different haplotypes were detected, diverging on 100 (18.12%) polymorphic nucleotide sites out of 552 characters obtained. There were 99 (17.93%) sites which are parsimony informative and no indels observed. According to codon position, the most informative was the third position (73.74%) followed by the first position (24.24%). Most nucleotide variation resulted from transitions followed by transversions with a ratio (Ts/Tv) of 4.8. The nucleotide frequencies were T = 28.5, C = 27.9, A = 31.5, G = 12.1. The nucleotide sequence characteristics of 552 bp partial sequence of cyt *b* gene across six populations including *P. denisonii* and *P. chalakkudiensis* were represented in Table 32 and the multiple alignment showing nucleotide variations among these haplotypes were presented in Appendix 1 – Table 5.

Amino Acid Translations - Nucleotide base pairs of the different haplotypes from all populations were translated into amino acid residues. Out of 183 total residues, 14 (7.65%) characters were variable, out of which 13 (7.10%) parsimony informative. All the variations observed where between the groups (*Pd* and *Pc*) and no amino acid sequence variations were observed within group 1 (*Pd*) .The multiple alignment showing amino acid variations among the haplotypes were presented in Appendix 1 – Table 6.

4.3.3.2. Genetic divergence

A haplotype each from each location was identified out of 12 samples sequenced from six different geographic locations. Group 2 (*Pc*) populations possessed two different haplotypes of which one was sharing between Chalakkudy and Periyar; and the other of Periyar. All the four populations of group 1 (*Pd*) possessed four different haplotypes (Table 33).

The mean pairwise distances between each population of group 1 (*Pd*) ranged from 0.0055 (between Kariangode and Valapattanam) to 0.0945 (between Chandragiri and Chaliyar). The mean pairwise distances between populations of the two groups - group 1 (*Pd*) and group 2 (*Pc*) - was found to be significantly higher (0.1468) (Table 34). The results observed here supported the above observations with ATPase and COI analysis indicated clear population structuring within group 1 and significant genetic divergence between the two groups.

4.3.3.3. Phylogenetic and geographical relationships among haplotypes

Phylogenetic trees constructed using the Neighbour joining (NJ) (Fig. 28) and Maximum parsimony (MP) (Fig. 29) methods show the same topology and the branching pattern was almost similar to that observed with ATPase and COI analyses. The two major groups (Group 1 and Group 2) found to be well separated with high bootstrap support in both analyses. The group 1 (*Pd*) was divided into three well separated clades - clade 1, clade 2 and clade 3 - all with high support values (bootstrap value 99 - 100) indicated clear population structuring. No subgroups or clades were recognized in group 2 (*Pc*). The Chandragiri (clade 1) and Chaliyar (clade 3) populations were found to be separated with high bootstrap support of 97 – 100% in both analyses; where as the haplotypes of Kariangode and Valapattanam populations together formed clade 2.

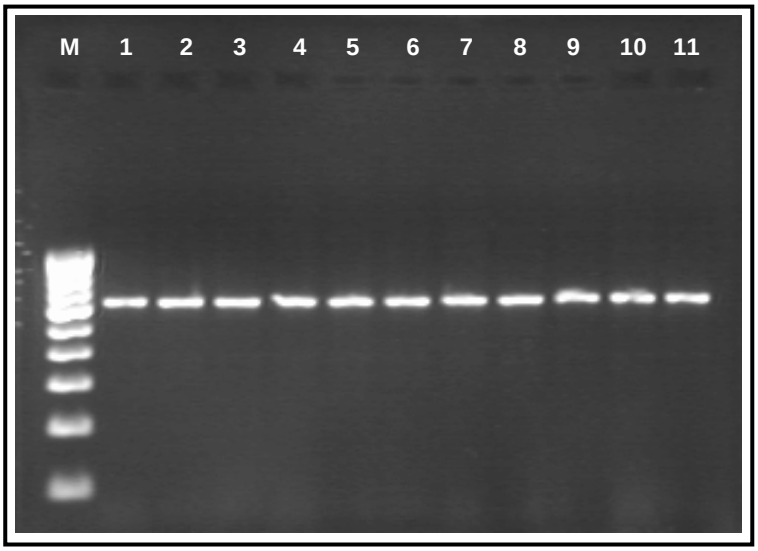


Fig. 26. Amplified products of cyt *b* gene separated on 1.5% agarose gel. Lane 1 to 11 – eleven samples and M – 100 bp DNA ladder.

```
1   ATGTAGGAGA TATATTAGTC CAATGAATCT GAGGAGGGTT CTCAGTAGAC AATGCAACAC
61  TAACACGATT CTTCGCATTC CACTTCTTCT TACCATTCAT CATTGCCGCA ATAACTATCC
121 TACACCTTCT ATTCCTACAC GAAACAGGAT CAAACAACCC AATTGGGCTA AACTCAGACG
181 CAGATAAAAT TCCATTTCAC CCCTACTTCA CCTACAAAGA CCTCCTTGGG TTTATAATTA
241 TACTATTAGC CCTAATACTA TTAGCACTAT TTACCCCCAA CCTCCTAGGA GACCCAGAAA
301 ACTTCACTCC TGCCAACCCA TTAGTTACTC CGCCACACAT CAAACCAGAG TGATATTTCC
361 TATTTGCTTA TGCCATCCTT CGATCAATCC CAAACAAACT CGGAGGTGTC CTCGCACTAC
421 TTTTTTCCAT CCTAGTATTA ATAATTGTAC CACTACTACA TACCTCAAAA CAACGAGGAC
481 TTACATTCCG CCCCATCACC CAGTTTTTAT TCTGAACCTT AGTAGCAGAT ATGATCATCT
541 TAACATGAAT CG
```

a. Cyt *b* – 552 bp partial sequence

```
1   VGDMLVQWIW GGFSVDNATL TRFFAFHFFL PFIIAAMTIL HLLFLHETGS NNPIGLNSDA
61  DKIPFHPYFT YKDLLGFMIM LLALMLLALF TPNLLGDPEN FTPANPLVTP PHIKPEWYFL
121 FAYAILRSIP NKLGGVLALL FSILVLMIVP LLHTSKQRGL TFRPITQFLF WTLVADMIIL
181 TWI
```

b. Cyt *b* - translated protein sequence

Fig. 27. DNA sequence of cyt *b* gene and translated protein sequence of a representative haplotype.

Table 32. Nucleotide sequence characteristics of cyt *b* gene across six populations including *P. denisonii* and *P. chalakkudiensis*.

	1st Codon	2nd Codon	3rd Codon	Totals
Characters / sites	184	184	184	552
Invariable (monomorphic) sites	159	182	111	452 (81.88%)
Variable (polymorphic) sites	25	2	73	100 (18.12%)
Singleton variable sites	1	-	-	01 (0.18%)
Parsimony informative sites	24	2	73	99 (17.93%)

Table 33. Distribution of haplotypes of 552 bp fragment of the cyt *b* gene among the populations of *P. denisonii* and *P. chalakkudiensis*.

Site	Haplotype (Representative Sample Name)						n_t	n_h
	A (CDR-b01)	**B** (KGD-b01)	**C** (VLP-b01)	**D** (CLR-b01)	**E** (CHD-b01)	**F** (PER-b02)		
Pd								
CDR	2	0	0	0	0	0	2	1
KGD	0	2	0	0	0	0	2	1
VLP	0	0	2	0	0	0	2	1
CLR	0	0	0	2	0	0	2	1
Pc								
CHD	0	0	0	0	2	0	2	1
PER	0	0	0	0	1	1	2	2*
Total	2	2	2	2	3	1	12	6

n_t - number of individuals analyzed per site; n_h - number of haplotypes per site.

* one haplotype shared between CHD and PER.

Table 34. Mean pairwise distances between populations of *P. denisonii* and *P. chalakkudiensis* based on 552 bp region of mtDNA cyt *b* gene.

	Pd				***Pc***	
	CDR	**KGD**	**VLP**	**CLR**	**CHD**	**PER**
Pd						
CDR						
KGD	0.0451					
VLP	0.0471	0.0055				
CLR	0.0945	0.0800	0.0821			
Pc		***0.1468***				
CHD	0.1492	0.1475	0.1451	0.1430		
PER	0.1504	0.1487	0.1463	0.1442	0.0018	

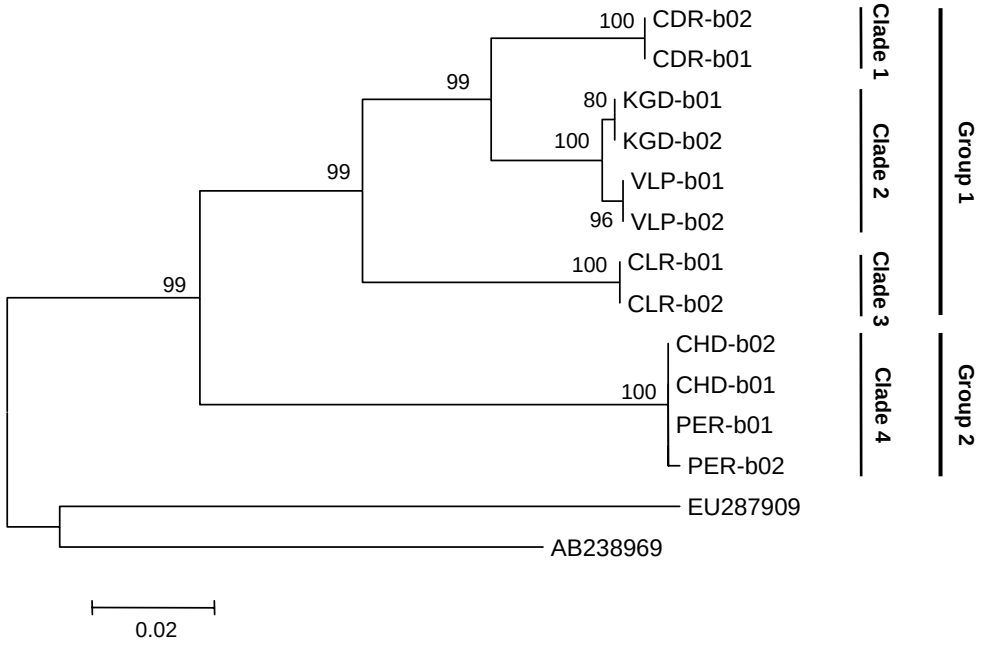


Fig. 28. Neighbour-joining tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA cyt *b* gene. Numbers at nodes indicate the bootstrap values. EU287909 (*Puntius tetrazona*) and AB238969 (*Puntius ticto*) are from genbank and are included as outgroup species.

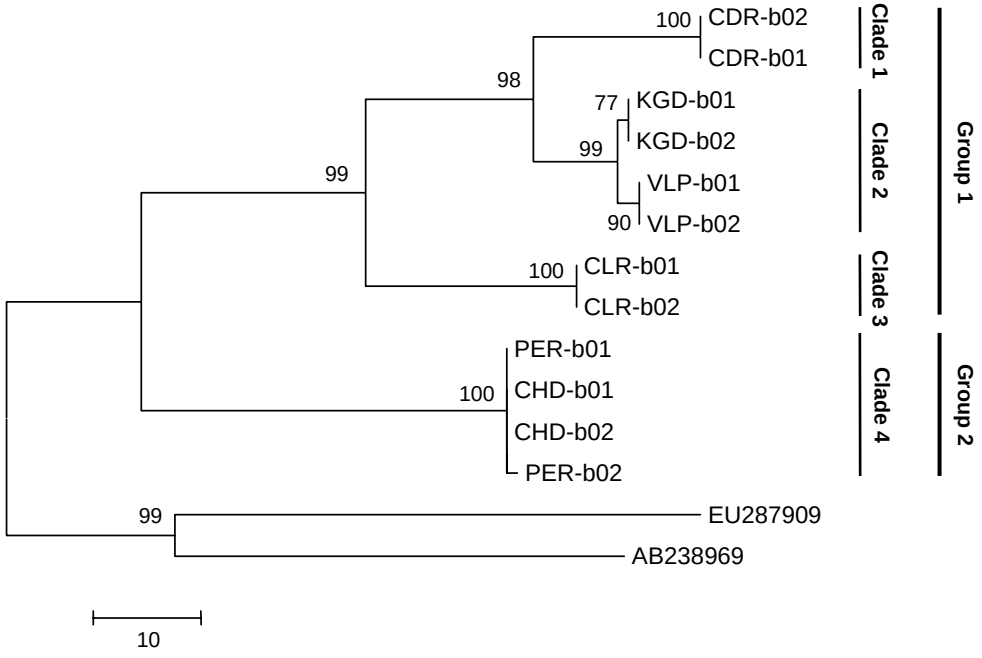


Fig. 29. Maximum parsimony tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial DNA cyt *b* gene. Numbers at nodes indicate the bootstrap values. EU287909 (*Puntius tetrazona*) and AB238969 (*Puntius ticto*) are from genbank and are included as outgroup species.

4.3.4. 16S rDNA region

A 562 bp sequence of 16S rDNA region was sequenced from 12 individuals including *P. denisonii* and *P. chalakkudiensis* from six different geographic locations. The size of amplified products was approximately 650 bp (Fig. 30). DNA sequence of a representative haplotype is given in Fig. 31.

4.3.4.1. 16 rDNA sequence variations

Comparison of the sequences revealed 6 different haplotypes out of 12 samples. As expected, sequence variation in the 16S rDNA region was less extensive than ATPase 8/6, COI and cyt *b* regions. In 562 bp, only 37 (6.58%) polymorphic nucleotide sites were observed among 12 individuals including *P. denisonii* and *P. chalakkudiensis* from six different geographic locations. Out of the 37 variable sites, 36 were found to be parsimony informative. There was one indel observed in Chaliyar haplotype at 397th bp position in the alignment. Most nucleotide variation resulted from transitions followed by transversions with a ratio (Ts/Tv) of 2.7. The average nucleotide frequencies were T = 21.8, C = 23.9, A = 32.7, G = 21.6. The multiple alignment showing nucleotide variations among these haplotypes were presented in Appendix 1 – Table 7.

4.3.4.2. Genetic divergence

Out of the six haplotypes observed, 5 were from the group 1 (*Pd*). All the 4 samples from group 2 (*Pc*) populations possessed the same haplotype (Table 35). The mean pairwise distances between each population of group 1 (*Pd*) ranged from 0.0018 (between Kariangode and Valapattanam) to 0.0211 (between Valapattanam and Chaliyar). The mean pairwise distances between populations of the two groups - group 1 (*Pd*) and group 2 (*Pc*) - was found to be significantly higher (0.0498) (Table 36). Even though the 16S rDNA region possessed less number of nucleotide variations as expected, it showed significant genetic divergence between populations of group 1 (*Pd*) especially with Chaliyar and

other three populations supported the observations with ATPase COI and cyt *b* analysis indicated clear population structuring within group 1 and significant genetic divergence between the two groups.

4.3.4.3. Phylogenetic and geographical relationships among haplotypes

The phylogenetic reconstruction using the Neighbour joining (NJ) (Fig. 32) and Maximum parsimony (MP) (Fig. 33) methods for *P. denisonii* and *P. chalakkudiensis* 16S rDNA region showed the same topology. The two major groups (Group 1 and Group 2) found to be well separated with high bootstrap support in both analyses and supported the results observed with ATPase 8/6, COI and cyt *b* analyses. The populations of group 1 (*Pd*) other than Chaliyar were not found to be well separated as separate clades, but no haplotypes found to be shared between them indicated population structuring. No subgroups or clades were recognized in group 2 (*Pc*) again indicated the homogeneity of Chalakkudy and Periyar populations.

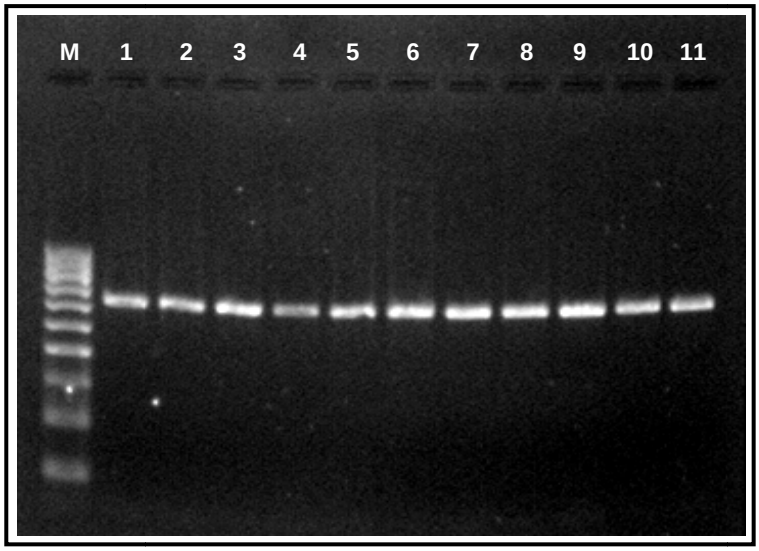


Fig. 30. Amplified products of 16S rDNA separated on 1.5% agarose gel. Lane 1 to 11 – eleven samples and M – 100 bp DNA ladder.

```
1   CCTGCCCAGT GACCATGAGT TAAACGGCCG CGGTATTTTG ACCGTGCGAA GGTAGCGCAA
61  TCACTTGTCT TTTAAATAAA GACCTGTATG AACGGTTAAA CGAGGGCTTA ACTGTCTCCC
121 ATCTCCAGTC AGTGAAATTG ATCTACCCGT GCAGAAGCGG GTATAATTAT ACAAGACGAG
181 AAGACCCTTT GGAGCTTAAG GTACAAAACT TAATCACGTC AAGCAACTCA ATAAAAAGCA
241 ATCCTTAAAC CTAGTGACAA ATAAGACCAT ACCTTCGGTT GGGGCGACCA AGGAGGAAAA
301 ACAAGCCTCC AAGTGGACTG GGGCAAACCA CCCTAAAACC AAGAGAGACA TCTCTAAGCC
361 ACAGAACATC TGACCATAAA TGATCCGATC AACCAAGATC GATCAACGAA CCAAGTTACC
421 CTAGGGATAA CAGCGCAATC CCCTCCAAGA GTCCATATCG ACGAGGGGGT TTACGACCTC
481 GATGTTGGAT CAGGACATCC TAATGGTGCA GCCGCTATTA AGGGTTCGTT TGTTCAACGA
541 TTAAAGTCCT ACGTGATCTG AG
```

Fig. 31. 16S rDNA - 562 bp partial sequence of a representative haplotype.

Table 35. Distribution of haplotypes of 16S rDNA among the populations of *P. denisonii* and *P. chalakkudiensis*.

Site	Haplotype (Representative Sample Name)						n_t	n_h
	A (CDR-s01)	**B** (KGD-s01)	**C** (VLP-s01)	**D** (CLR-s01)	**E** (CLR-s02)	**F** (CHD-s01)		
Pd								
CDR	2	0	0	0	0	0	2	1
KGD	0	2	0	0	0	0	2	1
VLP	0	0	2	0	0	0	2	1
CLR	0	0	0	1	1	0	2	2
Pc								
CHD	0	0	0	0	0	2	2	1*
PER	0	0	0	0	0	2	2	1*
Total	2	2	2	1	1	4	12	6

n_t - number of individuals analyzed per site; n_h - number of haplotypes per site.

* CHD and PER populations are having same haplotype

Table 36. Mean pairwise distances between populations of *P. denisonii* and *P. chalakkudiensis* based on partial sequence of 16S rDNA

	Pd				***Pc***	
	CDR	**KGD**	**VLP**	**CLR**	**CHD**	**PER**
Pd						
CDR						
KGD	0.0036					
VLP	0.0054	0.0018				
CLR	0.0192	0.0192	0.0211			
Pc			***0.0498***			
CHD	0.0427	0.0466	0.0485	0.0612		
PER	0.0427	0.0466	0.0485	0.0612	0.0018	

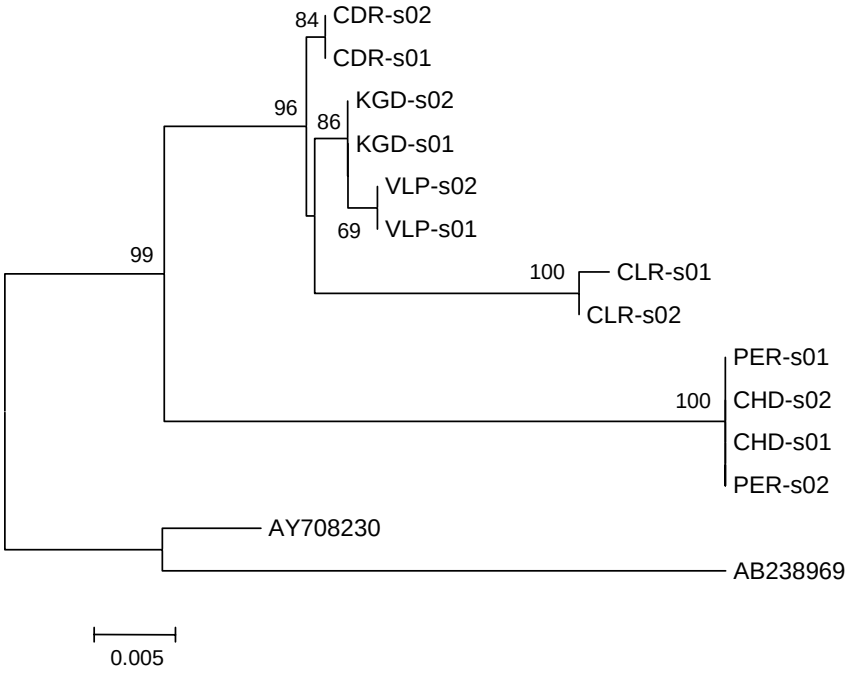


Fig. 32. Neighbour-joining tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial 16S rDNA. Numbers at nodes indicate the bootstrap values. AY708230 (*Puntius srilankensis*) and AB238969 (*Puntius ticto*) are from genbank and are included as outgroup species with original sequences for easy comparison.

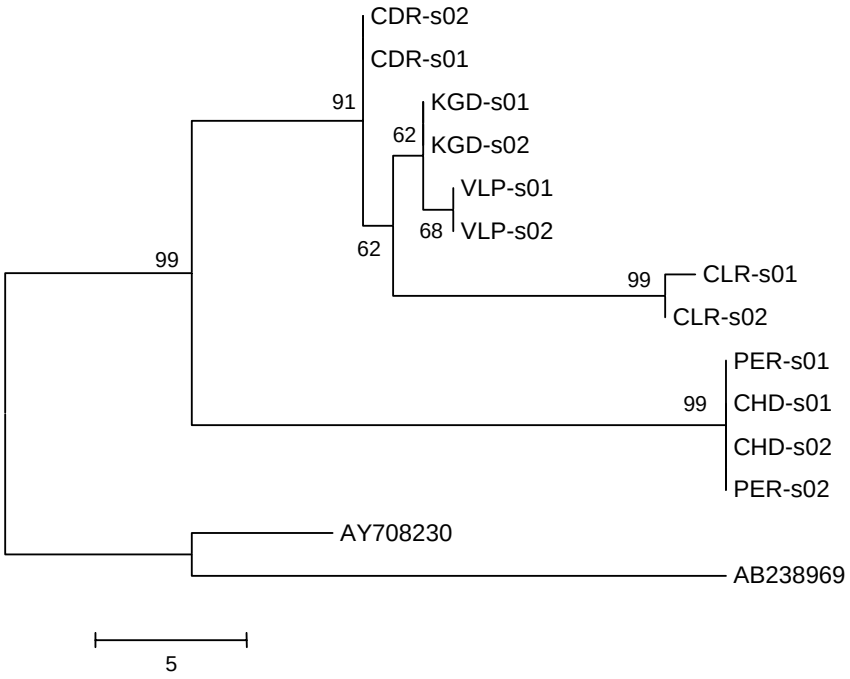


Fig. 33. Maximum parsimony tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the mitochondrial 16S rDNA. Numbers at nodes indicate the bootstrap values. AY708230 (*Puntius srilankensis*) and AB238969 (*Puntius ticto*) are from genbank and are included as outgroup species with original sequences for easy comparison.

4.3.5. Analysis of combined data of 2477 bp mtDNA region

A total of 2477 base pairs of mtDNA, including 521 bp of cytochrome oxidase subunit I, 552 bp of cytochrome *b*, 562 bp of 16S rDNA partial sequences and an 842 bp complete sequence of ATPase 8/6 genes, were aligned. Two samples each from each population which was sequenced for all the 5 genes were selected for this analysis.

4.3.5.1. Sequence variations

There were 10 different haplotypes out of 12 samples defined by 325 (13.12%) variable sites out of 2477 characters compared; in which 318 (12.84%) sites were found to be parsimony informative. The molecular characterization and phylogenetic information content of each gene and in the combined data set are presented in Table 37.

4.3.5.2. Genetic divergence

All the 8 samples of the group 1 (*Pd*) were 8 haplotypes. Group 2 (*Pc*) populations possessed two different haplotypes out of 4 samples, of which one was sharing between Chalakkudy and Periyar; and the other of Periyar. (Table 38). The mean pairwise distances between each population of group 1 (*Pd*) ranged from 0.0037 (between Kariangode and Valapattanam) to 0.0601 (between Kariangode and Chaliyar). The mean pairwise distances between populations of the two groups - group 1 (*Pd*) and group 2 (*Pc*) - was found to be 0.0998 (Table 39). All the observations were in concordance with that observed in above analyses. Clear genetic separation between *P. denisonii* and *P. chalakkudiensis* as two separate species was evident. The genetic homogeneity of specimens from Chalakkudy and Periyar was proved. The 4 different populations of *P. denisonii* from Chandragiri, Kariangode, Valapattanam and Chaliyar rivers exhibited significant genetic divergence.

4.3.5.3. Phylogenetic and geographical relationships among haplotypes

The analyses were performed according to the principles of Neighbour joining (NJ) (Fig. 34) and Maximum parsimony (MP) (Fig. 35) on the combined data set of 2477 bp of mitochondrial DNA region for *P. denisonii* and *P. chalakkudiensis*. Both the trees showed the same topology and supported the earlier results. The two major groups (Group 1 and Group 2) found to be well separated with high bootstrap support in both analyses and supported the results observed with ATPase 6/8, COI, cyt *b* and 16S rDNA analyses indicated clear genetic divergence between the two groups as two species *P. denisonii* and *P. chalakkudiensis*. In concordance with above observations, the group 1 (*Pd*) was well separated as 3 clades – clade 1, 2 and 3 - indicated clear population structuring with in *P. denisonii* populations. Whereas, in group 2 (*Pc*) formed a single cluster indicating genetic homogeneity between the two populations, Chalakkudy and Periyar.

Table 37. Molecular characterization and phylogenetic information content of the mitochondrial DNA regions in *P. denisonii* and *P. chalakkudiensis*.

	ATPase 8 (n)	ATPase 6 (n)	COI (n)	Cyt *b* (n)	16S (n)	Combined data set (n)
No. of bases analysed	165 (39)	684 (39)	521 (12)	552 (12)	562 (12)	2477 (12)
Nucleotide composition						
% A	37.3	31.8	26.3	31.5	32.7	31.1
% T	24.0	30.6	29.0	28.5	21.8	27.4
% G	11.1	12.6	16.8	12.1	21.6	15.3
% C	27.6	24.9	27.8	27.9	23.9	26.2
Invariable (monomorphic) sites	144 (87.27%)	582 (85.09%)	453 (86.95%)	452 (81.88%)	524 (93.24%)	2151 (86.84%)
Variable (polymorphic) sites	21 (12.73%)	102 (14.91%)	68 (13.05%)	100 (18.12%)	37 (6.58%)	325 (13.12%)
Singleton variable sites	2 (1.21%)	3 (0.44%)	01 (0.19%)	01 (0.18%)	1 (0.18%)	7 (0.28%)
Parsimony informative sites	19 (11.52%)	99 (14.47%)	67 (12.86%)	99 (17.93%)	36 (6.41%)	318 (12.84%)
Indel (insertion / deletion) sites	0	0	0	0	1 (0.18%)	1 (0.04%)
Ts/Tv	3.3	8.9	9.0	4.8	2.7	5.8

n = no. samples analyzed, Ts = transitions, Tv = transversions.

Table 38. Distribution of haplotypes of 2477 bp mtDNA region among the populations of *P. denisonii* and *P. chalakkudiensis*.

Site	Haplotype (Representative Sample Name)										n_t	n_h
	A (CDR-01)	**B** (CDR-02)	**C** (KGD-01)	**D** (KGD-02)	**E** (VLP-01)	**F** (VLP-02)	**G** (CLR-01)	**H** (CLR-02)	**I** (CHD-01)	**J** (PER-02)		
Pd												
CDR	1	1	0	0	0	0	0	0	0	0	2	2
KGD	0	0	1	1	0	0	0	0	0	0	2	2
VLP	0	0	0	0	1	1	0	0	0	0	2	2
CLR	0	0	0	0	0	0	1	1	0	0	2	2
Pc												
CHD	0	0	0	0	0	0	0	0	2	0	2	1
PER	0	0	0	0	0	0	0	0	1	1	2	2*
Total	1	1	1	1	1	1	1	1	3	1	12	10

n_t - number of individuals analyzed per site; n_h - number of haplotypes per site.

* one haplotype shared between CHD and PER.

Table 39. Mean pairwise distances between populations of *P. denisonii* and *P. chalakkudiensis* based on 2477 bp mtDNA region.

	Pd				***Pc***	
	CDR	**KGD**	**VLP**	**CLR**	**CHD**	**PER**
Pd						
CDR						
KGD	0.0276					
VLP	0.0276	0.0037				
CLR	0.0586	0.0601	0.0596			
Pc		***0.0998***				
CHD	0.1001	0.0984	0.0994	0.1010		
PER	0.1003	0.0986	0.0996	0.1013	0.0004	

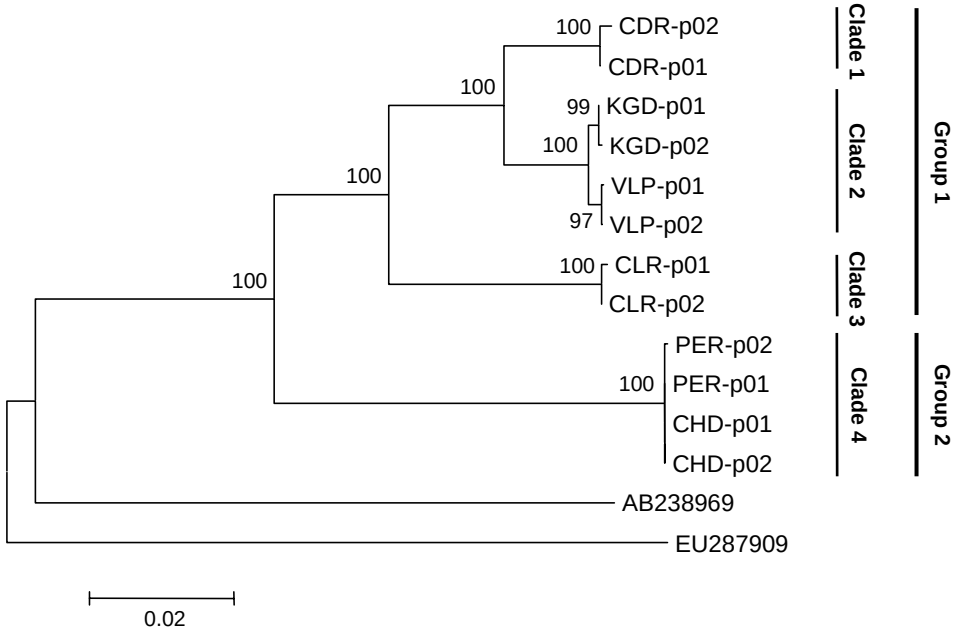


Fig. 34. Neighbour-joining tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the 2477 bp mtDNA region. Numbers at nodes indicate the bootstrap values. AB238969 (*Puntius ticto*) and EU287909 (*Puntius tetrazona*) are from genbank and are included as outgroup species.

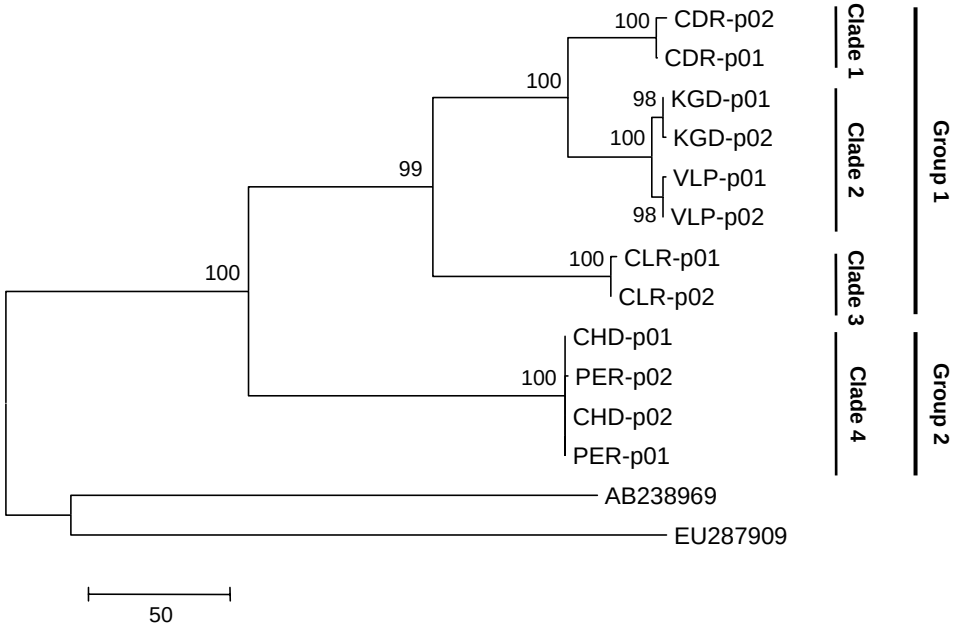


Fig. 35. Maximum parsimony tree of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype sequence variation of the 2477 bp mtDNA region. Numbers at nodes indicate the bootstrap values. AB238969 (*Puntius ticto*) and EU287909 (*Puntius tetrazona*) are from genbank and are included as outgroup species.

Chapter 5
DISCUSSION

To identify the geographical distribution and basic genetic characteristics of isolated populations is a basic need for the scientific management and conservation of a species. These isolated populations within a species, which are genetically distinct, are referred to as stocks. It is believed that a species may undergo micro-evolutionary processes and differentiate into genetically distinct sub-populations or stocks in the course of time, if reproductively and geographically isolated. The detection of stock differentiation would imply that the source groups comprise different stocks (Carvalho and Hauser, 1994) and should be treated as separate management units (Moritz, 1994). Failure to recognize or to account for stock complexity in management units has led to an erosion of spawning components, resulting in the loss of genetic diversity and other ecological consequences (Begg *et al*., 1999).

Morphometric and meristic studies; protein and DNA polymorphism studies etc., are the most popular approaches involved in stock identification. Morphological identification of stocks is the traditional approach in stock structure analysis in fishes and the use of modern tools in morphological identification make it more concrete. Multivariate statistical analyses like Principal Component Analysis (PCA) of morphometric characters are frequently used in stock differentiation. Although morphologic analysis is used as one of the tools for stock identification, it has some limitations; i.e., morphologic characters have a genetic basis but the environment may modify the expression of the character. The environmental component in morphologic characters is determined during the early larval stages when variation in temperature, salinity, oxygen, pH or food availability can modify the trait (Lindsey, 1988). This indicates the importance of genetic tools in stock structure studies which complements

phenotypic observations. The study of polymorphisms at DNA level has been made easy through Polymerase Chain Reaction (PCR) and sequencing. MtDNA sequence analysis is one of the most promising approaches used in modern days for genetic stock identification.

The present study provides a comprehensive phenotypic and genetic analysis of different geographically isolated populations of *P. denisonii* and *P. chalakkudiensis* in the Southern Region of Western Ghats. This study is the first attempt in determining population/stock structure and diversity of *P. denisonii* besides, genetic comparison of *P. denisonii* with *P. chalakkudiensis* to confirm the validity of the species *P. chalakkudiensis*.

5.1. Species validity of *P. chalakkudiensis*

Menon *et al*. (1999) described a new species *P. chalakkudiensis* from the River Chalakkudy of the Western Ghats. The species can be distinguished from all other *Puntius* species by its inferior mouth (terminal or subterminal in all other species) and a distinct black blotch on its dorsal fin from *P. denisonii* (Menon *et al*., 1999). In the present study, a series of phenotypic characters [18 out of 20 morphometric characters – other than L_S – examined (Table 4); and 6 out of 8 meristic characters (Table 13)] showed significant differences revealed by the univariate analysis of variance (ANOVA) to differentiate *P. denisonii* and *P. chalakkudiensis*. The multivariate analysis (Principal Component Analysis) using significant morphometric and meristic variables, leading to the formation of bivariate scatter plot of principal component 1 against component 2 indicated clear separation of the two species without any inter mixing individuals (Fig. 9 & 15). Structural variation observed on examination of gill rakers on the first gill arch (Fig. 13 & 14) of the two species also supported the distinct variation between the two species. The colour pattern (section 4.1. paragraph 1) observed in the adult specimens of *P. denisonii* (Fig. 1) and *P. chalakkudiensis* (Fig. 2) were also different. Moreover, the clearly distinguishable morphologic differences observed in the early juveniles (section 4.1. paragraph 3 and Fig. 8) of

both the species also emphasized the validity of *P. chalakkudiensis* as a different species from *P. denisonii*.

Molecular genetic information, including mtDNA sequences are very useful for clarifying species boundaries (Avise 1994, 2000). Molecular genetic analysis carried out in the present study using DNA sequences of five mitochondrial genes *viz.*, cytochrome oxidase subunit I, cytochrome *b*, 16S rDNA, ATPase 8 and ATPase 6 had shown unambiguous genetic divergence between these two species. The mean pairwise distances observed between these two species (4.98% with 16S rDNA, 9.03% with COI, 11.16% with ATPase 8/6 and 14.68% with cyt *b* sequences) were similar as reported for many other teleost species, including several species from the Western Ghats; Salini (2007) found a mean genetic divergence value ranging 3.43% to 14.66% among selected cyprinid species from the Western Ghats based on 16S rDNA sequences; Bose (2007) reported pairwise divergence ranging 6.28% to 8.87%, 13.48% to 16.0% and 15.95% to 18.77% based on 16S rDNA, COI and cyt *b* sequences respectively among three cyprinid species of the genus *Garra* from the Western Ghats; and Vineeth (2006) observed pairwise divergence ranging 4.79% to 6.84%, and 16.30% to 21.60% based on 16S rDNA, and COI sequences respectively among three Indian cichlid species. Garcia *et al.* (2000) reported 4.5 to 28% sequence divergence based on 324 bp of mitochondrial cyt *b* region in annual killifishes of the genus *Cynolebias*. Mean sequence divergence for all pairwise comparisons between *P. denisonii* and *P. chalakkudiensis* and the pairwise F_{ST} values based on ATPase 8/6 analysis in the present study were all significant enough to differentiate them as two distinct and separate species.

The two species *P. denisonii* and *P. chalakkudiensis* were clearly genetically distinct, as follows. 1) The amount of pairwise sequence divergence within each of the two species was much less than that between the two (Tables 23, 31, 34, 36 and 39); 2) the degree of sequence difference between the two species was comparable to that existing between many of the teleost species; and

3) in both the MP and NJ trees with all the mtDNA regions analyzed, the haplotypes of the two species were each monophyletic with 99 to 100% bootstrap values. These results, together with their difference in colouration, morphometric and meristic characters, indicated that *P. denisonii* and *P. chalakkudiensis* represent two distinct species.

According to Menon *et al*., (1999), *P. chalakkudiensis* is limited to the Chalakkudy River, while *P. denisonii* is distributed in several west flowing rivers of Kerala. In the present study, samples of *P. chalakkudiensis* collected from Chalakkudy River and the samples from Periyar River showed similarity in morphology. They also possessed only a single haplotype with all the four genes other than cyt *b* (2 haplotypes observed), showing little genetic divergence between them, which indicated that the particular species distributed in both Chalakkudy and Periyar Rivers are genetically similar. Thus, on the basis of the morphologic and genetic data presented in this study, *P. chalakkudiensis* can be considered as a valid species distributed in both Chalakkudy and Periyar Rivers. The species can be easily distinguished from *P. denisonii*, (specimens from Chandragiri, Kariangode, Valapattanam and Chaliyar Rivers) morphologically and by genetic tools as discussed in this study.

5.2. Morphologic differentiation between populations of *P. denisonii* and *P. chalakkudiensis*

Morphometric analysis showed a clear morphologic heterogeneity existing among populations of *P. denisonii* and between populations of *P. chalakkudiensis*. As mentioned earlier (section 4.1. paragraph 1), the size, body shape and colour differences between *P. denisonii* and *P. chalakkudiensis* were readily noticeable. The maximum total length recorded in the present study was 13.5 and 17.6 cm in *P. denisonii* and *P. chalakkudiensis* respectively (section 4.1. paragraph 1), which is much higher than the earlier report on *P. chalakkudiensis* (Menon *et al*., 1999). The total length recorded in the present study is the highest recorded for *P. chalakkudiensis* from the Western Ghats of India. Pharyngeal

teeth formula for both species (5,3,2-2,3,5) recorded in the present study (section 4.2.2. paragraph 2) is also a new finding. Pharyngeal teeth formula recorded earlier for *P. denisonii* was 4,3,2-2,3,4 by the British ichthyologist Francis Day in his classical works on the "Fishes of Malabar" and "Fishes of India". There was no report available earlier on pharyngeal teeth formula of *P. chalakkudiensis*.

It is expected that the morphometric traits show very high plasticity but are also influenced by environmental effects. Therefore, the differentiation of stocks solely based on the morphometric traits may be biased (especially, if they are not corrected for the size variation). Nevertheless their importance in the stock differentiation should not be overlooked as these traits have evolutionary importance and an indication of adaptability to their environment. There are many well documented studies on stock structure in fishes using morphological traits. Jayasankar *et al.* (2004) observed morphological homogeneity in Mackerals from three coasts of Peninsular India supported by genetic observations. Significant morphologic heterogeneity was observed in different regional populations of silver perch, *Leiopotherapon plumbeus* in the Philippines by Quilang *et al*. (2007). Elvira and Almodovar (2000) in *Acipenser sturio*; Smith *et al.* (2002) in black (*Allocytus niger*) and smooth oreos (*Pseudocyttus maculates*); Turan *et al.* (2005) in African catfish (*Clarias gariepinus*); Murphy *et al.* (2007) in river sturgeons (*Scaphirhynchus spp*.) and Nowak *et al.* (2008) in common gudgeon (*Gobio gobio*) are some other classical studies on stock structure in fishes using morphological traits.

5.2.1. Stock differentiation in *P. denisonii*

Both univariate and the multivariate analyses has clearly demonstrated an intraspecific morphological variation among the populations of *P. denisonii* from four different River systems of the Western Ghats of India. The variations observed are related to measurements of head like HD, HW, L_{PO}, MW besides, measurements like L_{CP}, L_{PRD}, and FBAN. Measurements of head were the most discriminating variable in this study. Accordingly, Chaliyar population possesses

the deepest head and lowest L_{PO} whereas, the Chandragiri population possesses the highest mean L_H (Table 5).

The coefficient of variation observed in the present study was comparatively lower ranging from 4.80% (CDR) to 5.38% (VLP). In fishes, the coefficients of variation within populations are usually far greater than 10% (Carvalho, 1993). The lower coefficient of variation indicates minimal or very low intra-population variation. Similar results were obtained by Mamuris *et al*. (1998) in the seven populations of red mullet (*Mullus barbatus*) and by Quilang *et al*. (2007) in four populations of Silver perch (*Leiopotherapon plumbeus*).

The morphological heterogeneity among populations of *P. denisonii* was further confirmed by the Principal Component Analysis. The bivariate scatter plots represented that the specimens from Kariangode and Valapattanam Rivers were in overlapping, while the specimens from Chandragiri and Chaliyar Rivers were in separate clusters. This clustering suggests closer morphological similarity between specimens from Kariangode and Valapattanam Rivers whereas, Chandragiri and Chaliyar populations were morphologically well distinct.

As mentioned earlier, environmental factors may affect morphological characters. In some studies, environmental conditions, particularly temperature, which prevail during some sensitive developmental stages, have been shown to have the greatest influence in morphological characters (Hubbs, 1922; Taning, 1952). Lindsey (1954) has explained the effect of temperature on morphological characters based on the study in Paradise fish (*Macropodus opercularis*). The eggs were collected from one pair of Paradise fish, reared them at different constant temperatures and noted the effects on some meristic characters. Some young ones were also transferred from low to high temperatures after varying periods to determine the duration of sensitivity of the morphologic characters to temperature. Different morphologic traits showed different reactions to sustained and also to transferred temperatures. The above study suggested that, the

differences in physico-chemical characteristics of the water in different isolated river systems may also explain the morphological variation in *P. denisonii*. However, in the present study, it may be difficult to point out which of the several biotic and abiotic factors contributed to this. But, the differentiation observed in the genetic stock structure pointed out that the morphological heterogeneity observed in *P. denisonii* is not probably results from phenotypic plasticity.

5.2.2. Stock differentiation in *P. chalakkudiensis*

Intraspecific morphological variation between Chalakkudy and Periyar populations of *P. chalakkudiensis* has been clearly demonstrated with the univariate and the multivariate analyses. The variations in the present study mainly concerns related to measures of L_{PRO}, HW, L_{PRV}, L_{O}, L_{PD}, and L_{PO}. As observed in *P. denisonii* the coefficient of variation observed in this also was comparatively lower (5.36% in CHD and 5.51% in PER) indicated minimal or very low intrapopulation variation. The bivariate scatter plot based on component 1 and component 2 resulted from the multivariate analysis showed separate clusters suggesting morphological heterogeneity between specimens from Chalakkudy and Periyar Rivers.

Intraspecific morphological heterogeneity observed in the present study may explain the effect of phenotypic plasticity as the genetic data do not allow the rejection of the null hypothesis of panmixia for *P. chalakkudiensis*. The physico-chemical parameters could have an effect on the development of *P. chalakkudiensis*, although studies on reproductive as well as foraging behavior especially during its larval stage are yet to be studied. The present study has shown the efficiency of using multivariate techniques in the population structure of *P. chalakkudiensis*. However, the true reasons for the observed morphologic variability needs further studies using appropriate sampling design and should include water quality parameters. The use of geometric morphometrics truss

network analysis may be tried in addition to the traditional method in future studies.

5.3. Genetic variation and distribution of *P. denisonii* and *P. chalakkudiensis*

Mitochondrial DNA is usually highly polymorphic than nuclear DNA. Polymorphism should be distinguished from heterogeneity. The former measures the multiplicity of genotypes within a sample whereas in the latter, the probability that two genotypes drawn at random from the sample will be different. Heterogeneity for haploid alleles is analogous to heterozygosity for diploid nuclear alleles (Carr and Crutcher, 1998). The present study has demonstrated that direct sequencing of variable mtDNA fragments has the potential to provide useful insights into the genetic diversity, divergence and genealogy of *P. denisonii* and *P. chalakkudiensis* populations.

Out of the five genes analysed in the present study, all the four protein coding genes *viz.*, cyt *b*, COI, ATPase 8 & 6, exhibited significantly higher genetic divergence than 16S rDNA gene as expected with higher rate of evolution exhibited by those genes. The cyt *b* (18.12% polymorphic sites) region appeared to be the most variable marker, followed by the ATPase 8/6 (14.61% polymorphic sites) and the COI (13.05% polymorphic sites). The alignment of these protein coding gene sequences was straight forward, as indels were not found; but indels were observed in 16S sequence alignments. Rarity of indels (Mardulyn and Whitfield 1999) was expected in this region and this was one of the reasons for the use of COI as a gene for 'barcoding' animals including teleost species (Hebert *et al*., 2003a).

Statistical analysis showed clear differences in molecular variation of *P. denisonii* populations and to *P. chalakkudiensis*. Nucleotide and haplotype diversity indices with ATPase gene in *P. denisonii* were consistent and are similar to many other freshwater fish species, including *Hypseleotris compressa*

(McGlashan and Hughes, 2001a), *Craterocephalus stercusmuscarum* (McGlashan and Hughes, 2001b) and common carp, *Cyprinus carpio* (Thai *et al*., 2004). The sequence divergence observed between different riverine populations of *P. denisonii* was considerably higher indicating a strong genetic structuring. The Chaliyar population was notably divergent from the other three populations (Valapattanam, Kariangode and Chandragiri), followed by Chandragiri. These observations were also in concordance to the observations made in several other endemic cyprinids like *Acrossocheilus paradoxus* in Taiwan (Wang *et al*., 2000); *Ladigesocypris ghigii* an endangered freshwater fish in Greece (Mamuris *et al*., 2005) and in a fresh water shrimp *Caridina indistincta* using COI and 16S rDNA sequences (Page and Hughes, 2007).

P. chalakkudiensis showed little or no genetic divergence at the intra- and inter population level. These patterns of genetic variation among populations of *P. chalakkudiensis* do not match traditional expectations for a freshwater fish, and it would appear that there has been some - at least historical - connectivity/mixing between populations inhabiting Chalakkudy and Periyar Rivers. These habitats (rivers) of *P. chalakkudiensis* populations are closely existing and having connections at the lower stretches. Even though this species exhibited a genetic homogeneity at intra-specific level, a clear morphological heterogeneity between the two populations (Chalakkudy and Periyar) was evident as discussed earlier (section 5.2.2). Many teleost populations exhibit morphological changes depending on the environment, still are genetically homogenous as reported in Brazilian populations of yellowtail snapper (*Ocyurus chrysurus*, Lutjanidae) (Vasconcellos *et al*., 2008) and in three different riverine populations of *Liza abu* a mugilid species from Turkey (Turan *et al*., 2004b). Baker *et al*. (2004b) also reported shell morphology of Australian freshwater mussels, *Alathyria jacksoni* and *Velesunio spp.* vary with local conditions which do not match the mitochondrial DNA based phylogenetic data. More detailed study has to be done to find out the actual reason behind the reduced genetic diversity within this species.

5.4. Population structure and divergence pattern in *P. denisonii* and *P. chalakkudiensis*

Mitochondrial DNA is sensitive to population genetic structure, is likely to result from the higher evolutionary rate and smaller effective population size. MtDNA heterogeneity is more sensitive to fluctuations in population size ("bottlenecks" and "founder events") than is nuclear heterozygosity (Wilson *et al*., 1985). Animal mitochondrial genomes are haploid and are almost always inherited maternally, decreasing the effective population size of mtDNA markers to 1/4 that of nuclear markers (Avise, 1994). This increases the stochastic effects of random mating on allele frequencies relative to this effect on nuclear DNA markers such as allozymes (Karl *et al.*, 1992).

Freshwater fish are expected to display greater levels of genetic differentiation and population subdivision than marine species due to the isolating nature of river systems and small effective population sizes (Ward *et al*., 1994; McGlashan and Hughes, 2001a). Due to the rugged landscape, most rivers of the Western Ghats region are separated from each other by mountain ridges at medium and high elevations, where these species (*P. denisonii* and *P. chalakkudiensis*) were distributed. Generally, neighbouring rivers are not connected to each other and substantial genetic subdivision among populations was expected. In the present study, the mtDNA data reveal that the levels of genetic differentiation among populations of *P. denisonii* between drainages were consistent with these expectations. Similar reports by Muneer (2005) on *Horabagrus brachysoma* and Musammilu (2008) on *Gonoproktopterus curmuca*, from the Western Ghats further support this view. In accordance to these genetic data, morphological comparisons among populations of *P. denisonii* revealed significant heterogeneity. The spatial structure of the populations in the present study, and the physical barriers among them, may partly explain the genetic differentiation observed.

This study clearly demonstrated that there is considerable genetic differentiation between different riverine populations of *P. denisonii.* A significant proportion of the variation in mtDNA haplotypes resulted from differences between populations, allowing a null hypothesis of panmixia in *P. denisonii* needs to be rejected. Considerable genetic divergence (maximum 9.45% between Chandragiri and Chaliyar populations with cyt *b*) was observed among populations of *P. denisonii*; whereas, comparatively low level of nucleotide diversity (maximum 0.0024 with ATPase 8/6 in Valapattanam population) was detected among the haplotypes within each population for all the mitochondrial DNA genes analyzed. 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 distribution analysis further supports a population expansion, for populations in the Chandragiri and the Valapattanam (Fig. 12). In such cases, the rapid growth of a population leads to the retention of new mutations especially in mitochondrial DNA sequences that is known to evolve several times faster than nuclear DNA (Brown *et al.*, 1979). Such patterns of diversity and mismatch equilibrium strongly suggest a historical influence on the genetic structure of *P. denisonii* populations as estimated by analysis of haplotype frequencies. At the same time, in the case of *P. chalakkudiensis*, surprisingly little divergence was observed within and between populations. The least genetic variability exhibited within the populations of *P. chalakkudiensis* has involved significant founder and population bottleneck events leading to localized loss of genetic variation.

Analysis of molecular variance, *F*-statistics and phylogenetic analysis indicate marked genetic structure in *P. denisonii* populations at the inter-regional scale. Population differentiation depends directly on gene flow. Species with higher capabilities of dispersal and migration across geographic barriers do not show much population differentiation whereas other species with lower dispersal and migration capabilities exhibits significant differentiation among population

over time. In the present study, the *Nm* (a maximum of 0.223 between Valapattanam and Kariangode populations) values observed were considerably lower indicating that gene flow among spatially distant populations of *P. denisonii* is very restricted. In fact, a value of *Nm* much lower than one is interpreted as evidence of gene flow insufficient to prevent differentiation due to genetic drift (Slatkin, 1987; Mills and Allendorf, 1996). However, a haplotype was found to be sharing between Valapattanam and Kariangode populations with COI analysis may represent occasional or even very rare migrants between these localities though the overall level of genetic exchange must be below that required to homogenise populations, or there may be a barrier to gene flow between these populations. It may also be noted that phylogenetic analysis for all the mitochondrial genes analyzed revealed separate clades of haplotypes representing each populations. This suggests some degree of reproductive isolation of *P. denisonii* populations leading geographical structuring with limited or no gene flow between populations.

The extent of genetic differentiation observed between *P. denisonii* populations is related to degree of geographic separation between river systems from where the samples were collected. The AMOVA analyses showed that populations from different regions are differentiated from each other (Table 25). A significant Φ_{SC} value indicated that a major amount of the variance could be ascribed to among population divergence. The pair wise F_{ST} (Table 26) values reached the same conclusion, which suggests that genetic differentiation can be largely explained by the limited dispersal of *P. denisonii* among regions due to geographical barriers. The degree of differentiation between populations is obviously associated with geographical distance. Space is not the only parameter that determines genetic population structure and gene flow. Instead, landscape features between populations can influence dispersal rates and migration success. Therefore, the divergence and genetic differentiation between the populations of major river systems in the Western Ghats can be explained with a model of isolation by distance (Slatkin, 1993). The reconstructed phylogenetic trees with

all the mtDNA genes also supported the high differentiation among geographical regions.

Out of the four populations studied in *P. denisonii*, the Chaliyar was the most separated (geographically; ~85km far from the nearest Valapattanam) population exhibited maximum pairwise differences from the other three whereas, Valapattanam and Kariangode populations exhibited least pairwise difference and were geographically closer (separated only by about ~40km). The samples from geographically proximal or neighboring localities are more similar genetically than samples from more geographically distant localities. According to Sokal and Oden (1978), this pattern is consistent with an isolation-by-distance model where migration of genes is inversely related to geographic distance. The pairwise difference values observed with all the genes analyzed in the present study were all concordant. The pattern of genetic divergence exhibited decreased gene flow among spatially distant populations supported the view of isolation due to distance. However, the least separated (~40km) populations (Valapattanam and Kariangode) were also exhibited a significant genetic differentiation (F_{ST} value 0.6919 at $p<0.005$). This suggested that, the geographic isolation due to the physical barriers among the rivers of the Western Ghats due to its rugged landscape is contributing more to the restricted gene flow between the river systems.

The species has shown a marked decline in abundance and population in recent years (Gopi, 2000; Kurup and Radhakrishnan, 2006a,b). Intensive collection efforts and fishing from the wild to supply the demand for the species in aquarium trade and the lack of commercial hatchery or captive breeding technology together contributed to the decline of abundance and population. 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. Populations with smaller effective population sizes also tend towards rapid fixation of haplotypes, which may be the reason for the reduced number of haplotypes found in *P. chalakkudiensis* population and higher number of private

haplotypes found in *P. denisonii* populations. As a conservation measure, Government of Kerala State has come out with a ban on fishing the species *P. denisonii* from the wild during its peak breeding season (G.O.(Rt)No.633/08/F&PD, dated, 5-11-2008). Recently, there were also news paper reports about the experimental success of captive breeding in *P. denisonii* by a fish hobbyist from Chennai, Tamil Nadu and also from College of Fisheries, Panangad, Kerala.

The fact that high genetic variation observed in *P. denisonii* may strongly influence the spatial genetic analysis cannot be neglected. As the occurrence of the species was found to be patchy, to determine the level of gene flow between streams within the catchments is of great importance and can be taken up as a natural extension of this study. Molecular markers with high variability (like microsatellites) may require bigger sampling numbers in order to "dilute" the genetic variability found. Mitochondrial markers such as cyt *b*, COI and ATPase regions revealed significant level of genetic variability in *P. denisonii* as observed in the present study can be utilized further.

In the case of *P. chalakkudiensis*, maybe some structuring could be found if higher levels of sampling could be acquired and nuclear DNA markers with high variability such as polymorphic microsatellite loci or mtDNA control region employed. The morphometric analysis revealed significant divergence between populations in this study is pointing out that possibility. Although none of the available information either on *P. denisonii* or *P. chalakkudiensis* biology seems to explain as such the divergent pattern observed, the incompleteness of current knowledge does not allow safely excluding or holding any of the above mentioned hypotheses.

These findings lead to the conclusion that it is important to study further the biology and ecology of these species, as it is likely that the life histories do exhibit some major differences important for the interpretation of these data.

5.5. Phylogenetic relationships and demographic evolution of *P. denisonii* and *P. chalakkudiensis* populations

Phylogenies from molecular data are often computed by pair-wise genetic distance based (numerical) methods like Neighbor Joining (NJ) tree, with branch lengths that are proportional to the amount of divergence. NJ tree making method is a widely use distance–clustering algorithm that allows unequal rates of divergence among lineages. Phylogenetic trees are also made based on "discrete methods" that operate directly on sequences like the Maximum Parsimony (MP) tree. MP chooses the tree (or trees) that require the fewest evolutionary changes (*i.e.* it makes trees from sequences exhibiting smallest evolutionary changes). In variant characters (bases) those that have the same state in all taxon are obviously unimportant (phylogenetically uninformative) and are ignored by the MP method. Both the numerical (NJ) and discrete (MP) tree making methods are used in the analysis in most of the species (Hall, 2004) as in the present study. Both analyses were carried out separately for the nucleotide data generated from each mitochondrial DNA genes such as ATPase 8/6, COI, cyt *b* and 16S rDNA. A combined analysis was also carried out by combining nucleotide data of all the genes together. The results of parsimony and neighbor joining analyses based on both type of data (combined and independent) were generated during this study: 1) established the validity of *P. chalakkudiensis* as a separate and distinct species from *P. denisonii*; 2) estimated the validity of subpopulations within *P. denisonii*; and 3) clarified the relationships of populations of *P. denisonii* with that of *P. chalakkudiensis*.

A monophyletic pattern was observed with all the analyses. All haplotypes of the two species separated clearly as two major distinct groups showed the phylogenetic separation between *P. denisonii* and *P. chalakkudiensis*. A distinct pattern could be visualized within group1 (group formed by the clusters of *P. denisonii* populations); from all the mtDNA genes analysed

reflected a clear region wise population sub-structuring within the species *P. denisonii*. Three major clades observed under group1 showed the sub-structuring. All members of Chandragiri population formed the clade 1 and clade 3 of Chaliyar population; whereas, Valapattanam and Kariangode with more similarity together formed clade 2. Even though these two populations formed single clade, some degree of isolation could be observed with all mitochondrial genes analysed other than COI. Overall, there was no evidence to unite or homogenize these populations; well support for some degree of structuring existing among populations of *P. denisonii* based on nucleotide data and geographical nature of the site. However, no distinct sub-structuring was observed for *P. chalakkudiensis*.

One of the fundamental questions in conservation genetics is to determine the historical demography of a population. It is needed to distinguish between small populations that naturally have limited genetic variation versus those that have reduced genetic variation due to a recent severe reduction in population size; on the other hand, a population may be large in current census size yet small in effective population size due to a past bottleneck (Crandall *et al*., 1999; Turner *et al*., 2002). In both the cases, influence of past demography on current genetic variability can have important management implications in terms of the genetic stability of populations and the potential impact of inbreeding depression on population viability (Vila *et al*., 2003). Mismatch distribution of pairwise nucleotide differences together with neutrality tests can be a source of data on recent evolutionary history by inferring past population sizes (Rogers and Harpeding, 1992). Episodes of population growth or decline leave a characteristic signature in the distribution of nucleotide site differences between pairs of individuals. In histograms showing the distribution of relative frequencies and pairwise mismatch distributions the shape of curves will depend on past demographic events (Rogers and Harpeding, 1992).

Observed distribution of pairwise differences between ATPase 8/6 haplotypes of *P. denisonii* fitted the distribution predicted for a population that has experienced genetic bottleneck events, with subsequent population expansion. Low nucleotide and high haplotype diversity values (Table 24) observed in the present study also pointed to a bottleneck followed by a sudden increase in population size (Grant and Bowen, 1998). This pattern of mtDNA variation has been observed in another highly endangered cyprinid species, *Anaecypris hispanica*, which is endemic to the Guadiana River basin in the Iberian Peninsula (Alves *et al*., 2001). The relationship between *h* (haplotype diversity) and π (nucleotide diversity) is informative about population demographic history. Taking into account the levels of diversity detected by these two indices, Grant and Bowen (1998) defined four categories of fish, using either mtDNA sequence data or RFLP: 1) low *h* and π are interpreted as recent population bottleneck or founder event by single or a few mtDNA lineages; 2) high *h* and low π are interpreted as population bottleneck followed by rapid population growth and accumulation of mutations; 3) low *h* and high π are interpreted as divergence between geographically subdivided populations and 4) high *h* and π are interpreted as large stable population with long evolutionary history of secondary contact between differentiated lineages.

Haplotype and nucleotide diversity values (high *h* and low π), the mismatch distribution pattern (poisson-shaped curve, Fig. 21) and the results of expansion model tested by Tajima's *D* (-ve values) together suggested a history of genetic bottleneck events, with subsequent population expansion especially with Chandragiri and Valapattanam populations of *P. denisonii*. But, the mismatch distributions were not fit to an expansion model curve for Chaliyar and Kariangode populations, suggesting a history of long-term population stasis and a lack of clear expansion. However, the Chaliyar population exhibited the highest raggedness index (R) (Table 27) pointing towards the stability of the population. Clear picture of this may be obtained by increasing the sample size further.

In the case of *P. chalakkudiensis* mismatch distribution could not be estimated as the variance was little. Genetic variability of these populations might have had been influenced by exploitation or still not have recovered from severe bottlenecks. The low *h* (haplotype diversity) and π (nucleotide diversity) values exhibited by *P. chalakkudiensis* populations also indicated a recent population bottleneck or founder event (Grant and Bowen, 1998). But, the question remains, how such related species have been so influenced in such different ways by climatic changes and historic events. The answer continues to hide probably behind unknown differences in the biology, ecology and behaviour of these two species. It is also important to consider the limitation of mtDNA based stock recognition when considering the case of *P. chalakkudiensis*, where no genetic divergence could be observed between populations, in contradiction to that observed in *P. denisonii* in the present study. Assuming an average rate of mtDNA evolution of 2% per million years (Brown, 2008); even with complete isolation, populations that have colonized habitats since the end of Pleistocene will show little divergence. The habitats of two populations of *P. chalakkudiensis*, Chalakkudy and Periyar Rivers are closely existing and having connections at the lower stretches. Hence, a combined approach using comparatively faster evolving mtDNA control region and highly variable nuclear DNA regions such as microsatellites with adequate sample size could be more appropriate to get a realistic picture of stock discrimination and demographic evolution in this species.

The climate and sea-level changes of the last 18,000 years (last glacial maximum, 'LGM') wrought great changes on the landscapes of the world in general. The most recent glaciation and the subsequent warming and sea-level rise (17,000–6500 years ago; Neal and Stock, 1986) had significant effects on the intra-specific genetic structuring of many terrestrial and aquatic fauna throughout the world (Hewitt, 2000). Based on this study and several other studies carried out in several fish species of Western Ghats (Muneer, 2005; Johnson *et al*., 2007, Musammilu, 2008) in which significant intra-specific genetic structuring were

reported; it may be suggested that the LMG or glaciations prior to the LMG may have had strong impacts on the diversity of aquatic fauna of this region. But, the divergence time has to be estimated for these species to come into a conclusion about these views.

Fully freshwater animals are excellent model organisms to study the relationship between biota and landscape because of their dispersal limitations due to geographical barriers made by intervening dry land or salt water. A powerful method for inferring biological history, both evolutionary and biogeographic, is the phylogeographic analysis of DNA sequences, and subsequent dating of genetic divergences. This can then be related to current distributions and known geological and geomorphological history to understand their relationships.

5.6. Implications for fisheries management and conservation

In freshwater ecosystems, if populations are depleted in one region through fishing activities then it is unlikely that they will be recolonised from adjacent geographic regions by immigration over the short to medium timescale. If overexploitation occurs within a very limited area on a local scale, then targeted populations are likely to be replenished from other proximate sub-populations. This suggests that a useful management strategy is one that protects at least some subpopulations within each region in which the species is found. Only such a strategy will be likely to assist in the survival of the species and preservation of intraspecific genetic diversity. Management measures that attempt to limit fishing pressure across a wide geographic area will reduce the genetic diversity of the entire regional population. Such a strategy can have disastrous consequences if control of exploitation is ineffective or quotas are set at the wrong level. 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. denisonii*, *P. chalakkudiensis* and other freshwater fish populations of the Western Ghats with similar characteristics.

P. denisonii and *P. chalakkudiensis* are the most exploited species of the Western Ghats from the wild to meet the high demand from domestic and international aquarium trade. Both these species are normally considered together in terms of fishery. Both the species are traded under the same trade name/ common name as 'Denison's Barb' or 'Red Lined Torpedo fish' and has recently become India's largest exported ornamental fish (Anon, 2008). As reported by Sekharan and Ramachandran (2006) 'Red Lined Torpedo fish' is the species in demand and is being exported in consistent numbers on regular basis from India. Both *P. denisonii* and *P. chalakkudiensis* are significant components of recreational and/or commercial fisheries, and the issue of population structure/gene flow is critical to their conservation and management.

The results of the present study strongly indicated that, although both species appear similar in some respects, management strategies should be developed for each species separately. Analysis of *P. denisonii* revealed a high level of genetic variation with four independent stocks inhabiting the four geographically isolated habitats of the Western Ghats. 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 loci (Moritz, 1994). Under this criterion, the four riverine populations of *P. denisonii*, *viz*. Chandragiri, Kariangode, Valapattanam and Chaliyar, can be proposed as distinct MUs, which require separate monitoring and management. In future, a conservation policy based on these MUs is advisable.

Secondly, the highly divergent clades (clade 1&3; Chandragiri & Chaliyar populations) detected in *P. denisonii* suggests that this stock (Chaliyar) has been

evolving independently from the other stocks for a considerable length of time. Thus from a management perspective, the data suggests that it would be unwise to mix these stocks in propagation-assisted river-ranching programs as there is a potential for loss of genetic diversity of distinct stocks or out breeding depression to occur or one of the stocks could go extinct. Out-breeding depression is essentially where the resulting hybrid offspring of genetically divergent parents have a lower fitness than both parental stocks. This may be due to the disruption of co-evolved gene complexes or by driving locally adapted phenotypes from their optimum.

In contrast, the analysis for *P. chalakkudiensis* shows a very different picture. The lack of genetic divergence found in this species between the Chalakkudy and Periyar populations is consistent with a "one stock" management whereas, the morphometric analysis revealed significant divergence between these populations in this study indicates the possibility of some structuring if higher levels of sampling could be acquired. Secondly, the extremely low level of genetic variation present for this species suggests that the population as a whole has undergone a dramatic reduction in size in the recent evolutionary past. As a consequence, the reduced level of variation may compromise the 'genetic health' of the population (it is generally accepted that low variation is associated with low evolutionary potential) and thus may affect its long term viability. Effort should be directed to ensuring the level of genetic variation that currently exists for this species is not eroded further. Hence, conservation actions should concentrate on both species separately, especially as the species *P. chalakkudiensis* appears to have a smaller population size than *P. denisonii*. A propagation assisted-rehabilitation of the natural populations of *P. chalakkudiensis* is advisable. Habitat protection for natural breeding should be aimed in all cases.

These results together with observation of decline in abundance of the species along the rivers of Western Ghats in recent years (Gopi, 2000; Kurup and Radhakrishnan, 2006a,b) reinforce the need for more studies/proposals for

conservation of these habitats against overexploitation. More studies on the biology, behaviour and population genetics to determine the diversity and level of gene flow between streams within the catchments of the two species, and especially on *P. denisonii* (as its distribution is found to be patchy) are needed for better understanding on the differences revealed in the present study.

Chapter **6**

SUMMARY AND CONCLUSION

Puntius denisonii (Day) is brilliantly coloured ornamental teleost belonging to family Cyprinidae, endemic to selected west flowing rivers originating from the Western Ghats. This species has acquired high demand in domestic as well as international aquarium trade and is being exploited from the wild in large quantities. It is categorized as "endangered" based on latest IUCN criteria, due to restricted distribution, loss of habitat, over exploitation, destructive fishing practices and aquarium trade (Anon., 1998). The species is also having taxonomic ambiguity with another similarly looking species *Puntius chalakkudiensis*, described from Chalakkudy River of the Western Ghats. It is apparent that, understanding of correct taxonomic identity, degree of population/stock structuring and evolutionary story is essential for the judicious exploitation and conservation of fishery resources. Therefore, the present work was undertaken to study the genetic diversity and population/stock structure in *P. denisonii* from different geographically isolated environments and to validate the taxonomic status of *P. chalakkudiensis* using morphologic (morphometric and meristic analysis) and genetic (mtDNA analysis) tools.

- Specimens for this study were collected from selected six geographically isolated river systems of the Western Ghats *viz.*, Periyar, Chalakkudy, Chaliyar, Valapattanam, Kariangode and Chandragiri, which includes the habitats of *P. denisonii* and *P. chalakkudiensis*.

- A close morphological observation revealed noticeable differences in size, body shape and colour, between the specimens of *P. denisonii* and *P. chalakkudiensis*.

- Body of *P. chalakkudiensis* specimens was moderately deeper (especially in larger specimens) with slightly rounded snout and comparatively more greenish dorsum. A prominent black bloch on the dorsal fin were also present in *P. chalakkudiensis*. In *P. denisonii*, body was comparatively slender and streamlined with a pointed snout. The black longitudinal stripe was more clearly defined in *P. denisonii* than in *P. chalakkudiensis*.

- Chalakkudy River was described as type locality of *P. chalakkudiensis* (Menon *et al*., 1999). However, in the present study, specimens collected from Periyar River resembled *P. chalakkudiensis* in their colour characteristics.

- Shape and colour pattern of early juveniles of *P. denisonii* and *P. chalakkudiensis* were also distinguishable between each other and from their adults too. The prominent red or scarlet horizontal band observed in adult specimens was not prominent in early juveniles, whereas three prominent black vertical bands were observed. These vertical bands on sides of body were broader and more prominent in early juveniles of *P. chalakkudiensis* and that of *P. denisonii* were comparatively narrow and diffuse.

- Microscopic examination of pharyngeal teeth revealed the presence of an additional pair of teeth in the inner most rows on pharyngeal bones in *P. denisonii* than earlier report of Day (1865). Arrangement of pharyngeal teeth for both species were observed and recorded the formula as 5,3,2-2,3,5.

- Univariate and multivariate analysis using 8 meristic characters clearly indicated that both *P. denisonii* and *P. chalakkudiensis* are distinct species. Structural variation observed on the microscopic examination of gill rakers on the first gill arch of the two species (slender and villiform in

P. denisonii and comparatively stout with blunt tip in *P. chalakkudiensis*) further supported the differentiation between them.

- In morphometrics of *P. denisonii* and *P. chalakkudiensis*, 21 (including L_S) reliably measurable morphometric characters were selected. Univariate analysis of variance (ANOVA) and multivariate, Principal Component Analysis (PCA) clearly indicated that both *P. denisonii* and *P. chalakkudiensis* are distinct. Besides, all the fish samples from different sites were also differed significantly in both analyses, leading to the rejection of the null hypothesis of 'no heterogeneity in fish morphology among riverine populations' of these species.

- In *P. denisonii*, the samples collected from Kariangode River and Valapattanam River showed similarity; whereas, the samples from Chandragiri River and Chaliyar River were morphologically distinct from others. In *P. chalakkudiensis*, the samples from Chalakkudy River and Periyar River were morphologically distinct.

- Measurements of head, like HD, HW, L_{PO}, MW and other measures like L_{CP}, L_{PRD} & FBAN were the most discriminating variables observed among populations of *P. denisonii* in this study. Whereas in *P. chalakkudiensis* this variation mainly concerns measures related to that of L_{PRO}, HW, L_{PRV}, L_O, L_{PD} and L_{PO}.

- The CV and CVp values obtained for the morphometric characters with in each population were generally low for both *P. denisonii* (CV, 1.30 – 9.49%; CVp, 4.80 - 5.38%) and *P. chalakkudiensis* (CV, 1.87 – 8.75%; CVp, 5.36 - 5.51%). This indicated minimal or very low intra-population variation in morphometric characters.

- The application of molecular genetic markers (mtDNA regions such as ATPase 8/6, COI, cyt *b* and 16S) in the present study has clearly indicated their usefulness in discriminating the species and populations/stocks within species. MtDNA have performed as reliable taxonomic tool in confirming both *P. denisonii* and *P. chalakkudiensis* as distinct and separate species thereby resolving the taxonomic ambiguity.

- In mtDNA (ATPase 8/6, COI, cyt *b* and 16S) analysis, the genetic divergent value obtained in the present study between *P. denisonii* and *P. chalakkudiensis* was significantly higher than that within each of the two species. The degree of sequence divergence between the two species (4.98% with 16S rDNA, 9.03% with COI, 11.16% with ATPase 8/6 and 14.68% with cyt *b* sequences) was also comparable to that existing between many of the teleost species. Pairwise F_{ST} values between the populations of *P. denisonii* and *P. chalakkudiensis* were extremely higher (ranging from 0.9875 to 0.9973; $P<0.005$). Moreover, in both the MP and NJ trees with all the mtDNA regions analyzed, the haplotypes of the two species were each monophyletic with 99 to 100% bootstrap values supported the separation of *P. denisonii* and *P. chalakkudiensis* as two distinct species.

- ATPase 8/6 gene was sequenced from 39 samples including *P. denisonii* (25 samples) and *P. chalakkudiensis* (14 samples) from six different geographic locations. Out of 25 samples of *P. denisonii*, 6 each from Chandragiri & Chaliyar; 5 from Kariangode; and 8 from Valapattanam. Whereas, in *P. chalakkudiensis*, 9 samples from Chalakkudy and 5 from Periyar.

- Sequence comparison of 842 bp complete mitochondrial ATPase 8/6 gene, revealed 15 (14 of *P. denisonii* and 1 of *P. chalakkudiensis*) different haplotypes out of 39 individuals, defined by 123 (14.61%)

divergent nucleotide sites. No haplotypes were found to be sharing among any populations of *P. denisonii* indicated significant genetic separation between these populations. Whereas, the genetic homogeneity of Chalakkudy and Periyar populations of *P. chalakkudiensis* was revealed by the presence of same single haplotype in all the 14 samples examined.

- The mean pairwise distances estimated by the Kimura 2-parameter method among populations of *P. denisonii* ranged from 0.0056 (between Kariangode and Valapattanam) to 0.0622 (between Kariangode and Chaliyar) and a high proportion of the total variance (95.97%) attributed to differences between the geographically isolated populations (F_{ST}=0.9597, P <0.001) with AMOVA indicated clear population structuring within the species. Moreover, significantly lower *Nm* values (0.006 – 0.223) observed among populations of *P. denisonii* suggested, little or no effective gene flow between populations, further supported the population structuring within the species.

- Pairwise F_{ST} values were extremely high for all comparisons among populations of *P. denisonii*, (ranging from 0.6919 to 0.9889; P<0.005) indicates high genetic differentiation among geographically isolated populations of this species.

- Comparatively high values for haplotype diversity (*h*) within populations, ranging from 0.8667 (Chandragiri population) to 0.3333 (Chaliyar population) and low nucleotide diversity (π) values ranging from 0.0005 (Kariangode population) to 0.0024 (Valapattanam population) was observed in *P. denisonii*. Whereas, both *h* and π were found to be 0.0 (only 1 haplotype found out of 14 individuals) in *P. chalakkudiensis*.

- The mismatch distributions for the Chandragiri and Valapattanam populations were appeared close to an estimated Poisson-shaped curve

expected for a population that has undergone a sudden expansion in effective population size; whereas, that for Chaliyar and Kariangode populations were not fit to an expansion model curve. Tajima's D-values were negative but not significant. Comparatively high raggedness index (R) value observed (0.667) for Chaliyar population signifies the stability of the population.

- A total of 12 samples including *P. denisonii* and *P. chalakkudiensis* from six different geographic locations were selected for COI, cyt *b* and 16S rDNA analyses. Out of twelve, 8 samples were that of *P. denisonii* and 4 of *P. chalakkudiensis* from four (Chandragiri, Kariangode, Valapattanam and Chaliyar) and two (Chalakkudy and Periyar) different geographic locations respectively (2 samples each from each site).

- A 521 bp partial sequence of COI gene revealed 7 different haplotypes out of 12 samples, defined by 68 (13.05%) divergent nucleotide sites. All 4 samples of *P. chalakkudiensis* populations possessed the same haplotype; whereas, six different haplotypes were observed out of eight samples from four populations of *P. denisonii*. Specific haplotypes were observed for Chandragiri and Chaliyar populations and at same time a haplotype was found to be sharing between Kariangode and Valapattanam populations. The mean pairwise distance among populations (0.0019 to 0.0821) was comparatively higher, indicating significant genetic differentiation among geographically isolated populations of *P. denisonii*.

- Analysis of 552 bp region of mtDNA cyt *b* gene from 12 individuals including *P. denisonii* and *P. chalakkudiensis* from six different geographic locations identified a haplotype each from each location. Out of the 2 haplotypes observed for *P. chalakkudiensis* populations, one was shared between Chalakkudy and Periyar; and the other of Periyar. All the four populations of *P. denisonii* possessed 1 each population specific

haplotypes. However, the mean pairwise distances among populations of *P. denisonii* (0.0055 - 0.0945) was higher than that observed within *P. chalakkudiensis* (0.0018).

- 16S rDNA analysis was also carried out with 562 bp region sequenced from 12 individuals including *P. denisonii* and *P. chalakkudiensis* from six different geographic locations. A total of 6 different haplotypes were observed, defined by 37 (6.58%) polymorphic nucleotide sites. All 4 samples of *P. chalakkudiensis* populations possessed the same haplotype; whereas, in *P. denisonii*, population specific haplotypes were observed. The mean pairwise distances observed among populations of *P. denisonii* (0.0018 - 0.0211) with 16S rDNA analysis was comparatively low than that observed with other mtDNA regions analysed in this study.

- A collective analysis was also carried out by combining sequence information of all the 5 genes sequenced in the above 12 samples. There were 10 different haplotypes out of 12 samples, defined by 325 (13.12%) variable sites out of 2477 characters compared. All the 8 samples of the *P. denisonii* were 8 haplotypes. *P. chalakkudiensis* populations possessed two different haplotypes out of 4 samples, of which one was sharing between Chalakkudy and Periyar; and the other of Periyar. The mean pairwise distances among populations of *P. denisonii* ranged 0.0037 - 0.0601; whereas, that within *P. chalakkudiensis* was 0.0004.

- Phylogenies were constructed using the Neighbour joining (NJ) and Maximum parsimony (MP) methods with the sequence information of each gene separately and also using combined data. Both trees showed almost the similar topology with all analysis and indicated that two major groups of *P. denisonii* and *P. chalakkudiensis* could be recognized. All four subpopulations of *P. denisonii* formed a monophyletic group further divided into three major clades with high support values (bootstrap value

97 – 100%) indicated clear population structuring within this species. No subgroups or clades were recognized within *P. chalakkudiensis* indicated genetic homogeneity existing within this species.

Conclusion

In general, the present study unambiguously confirmed the existence of two distinct species *P. denisonii* and *P. chalakkudiensis* distributed in selected river systems of Southern region of Western Ghats. Clear genetic and morphologic separation between *P. denisonii* and *P. chalakkudiensis* as two separate species was evident. The genetic homogeneity of *P. chalakkudiensis* specimens from Chalakkudy and Periyar was proved; however, it exhibited morphological diversity. The 4 different populations of *P. denisonii* from Chandragiri, Kariangode, Valapattanam and Chaliyar rivers exhibited significant genetic and morphologic divergence indicated clear population structuring within species. In *P. denisonii*, geographic isolation due to the physical barriers among the rivers of the Western Ghats is likely to be the factor that contributed to the restricted gene flow between the river systems. The pattern of genetic diversity (high *h* and low π) and results of mismatch distribution analysis together supported a population expansion after a low effective population size in this species. Whereas, in the case of *P. chalakkudiensis*, surprisingly little divergence were observed within and between populations might be due to inbreeding as a result of over-exploitation. The study emphasizes the need for a conservation policy based on MUs (Management Units) in the species *P. denisonii i.e.,* stock-specific propagation assisted-rehabilitation and building up of brood stock. Captive breeding and river ranching of the natural populations of *P. chalakkudiensis* needs consideration and in all cases, habitat protection for natural breeding should be aimed.

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Appendices

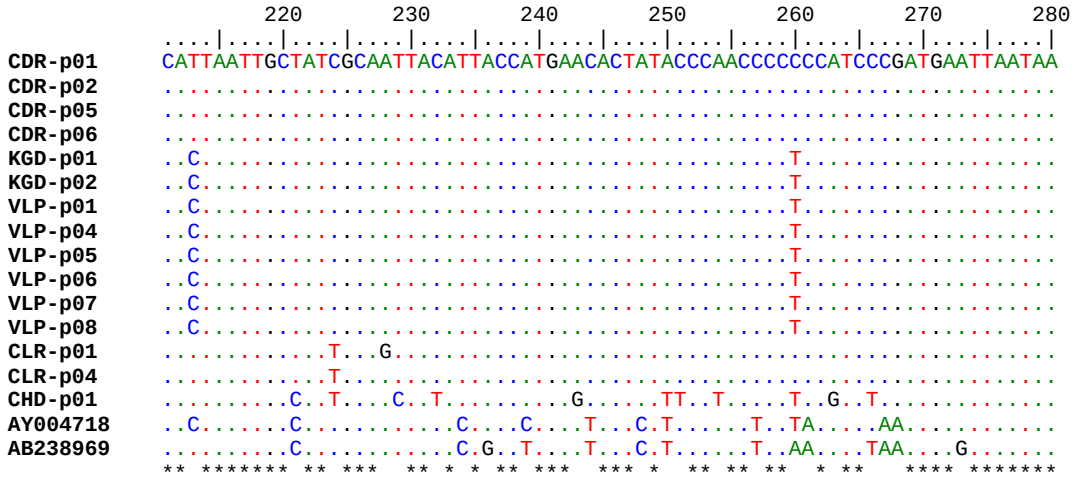
Sequence alignments:

Table. 1. Multiple alignment of the nucleotide sequences of the *ATPase 8/6* genes of 15 haplotypes of *P. denisonii* from six locations and outgroup species. AY004718 (*Puntius conchonius*) and AB238969 (*Puntius ticto*) are from genbank and are included as outgroup species with original sequences for easy comparison.

```
                    10        20        30        40        50        60        70
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
CDR-p01     ATGCCACAACTAAACCCAGACCCGTGATTTGCAATTTTAACATTCTCCTGACTAGTTTTTTTAACCATTA
CDR-p02     ...........................................................G..................
CDR-p05     ...........................................................................
CDR-p06     ...................................................T.......................
KGD-p01     ...........................................................................
KGD-p02     ........G..................................................................
VLP-p01     ...........................................................................
VLP-p04     ...........................................................................
VLP-p05     ...........................................................................
VLP-p06     ...........................................................................
VLP-p07     ...........................................................................
VLP-p08     ...........................................................................
CLR-p01     ..............T..C.....................................................C.
CLR-p04     ..............T..C.....................................................C.
CHD-p01     ................C.....A.................T.........A.........T.....
AY004718    ..........T.....T.G...T......TT.......GT...T..T...T........C.........
AB238969    ..........G.....C.G...T......TT.......GT...T......T....C...C.........
            ******** ** ** ** * *** ******  *******  *** ** **  ** * *** *** *** *

                    80        90       100       110       120       130       140
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
CDR-p01     TCCCAACTAAAACTTTAAACCACATCTCACCAAACGAACCAGCCCCAGTAAGTGCCGAAAAACACAAAAC
CDR-p02     ...........................................................................
CDR-p05     ...........................................................................
CDR-p06     ...........................................................................
KGD-p01     .T.........................................AA.............................
KGD-p02     .T.........................................AA.............................
VLP-p01     ...........................................AA.............................
VLP-p04     ...........................................AA.............................
VLP-p05     ...........................................AA.............................
VLP-p06     ...........................................AA.............................
VLP-p07     ...........................................AA.............................
VLP-p08     ...........................................AA.............................
CLR-p01     ..........T.C.....................G...A.....................................
CLR-p04     ..........T.C.....................G...A.....................................
CHD-p01     .T..........CC..........AT......T...T.A.....................................
AY004718    ....C.......T............A.............A..T.........A.....................
AB238969    .T..C.......T............A.............A..T.....................................
            * ** *******   ***********  ****** ** * *  * ********** **************

                   150       160       170       180       190       200       210
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
CDR-p01     TGAAGCCTGAGACTGACCATGATAGCAAACTTCTTTGATCAATTTGCAAGCCCATATTTCCTAGGAATTC
CDR-p02     ...........................................................................
CDR-p05     ...........................................................................
CDR-p06     ...........................................................................
KGD-p01     ...........................................................................
KGD-p02     ...........................................................................
VLP-p01     ...........................................................................
VLP-p04     ...........................................................................
VLP-p05     ...........................................................................
VLP-p06     ...........................................................................
VLP-p07     ...........................................................................
VLP-p08     ...........................................................................
CLR-p01     ...................................C.......................................C.
CLR-p04     ...................................C.......................................C.
CHD-p01     ....T.....................G.....................T.....C.....................
AY004718    ....T.....A.............T..G.........C.....C.........G....A.....C.....
AB238969    ....T.....A.T...........T..G.........C.....C..........TC.A.........C.
            **** ***** * ************ ** ********* ***** ***** ** *  * ********* *
```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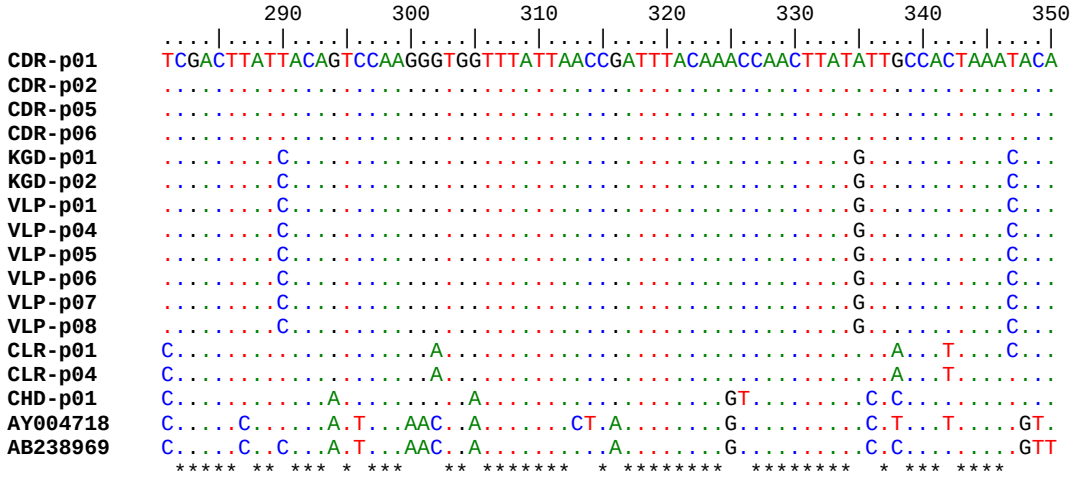
CDR-p01 CATTAATTGCTATCGCAATTACATTACCATGAACACTATACCCAACCCCCCCATCCCGATGAATTAATAA
CDR-p02
CDR-p05
CDR-p06
KGD-p01
KGD-p02
VLP-p01
VLP-p04
VLP-p05
VLP-p06
VLP-p07
VLP-p08
CLR-p01
CLR-p04
CHD-p01
AY004718
AB238969


290 300 310 320 330 340 350
CDR-p01 TCGACTTATTACAGTCCAAGGGTGGTTTATTAACCGATTTACAAACCAACTTATATTGCCACTAAATACA
CDR-p02
CDR-p05
CDR-p06
KGD-p01
KGD-p02
VLP-p01
VLP-p04
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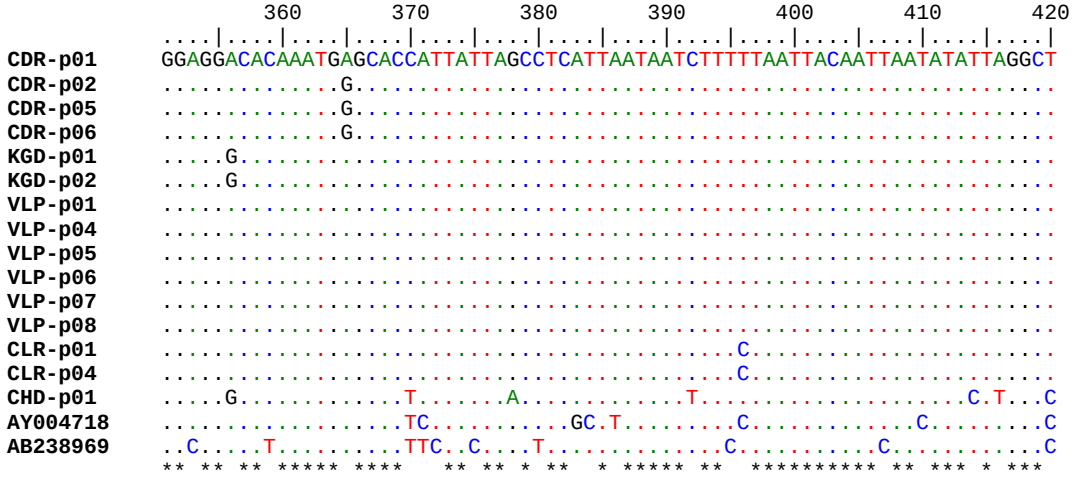
CLR-p01
CLR-p04
CHD-p01
AY004718
AB238969


360 370 380 390 400 410 420
CDR-p01 GGAGGACACAAATGAGCACCATTATTAGCCTCATTAATAATCTTTTTAATTACAATTAATATATTAGGCT
CDR-p02
CDR-p05
CDR-p06
KGD-p01
KGD-p02
VLP-p01
VLP-p04
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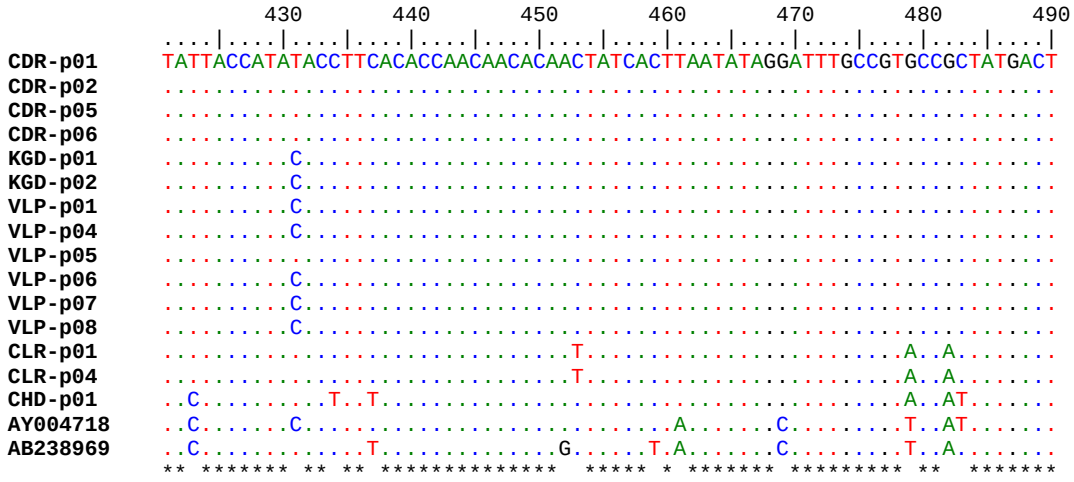
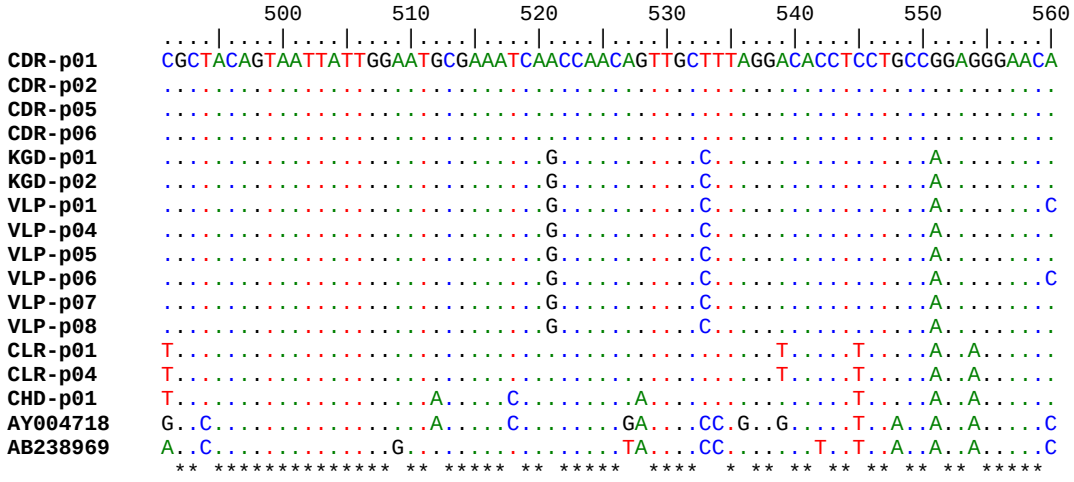
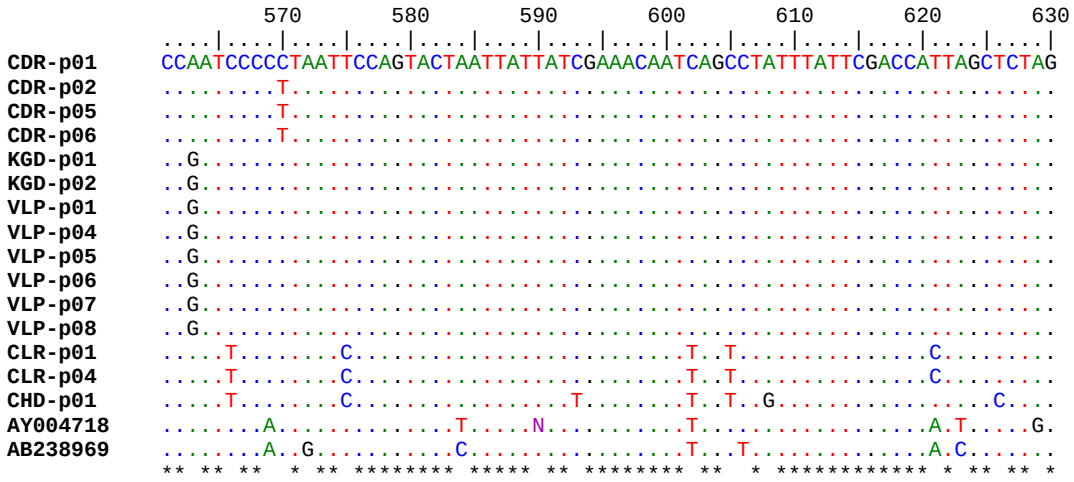
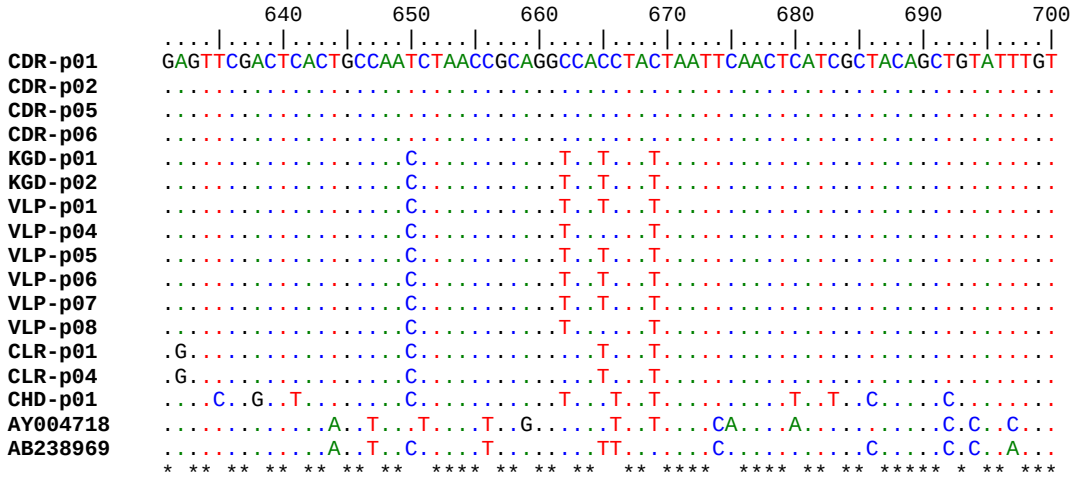
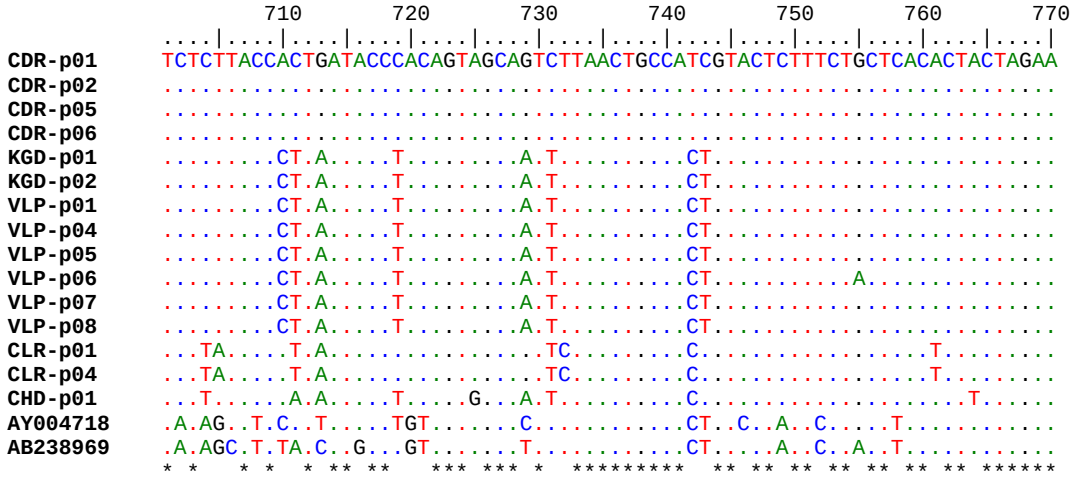
CLR-p04
CHD-p01
AY004718
AB238969


430 440 450 460 470 480 490
CDR-p01 TATTACCATATACCTTCACACCAACAACACAACTATCACTTAATATAGGATTTGCCGTGCCGCTATGACT
CDR-p02
CDR-p05
CDR-p06
KGD-p01
KGD-p02
VLP-p01
VLP-p04
VLP-p05
VLP-p06
VLP-p07
VLP-p08
CLR-p01
CLR-p04
CHD-p01
AY004718
AB238969


500 510 520 530 540 550 560
CDR-p01 CGCTACAGTAATTATTGGAATGCGAAATCAACCAACAGTTGCTTTAGGACACCTCCTGCCGGAGGGAACA
CDR-p02
CDR-p05
CDR-p06
KGD-p01
KGD-p02
VLP-p01
VLP-p04
VLP-p05
VLP-p06
VLP-p07
VLP-p08
CLR-p01
CLR-p04
CHD-p01
AY004718
AB238969


570 580 590 600 610 620 630
CDR-p01 CCAATCCCCCTAATTCCAGTACTAATTATTATCGAAACAATCAGCCTATTTATTCGACCATTAGCTCTAG
CDR-p02
CDR-p05
CDR-p06
KGD-p01
KGD-p02
VLP-p01
VLP-p04
VLP-p05
VLP-p06
VLP-p07
VLP-p08
CLR-p01
CLR-p04
CHD-p01
AY004718
AB238969


640 650 660 670 680 690 700
CDR-p01 GAGTTCGACTCACTGCCAATCTAACCGCAGGCCACCTACTAATTCAACTCATCGCTACAGCTGTATTTGT
CDR-p02
CDR-p05
CDR-p06
KGD-p01
KGD-p02
VLP-p01
VLP-p04
VLP-p05
VLP-p06
VLP-p07
VLP-p08
CLR-p01
CLR-p04
CHD-p01
AY004718
AB238969


710 720 730 740 750 760 770
CDR-p01 TCTCTTACCACTGATACCCACAGTAGCAGTCTTAACTGCCATCGTACTCTTTCTGCTCACACTACTAGAA
CDR-p02
CDR-p05
CDR-p06
KGD-p01
KGD-p02
VLP-p01
VLP-p04
VLP-p05
VLP-p06
VLP-p07
VLP-p08
CLR-p01
CLR-p04
CHD-p01
AY004718
AB238969

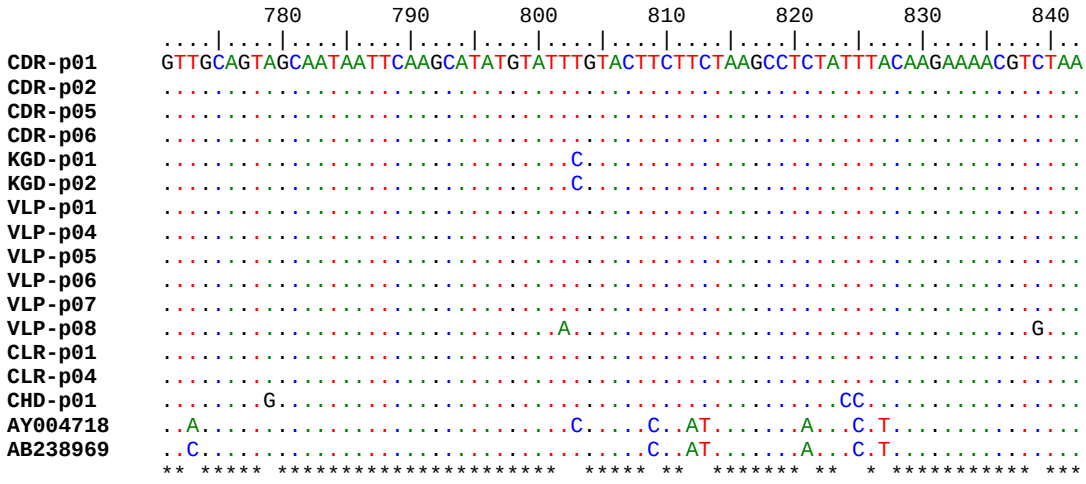

780 790 800 810 820 830 840
CDR-p01 GTTGCAGTAGCAATAATTCAAGCATATGTATTTGTACTTCTTCTAAGCCTCTATTTACAAGAAAACGTCTAA
CDR-p02
CDR-p05
CDR-p06
KGD-p01
KGD-p02
VLP-p01
VLP-p04
VLP-p05
VLP-p06
VLP-p07
VLP-p08
CLR-p01
CLR-p04
CHD-p01
AY004718
AB238969

Table. 2. Multiple alignment of amino acid residues translated from the nucleotide sequences of the *ATPase 8* (residues from 1 to 54) and *ATPase 6* (residues from 55 to 281) genes of different haplotypes of *P. denisonii*.

```
                10        20        30        40        50        60        70
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
CDR-p01    MPQLNPDPWFAILTFSWLVFLTIIPTKTLNHISPNEPAPVSAEKHKTEAWDWPWMMANFFDQFASPYFLG
CDR-p02    ..............................................................................
CDR-p05    ..............................................................................
CDR-p06    ..............................................................................
KGD-p01    ..............................N...............................................
KGD-p02    ..............................N...............................................
VLP-p01    ..............................N...............................................
VLP-p04    ..............................N...............................................
VLP-p05    ..............................N...............................................
VLP-p06    ..............................N...............................................
VLP-p07    ..............................N...............................................
VLP-p08    ..............................N...............................................
CLR-p01    ........................I.........T...........................................
CLR-p04    ........................I.........T...........................................
CHD-p01    ................I..I.........M...LT..........S........S.....................
PER-p01    ................I..I.........M...LT..........S........S.....................
           ****************** ** ***** **** ***  ********** ******** ************

                80        90       100       110       120       130       140
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CDR-p01    IPLIAIAITLPWTLYPTPPSRWINNRLITVQGWFINRFTNQLMLPLNTGGHKWAPLLASLMIFLITINML
CDR-p02    ..............................................................................
CDR-p05    ..............................................................................
CDR-p06    ..............................................................................
KGD-p01    ..............................................................................
KGD-p02    ..............................................................................
VLP-p01    ..............................................................................
VLP-p04    ..............................................................................
VLP-p05    ..............................................................................
VLP-p06    ..............................................................................
VLP-p07    ..............................................................................
VLP-p08    ..............................................................................
CLR-p01    .......V......................................................................
CLR-p04    ..............................................................................
CHD-p01    .......TM...A.F.............I.........S.............L..T.....................
PER-p01    .......TM...A.F.............I.........S.............L..T.....................
           *******  *** * ************** ********* ************** ** ************

                150       160       170       180       190       200       210
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CDR-p01    GLLPYTFTPTTQLSLNMGFAVPLWLATVIIGMRNQPTVALGHLLPEGTPIPLIPVLIIIETISLFIRPLA
CDR-p02    ..............................................................................
CDR-p05    ..............................................................................
CDR-p06    ..............................................................................
KGD-p01    ..............................................................................
KGD-p02    ..............................................................................
VLP-p01    ..............................................................................
VLP-p04    ..............................................................................
VLP-p05    ..............................................................................
VLP-p06    ..............................................................................
VLP-p07    ..............................................................................
VLP-p08    ..............................................................................
CLR-p01    ..............................................................................
CLR-p04    ..............................................................................
CHD-p01    ..............................I...............................................
PER-p01    ..............................I...............................................
           ********************************** ********************************
```

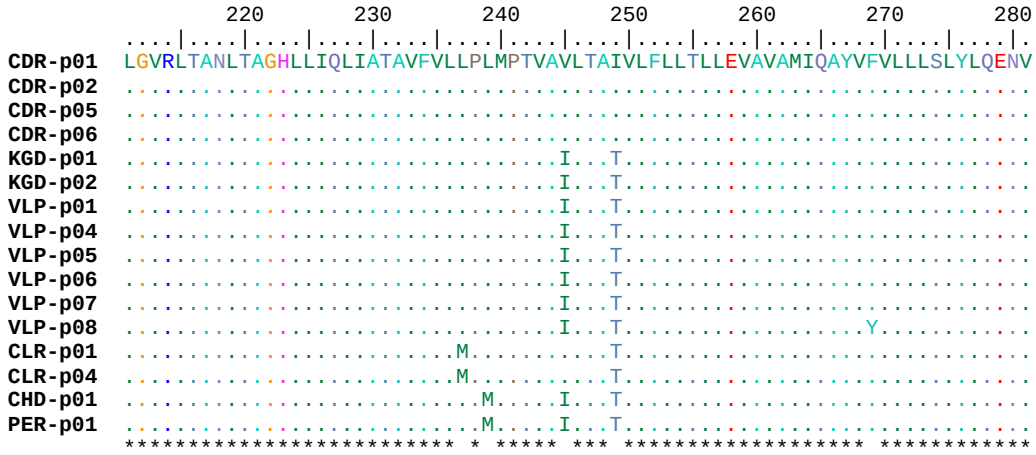
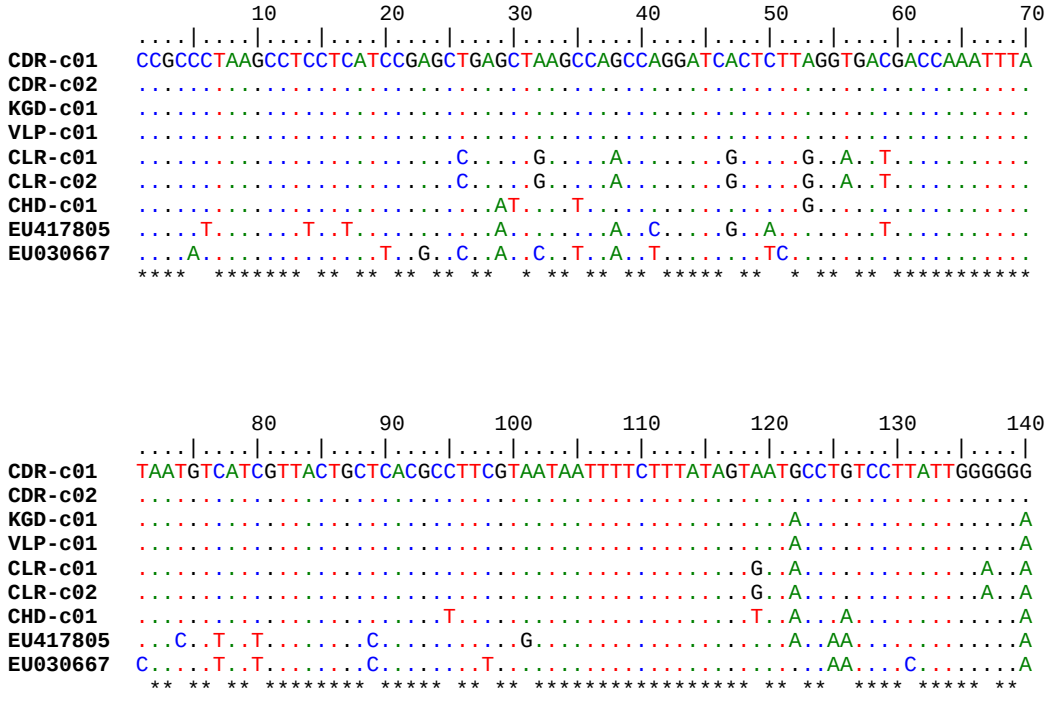
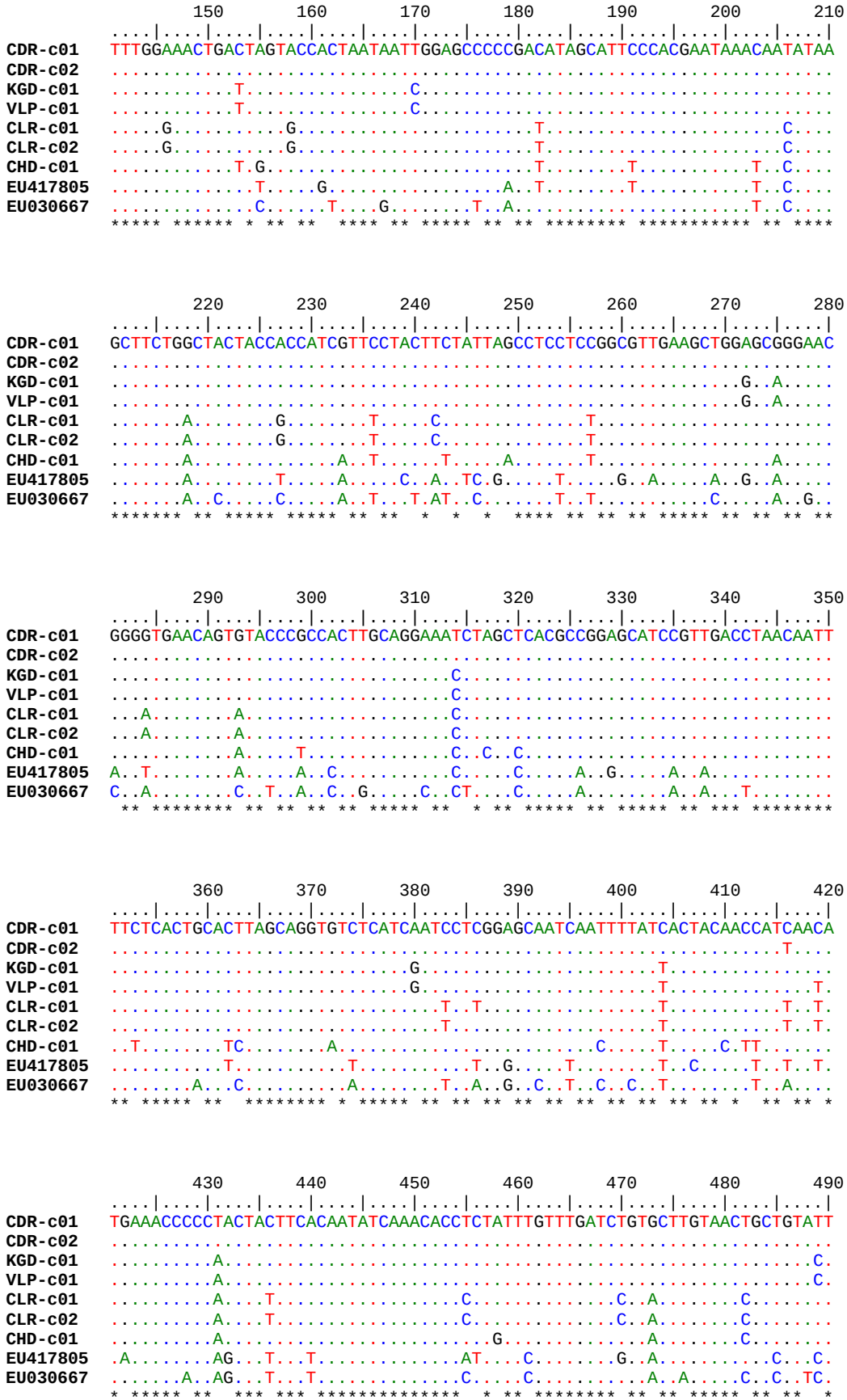


Table 3. Multiple alignment of the nucleotide sequences of the *COI* gene of 7 haplotypes from six locations and outgroup species. EU417805 (*Puntius sarana*) and EU030667 (*Labeo bata*) are from genbank and are included as outgroup species with original sequences for easy comparison.




150 160 170 180 190 200 210
CDR-c01 TTTGGAAACTGACTAGTACCACTAATAATTGGAGCCCCCGACATAGCATTCCCACGAATAAACAATATAA
CDR-c02
KGD-c01
VLP-c01
CLR-c01
CLR-c02
CHD-c01
EU417805
EU030667
220 230 240 250 260 270 280
CDR-c01 GCTTCTGGCTACTACCACCATCGTTCCTACTTCTATTAGCCTCCTCCGGCGTTGAAGCTGGAGCGGGAAC
CDR-c02
KGD-c01
VLP-c01
CLR-c01
CLR-c02
CHD-c01
EU417805
EU030667
290 300 310 320 330 340 350
CDR-c01 GGGGTGAACAGTGTACCCGCCACTTGCAGGAAATCTAGCTCACGCCGGAGCATCCGTTGACCTAACAATT
CDR-c02
KGD-c01
VLP-c01
CLR-c01
CLR-c02
CHD-c01
EU417805
EU030667
360 370 380 390 400 410 420
CDR-c01 TTCTCACTGCACTTAGCAGGTGTCTCATCAATCCTCGGAGCAATCAATTTTATCACTACAACCATCAACA
CDR-c02
KGD-c01
VLP-c01
CLR-c01
CLR-c02
CHD-c01
EU417805
EU030667
430 440 450 460 470 480 490
CDR-c01 TGAAACCCCCTACTACTTCACAATATCAAACACCTCTATTTGTTTGATCTGTGCTTGTAACTGCTGTATT
CDR-c02
KGD-c01
VLP-c01
CLR-c01
CLR-c02
CHD-c01
EU417805
EU030667

```
                500       510       520
          ....|....|....|....|....|....|.
CDR-c01   ATTACTACTCTCACTTCCAGTCTTAGCTGCC
CDR-c02   ...............................
KGD-c01   ....T..........................
VLP-c01   ....T..........................
CLR-c01   .C.......T..........TC........
CLR-c02   .C.......T..........TC........
CHD-c01   .C.......T..........TC........
EU417805  .C.T..........A.....AC....C...
EU030667  .C.C.....A.....A.....T.....C...
          * *  **** ***** *****  **** ***
```

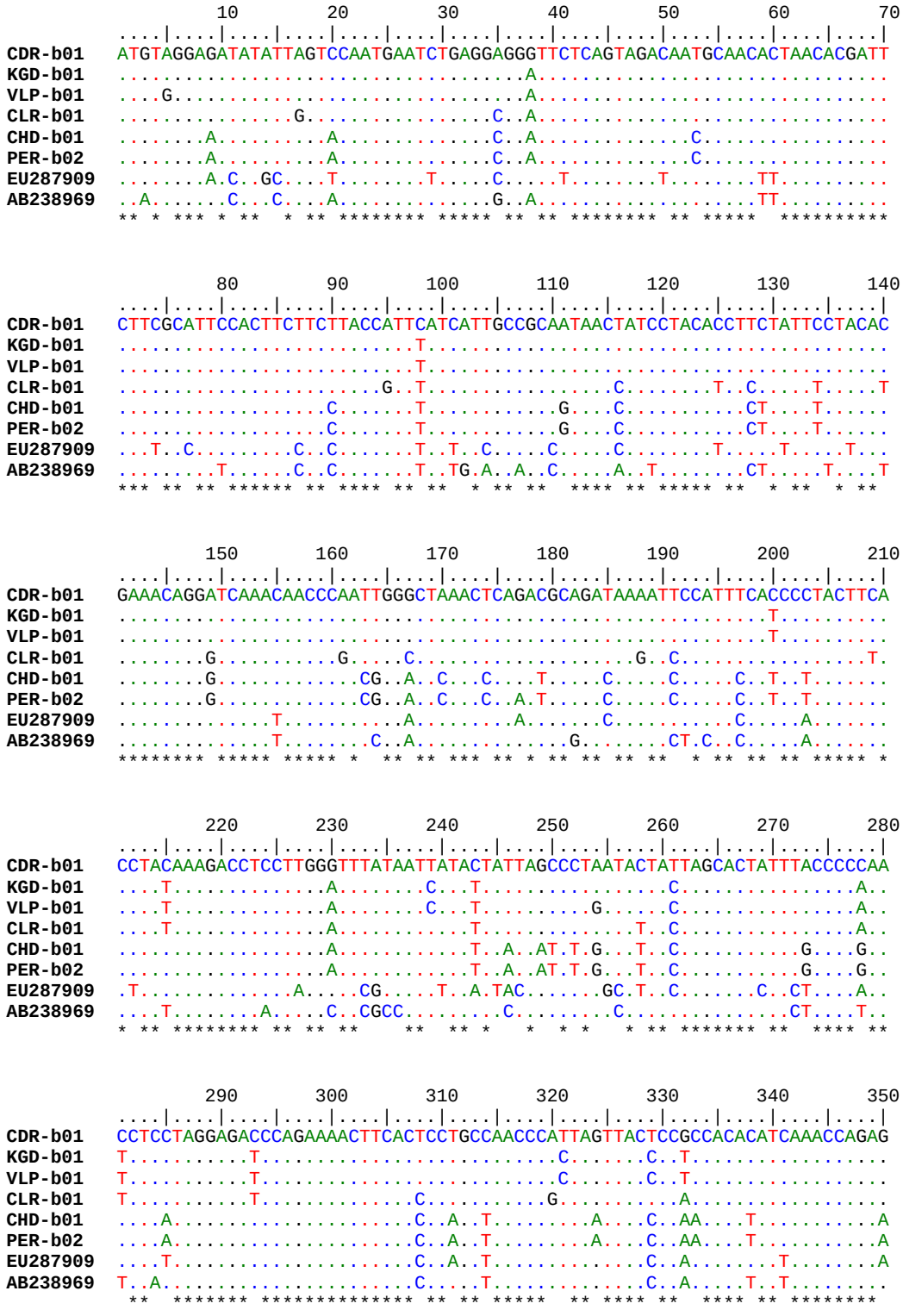
Table 4. Multiple alignment of amino acid residues translated from the nucleotide sequences of COI gene of different haplotypes of *P. denisonii* and *P. chalakkudiensis*.

```
                10        20        30        40        50        60        70
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Cdr-c01   ALSLLIRAELSQPGSLLGDDQIYNVIVTAHAFVMIFFMVMPVLIGGFGNWLVPLMIGAPDMAFPRMNNMS
Cdr-c02   ...............................................................................
Kgd-c01   ...............................................................................
Vlp-c01   ...............................................................................
Clr-c01   ...............................................................................
Clr-c02   ...............................................................................
Chd-c01   ...........................................I...................................
Per-c01   ...........................................I...................................
          ************************************** ****************************

                80        90       100       110       120       130       140
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Cdr-c01   FWLLPPSFLLLLASSGVEAGAGTGWTVYPPLAGNLAHAGASVDLTIFSLHLAGVSSILGAINFITTTINM
Cdr-c02   ...............................................................................
Kgd-c01   ...............................................................................
Vlp-c01   ...............................................................................
Clr-c01   ...............................................................................
Clr-c02   ...............................................................................
Chd-c01   ...........T...................................................I...........I...
Per-c01   ...........T...................................................I...........I...
          ************ ************************************** ************ ***

                150       160       170
          ....|....|....|....|....|....|...
Cdr-c01   KPPTTSQYQTPLFVWSVLVTAVLLLLSLPVLAA
Cdr-c02   ................................
Kgd-c01   ................................
Vlp-c01   ................................
Clr-c01   ....I...........................
Clr-c02   ....I...........................
Chd-c01   ................................
Per-c01   ................................
          **** ****************************
```

Table 5. Multiple alignment of the nucleotide sequences of the cyt *b* gene of 6 haplotypes from six locations and outgroup species. EU287909 (*Puntius tetrazona*) and AB238969 (*Puntius ticto*) are from genbank and are included as outgroup species with original sequences for easy comparison.



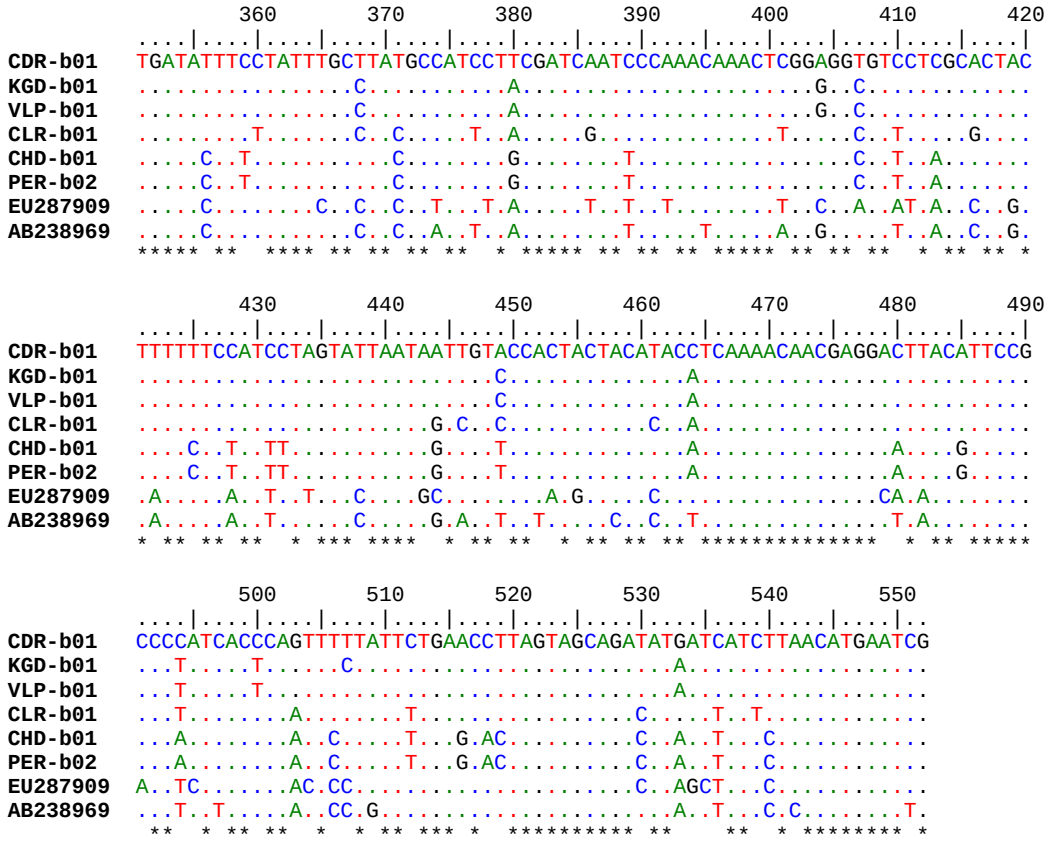


Table 6. Multiple alignment of amino acid residues translated from the nucleotide sequences of cyt *b* gene of different haplotypes of *P. denisonii* and *P. chalakkudiensis*.

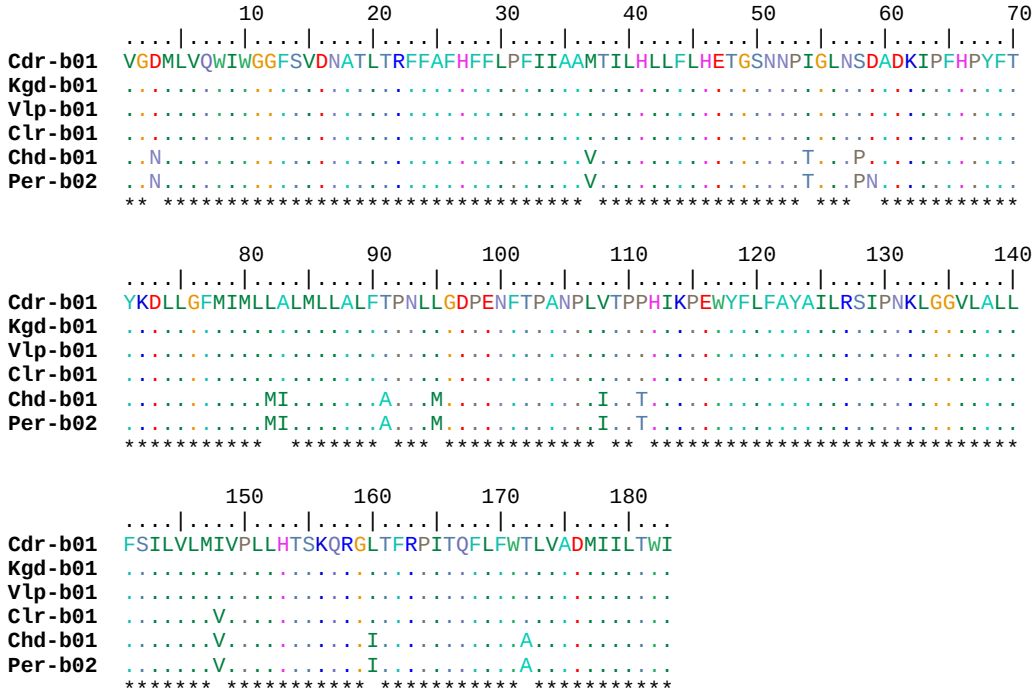
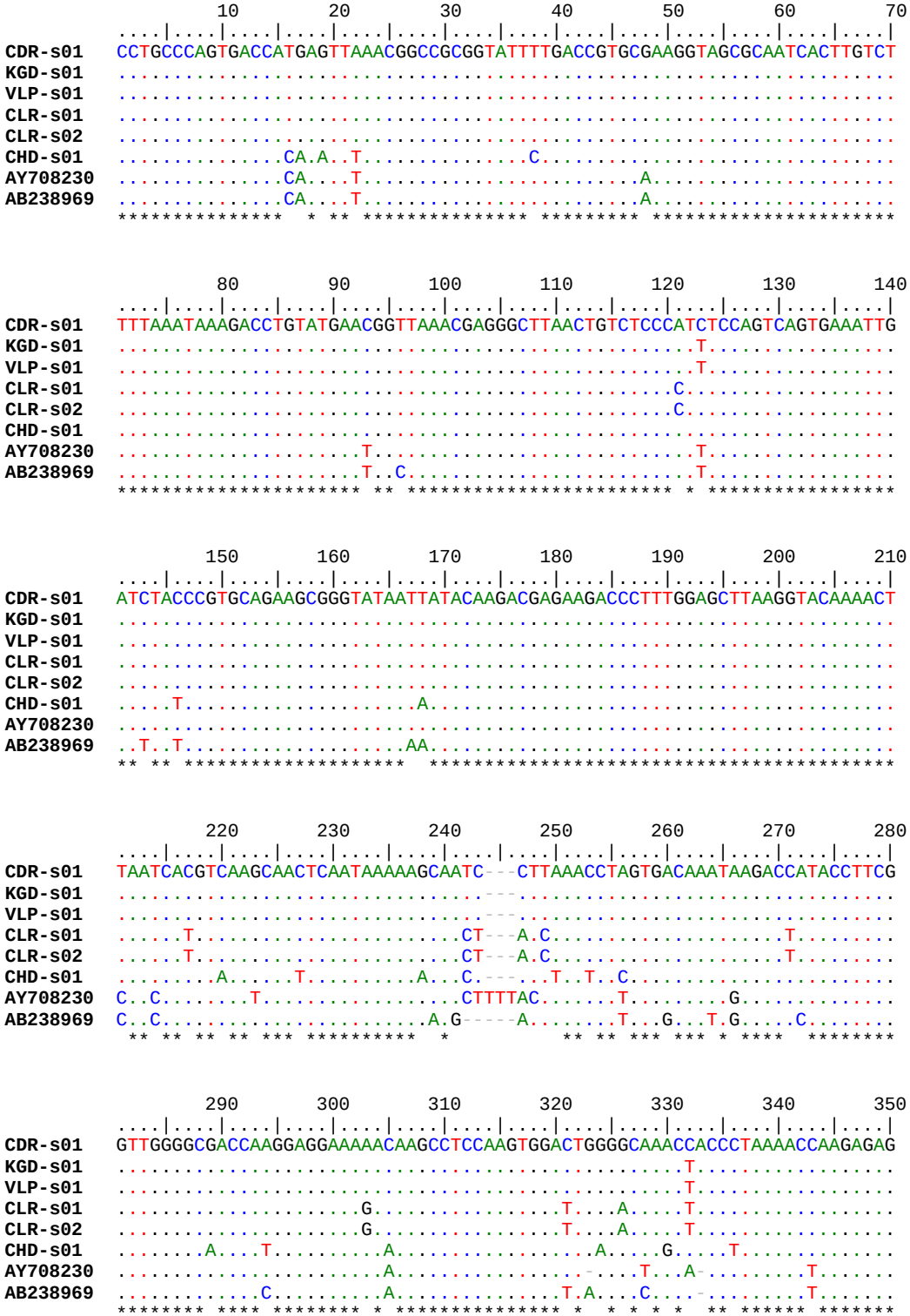


Table 7. Multiple alignment of the nucleotide sequences of the 16S rDNA of 6 haplotypes from six locations and outgroup species. AY708230 (*Puntius srilankensis*) and AB238969 (*Puntius ticto*) are from genbank and are included as outgroup species with original sequences for easy comparison.



```
                360       370       380       390       400       410       420
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CDR-s01    ACATCTCTAAGCCACAGAACATCTGACCATAAATGATCCGATCAACCAAGATCGATCAACGAACCAAGTT
KGD-s01    ....................................................................
VLP-s01    ...................................................G................
CLR-s01    ....T...............................................-................
CLR-s02    ....................................................-................
CHD-s01    ...................................................T................
AY708230   ..................................G.....T....C.....................
AB238969   .......C....A..................C........GCT....C..-C.....................
           **** ** **** ****************** ********   ***   *  ******************
```

```
                430       440       450       460       470       480       490
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CDR-s01    ACCCTAGGGATAACAGCGCAATCCCCTCCAAGAGTCCATATCGACGAGGGGGTTTACGACCTCGATGTTG
KGD-s01    ....................................................................
VLP-s01    ....................................................................
CLR-s01    ....C...............................................................
CLR-s02    ....C...............................................................
CHD-s01    ....A.........................C.....................................
AY708230   ........................C...........................................
AB238969   ........................C...........................................
           **** ********************** **** **********************************
```

```
                500       510       520       530       540       550       560
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CDR-s01    GATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGGTTCGTTTGTTCAACGATTAAAGTCCTACGTGAT
KGD-s01    ....................................................................
VLP-s01    ....................................................................
CLR-s01    ....................................................................
CLR-s02    ....................................................................
CHD-s01    ................C...A................................................
AY708230   ....................................................................
AB238969   ....................................................................
           ******************* *** ********************************************
```

```
           ....|
CDR-s01    CTGAG
KGD-s01    .....
VLP-s01    .....
CLR-s01    .....
CLR-s02    .....
CHD-s01    .....
AY708230   .....
AB238969   .....
           *****
```

Publications:

Asian Fisheries Science **22** (2009): 359-391

Asian Fisheries Society, Selangor, Malaysia
Available online at www.asianfisheriessociety.org

Low Genetic Differentiation in the Populations of the Malabar Carp *Labeo dussumieri* as revealed by Allozymes, Microsatellites and RAPD

A. GOPALAKRISHNAN,[1]*, K. K. MUSAMMILU[1], V.S. BASHEER[1], LIJO JOHN[1], K.G. PADMAKUMAR[3], K.K. LAL[2], V. MOHINDRA[2], P. PUNIA[2], K. DINESH[4], HASHIM MANJEBRAYAKATH[5], A.G. PONNIAH[6] and W.S. LAKRA[2]

[1] National Bureau of Fish Genetic Resources (NBFGR) Cochin Unit, CMFRI Campus, Kochi – 682 018, Kerala, India.
[2] NBFGR, Canal Ring Road, Dilkusha P.O., Lucknow – 226 002, U.P., India.
[3] Regional Agricultural Research Station (RARS), Kumarakom, Kottayam – 686 566, Kerala, India.
[4] College of Fisheries, Panangad, Kochi – 682 506, Kerala, India.
[5] Central Marine Fisheries Research Institute (CMFRI), Kochi – 682 018, Kerala, India.
[6] Central Institute of Brackishwater Aquaculture (CIBA), Chennai – 600 028, Tamil Nadu, India.

Abstract

The population structure of *Labeo dussumieri*, an endangered and endemic cyprinid from three riverine locations in the Western Ghats, India was investigated using allozyme, microsatellite and RAPD markers. *L. dussumieri* samples were obtained from Meenachil, Manimala and Pamba River basins, Kerala. Fourteen (46.7%) out of 30 allozyme loci, seven microsatellite loci and 12 RAPD Operon decamers gave polymorphic pattern. Six allozyme loci (*AAT-2*, EST-4*, GLDH*, GPI-2*, G_6PDH** and *LDH-2**) and three microsatellite loci (*LdussG1*, *MFW19* and *Bgon22*) exhibited consistent significant deviation from Hardy-Weinberg Equilibrium expectations in different populations after probability level ($P<0.05$) was adjusted for sequential Bonferroni correction. All the three marker types demonstrated concordant results and various estimates revealed genetic variability within the subpopulations but surprisingly low level ($\theta = 0.0034$ to 0.0081) of genetic differentiation among *L. dussumieri* from different river samples. AMOVA analysis also indicated low differentiation among subpopulations. No evidence for a recent genetic bottleneck was observed in *L. dussumieri* populations based on allozyme and microsatellite data set analysis. Meenachil, Manimala and Pamba Rivers open in to the southern end of Vembanad Lake in Kerala and are connected to each other in the lower reaches through an extensive network of natural canals. Common ancestry in the prehistoric period; and possible mixing of fish populations resulting in high gene flow across the rivers through the lake and interconnecting canals could have been responsible for the lack of significant allelic heterogeneity among the *L. dussumieri* populations. The stocks from the three rivers do not require different management strategies and for propagation assisted river ranching programme of this species, large effective breeding population can be developed by mixing individuals from three rivers.

Indian J. Fish., 56(3) : 223-226, 2009

Note

Mitochondrial DNA (Cytochrome c oxidase I) sequencing of Indian marine mussels

P. R. DIVYA, A. GOPALAKRISHNAN, LIJO JOHN, P. C. THOMAS[1], AND W. S. LAKRA[2]

National Bureau of Fish Genetic Resources , Cochin Unit, CMFRI Campus, Cochin - 682 018, India
[1]Marine Biotechnology Division, Central Marine Fisheries Research Institute, Cochin - 682 018, India
[2]National Bureau of Fish Genetic Resources, Lucknow - 226 002, India
e-mail: divyanbfgr@gmail.com

ABSTRACT

Two species of marine mussels, the green mussel *Perna viridis* (Linnaeus, 1758) and the brown mussel *Perna indica* (Kuriakose and Nair, 1976) are found along the Indian coast. It had been suggested that *P. indica*, which occurs only along the Indian coast, is a synonym of the globally distributed *Perna perna*. Along the south-west coast of India, where both *P. viridis* and *P. indica* co-exist, a third type referred to as parrot mussel, which has shell shape of brown mussel and color of green mussel (suspected to be their hybrid/morphotype) also occurs. The present investigation is a preliminary attempt for resolving the taxonomic ambiguity among Indian marine mussel species using the mitochondrial cytochrome oxidase I (COI) gene. *P. indica* revealed 95% sequence similarity to *P. perna*. The sequence divergence between *P. indica* and parrot mussel was negligibly low (< 2%). Green mussel *P. viridis* showed 20.87% of sequence divergence with brown mussel *P. indica* as well as with the parrot mussel.

Keywords : Brown mussel, Cytochrome oxidase I (COI) gene, Green mussel, *Perna indica, Perna virdis*

*Indian J. Fish., **52**(2) : 125-140, Apr.-June, 2005*

Genetic identity of *Tor malabaricus* (Jerdon) (Teleostei : Cyprinidae) as revealed by RAPD markers

E. G. SILAS, A.GOPALAKRISHNAN*, LIJO JOHN* AND C. P. SHAJI **

Managing Trustee, E. G. Silas Foundation for Nature Conservation, 37 Ambady Retreat, Cochin - 682 020, Kerala, India.
** National Bureau of Fish Genetic Resources (NBFGR) Cochin Unit, Cochin - 682 018, <nbfgrcochin@eth.net>*
***Central Marine Fisheries Research Institute, Cochin - 682 018, India.*

ABSTRACT

Tor malabaricus (Jerdon) is a mahseer species endemic to the Western Ghats. Since its original description, taxonomic position of the species has been extremely confusing. In the present study, Random Amplified Polymorphic DNA (RAPD) markers were used to determine the taxonomic status of *T. malabaricus* collected from Balamore River, Tamil Nadu, India, by comparing its RAPD profile with that of *Tor khudree*. 15 random oligodecamers were used to amplify DNA from *Tor malabaricus* and *Tor khudree* (n=30 each) collected from two geographically isolated localities and a total of 119 amplicons were detected. The RAPD fingerprints generated were consistent, reproducible and yielded 22 species-specific markers (6 for *Tor malabaricus* and 16 for *Tor khudree*). The genetic distance of 0.3429 and the UPGMA dendrogram between the two species indicate that both are not the part of the same gene pool and have to be treated as two distinct species. The application of the result to the taxonomic status and conservation of *Tor malabaricus* is also discussed.

List of NCBI accessions* (relevant to the present study only are listed)

S.No.	NCBI Accession no.	Details
1.	GQ247528	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius chalakkudiensis* 16S ribosomal RNA gene, partial sequence; mitochondrial.
2.	GQ247529	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius denisonii* haplotype A 16S ribosomal RNA gene, partial sequence; mitochondrial.
3.	GQ247530	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius denisonii* haplotype B 16S ribosomal RNA gene, partial sequence; mitochondrial.
4.	GQ247531	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius denisonii* haplotype C 16S ribosomal RNA gene, partial sequence; mitochondrial.
5.	GQ247532	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius denisonii* haplotype D 16S ribosomal RNA gene, partial sequence; mitochondrial.
6.	GQ247533	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius denisonii* haplotype E 16S ribosomal RNA gene, partial sequence; mitochondrial.
7.	GQ247534	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius chalakkudiensis* ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.
8.	GQ247535	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype A ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.
9.	GQ247536	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype B ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.
10.	GQ247537	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype C ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.
11.	GQ247538	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype D ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.
12.	GQ247539	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype M ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes,

complete cds; mitochondrial.

13. GQ247540 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype N ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.

14. GQ247541 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype E ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.

15. GQ247542 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype F ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.

16. GQ247543 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype G ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.

17. GQ247544 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype H ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.

18. GQ247545 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype I ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.

19. GQ247546 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype J ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.

20. GQ247547 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype K ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.

21. GQ247548 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype L ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes, complete cds; mitochondrial.

22. GQ247549 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius chalakkudiensis* cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial.

23. GQ247550 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype A cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial.

24. GQ247551 **Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype B cytochrome

		c oxidase subunit I (COI) gene, partial cds; mitochondrial.
25.	GQ247552	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype E cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial.
26.	GQ247553	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype F cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial.
27.	GQ247554	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype C cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial.
28.	GQ247555	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Reynold, P., Siby, P., and Lakra, W.S. *Puntius denisonii* haplotype D cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial.
29.	GQ247556	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius chalakkudiensis* voucher Pc CHD 1 cytochrome b (Cytb) gene, partial cds; mitochondrial.
30.	GQ247557	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius chalakkudiensis* voucher Pc PER 2 cytochrome b (Cytb) gene, partial cds; mitochondrial.
31.	GQ247558	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius denisonii* haplotype A cytochrome b (Cytb) gene, partial cds; mitochondrial.
32.	GQ247559	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius denisonii* haplotype B cytochrome b (Cytb) gene, partial cds; mitochondrial.
33.	GQ247560	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius denisonii* haplotype D cytochrome b (Cytb) gene, partial cds; mitochondrial.
34.	GQ247561	**Lijo, J.**, Gopalakrishnan, A., Basheer, V.S., Jeena, N.S., Vidya, R., and Lakra, W.S. *Puntius denisonii* haplotype C cytochrome b (Cytb) gene, partial cds; mitochondrial.

*Sequences will be released to public domain only on 01st March 2010.