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Population Structure of Denison's barb, *Puntius denisonii* (Pisces: Cyprinidae): A Species Complex Endemic to the Western Ghats of India

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Abstract

Genetic and morphologic variation, haplotype relationships, and structuring of populations within *Puntius denisonii* and its close related species *Puntius chalakkudiensis* have been tested using molecular and biometric data, to infer phylogeographic patterns. Sequences of mitochondrial DNA ATPase 8 and 6 genes, and morphometric data, were used to find population structuring. Specimens were collected from 7 locations in the southern region of Western Ghats, a global biodiversity hotspot in India. Biometric analysis revealed apparent heterogeneity in the morphology and color pattern between the species at juvenile and adult stages, and among different geographically separated populations of these species. High values for mean pair wise distances and a high proportion of the total variance attributed to differences between the geographically isolated populations with AMOVA, indicated clear population structuring within these species. Extremely high values for Pair wise F_{ST} and significantly lower *Nm* values observed among the populations studied, suggested little or no effective gene flow among them. Constructed phylogenies further confirmed a high degree of population structuring within the species, showing local endemism with population specific haplotypes forming a species complex. The present study thus estimates the validity of subpopulations within *P. denisonii* and *P. chalakkudiensis*; clarifies the relationships of populations of *P. denisonii* with that of *P. chalakkudiensis,* and also indicates the presence of four different independent evolutionary lineages forming cryptic species within *P. denisonii*. The study further emphasizes the need for a conservation policy to be developed for each population of both species, separately based on MUs (Management Units).

Keywords: *Puntius denisonii*; Western Ghats; Cryptic species; Population structure; Conservation

Introduction

Puntius denisonii (Pisces: Cyprinidae) is a vibrantly colored, globally traded ornamental teleost, endemic to the southern part of the Western Ghats of India. It is found in selected west flowing rivers originating from the Western Ghats [1-3]. The species was described by Francis Day, in 1865 [4], from Kerala state of India, and was of no interest to commercial fisheries [5], until 1996. In 1997, in Singapore's Aquarama exhibition, the species won an award in the 'new species' category [3,6], and became very popular in both national and international aquarium trade. Later on Menon et al. [1] described another species, *Puntius chalakkudiensis*, from the River Chalakkudy in the Western Ghats, which closely resembles *P. denisonii* in appearance. Both the species then achieved high demand in domestic, as well as in international aquarium trade, and is being exploited from the wild in large quantities [7,8]. 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 [9]. Ponniah and Gopalakrishnan [2] reported an alarming rate of depletion of fish diversity of the region due to overexploitation. There is an urgent need for the development of scientific management strategies for the sustainable utilization of these natural resources.

Mitochondrial DNA (mtDNA) sequencing has become the molecular marker of choice, when studying cospecific populations (eg. [10-15]). In recent years, mtDNA, because of its fast evolution [16], 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 (eg. [16-21]).

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 [22-24]. Therefore, molecular markers alone may not be sufficient to detect existing genetic variation among populations, and also only a small proportion of DNA is analyzed by molecular markers. Morphometric and meristic characters have been commonly used in fisheries biology, as powerful phenotypic tool for measuring discreteness and relationships among various taxonomic categories [25-28]. 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 [24], even without showing genetic differentiation [29]. Morphometric and meristic analysis can thus, be a first step in investigating the stock structure of species with large population sizes [29]. As a potential indicator of phenotypic stocks, analysis of morphometric landmarks is a valuable tool that compliments other stock identification methods. The present study provides a comprehensive phenotypic and genetic analysis of different geographically isolated populations of *P. denisonii* and *P. chalakkudiensis*. The study is a pioneering attempt in determining population/stock structure and diversity of *P. denisonii,* besides genetic comparison of *P. denisonii* with *P. chalakkudiensis*.

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Materials and Methods

Sampling

Fish samples were collected from seven geographically isolated locations, comprising the rivers Chandragiri, Kariangode, Valapattanam, Chaliyar, Chalakkudy, Periyar and Pamba (Figure 1), throughout the species distribution (Table 1). Specimens of *P. denisonii* and *P. chalakkudiensis* were identified up to species level in the field, following Day [4] and Menon et al. [1]. A piece of tissue (fin clips of approximately 5×5 mm size from pectoral and pelvic fins of right side of the specimens) was excised and placed in 95% ethanol for isolating DNA, and further the specimens were fixed in 30% formalin and preserved in 10% formalin for biometric analysis.

Biometric analysis

In biometric analysis, color pattern of the juveniles and adult specimens, structure and arrangement of pharyngeal teeth and gill rakers, and morphometric measurements, were thoroughly examined. Except morphometrics, all other characters were examined at species level; whereas, morphometric analysis has been done up to population level. Morphometric measurements following Kottelat [30], were made on 210 specimens comprising 30 each from 7 different watersheds, using 21 reliably measurable morphometric characters described in table 2. 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).

The pharyngeal teeth were counted (8 specimens), and represented by a formula adopting the method of Hubbs and Lagler [22]. The structure and arrangement of pharyngeal teeth and gill rakers were observed under a binocular microscope (Nikon DS-L2), with a magnification of 4X, and images were digitally captured.

In morphometric analyses, size-dependent variation for morphometric characters was excluded [31]. 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 software (ver. 13.0). The Coefficient of Variation (CV) was computed for each character, following van Valen [32]. In each species' sample group, morphological variability was estimated by the multivariate generalization of the coefficient of variation (CVp), using the formula of van Valen [32]. To identify whether there are any statistically significant differences between the species/population for each character, one-way Analysis of Variance (ANOVA) was performed [33], using SPSS software (ver. 13.0). Finally, the size-adjusted morphometric data showing statistically significant differences among groups (species or populations) were submitted to Principal Component Analysis (PCA), and scatter plots generated using the software PAST (ver. 1.89).

Genetic analysis

DNA isolation and PCR amplification: Total DNA was extracted

River basin	Site	Site code	Geographic coordinates	n_t	Mt DNA (ATPase 8/6)	Morphometrics
Chandragiri	Sullya	CDR	12°34' N 75°23' E	50	10	30
Kariangode	Cherupuzha	KGD	12°17' N 75°23' E	32	8	30
Valapattanam	Koottupuzha	VLP	12°04' N 75°43' E	52	8	30
Chaliyar	Pullooranpara	CLR	11°23' N 76°01' E	36	9	30
Chalakkudy	Athirapilly	CHD	10°17' N 76°32' E	35	9	30
Periyar	Pooyamkutty	PER	10°08' N 76°47' E	55	17	30
Pamba	Kuzhimavu	PMB	09°27' N 76°57' E	32	13	30

n_t-Total no. of specimens (including juveniles) collected from each location.

Table 1: Details of sampling with geographic locations and numbers of individuals used in analyses.

Character	Code	Description
Standard length	L_S	snout tip to the midpoint of caudal fin origin
Head length	L_H	snout tip to the posterior edge of operculum
Maximum body depth	MBD	distance between points at deepest part of body (measured vertically)
Pre dorsal length	L_{PRD}	snout tip to the origin of dorsal fin
Post dorsal length	L_{PD}	length from the last ray of the dorsal fin to origin of caudal fin
Pre ventral length	L_{PRV}	snout tip to the origin of ventral fin
Pre anal length	L_{PRA}	snout tip to the origin of anal fin
Pectoral to pelvic origin	L_{PTFPLF}	length from the origin of pectoral fin to that of pelvic fin
Pelvic to anal	L_{PLFAF}	length from the origin of pelvic fin to that of anal fin
Length of body cavity	L_{BC}	length from the first ray of pectoral fin to vent
Dorsal fin base	FBDO	length between the visible origins of the first spine and the last ray of the dorsal fin
Anal fin base	FBAN	length between the visible origins of the first spine and the last ray of the anal fin
Length of caudal peduncle	L_{CP}	length from the anal fin insert to the midpoint of the caudal peduncle
Depth of caudal peduncle	CPD	least depth measured vertically
Head depth	HD	depth at the nape measured vertically
Width of head	HW	Width behind orbit perpendicular to the longitudinal axis
Width of mouth	MW	length between the points of lateral edges of jaws of the mouth
Orbital length	L_O	length (along axis) of the orbit
Pre orbital length	L_{PRO}	mouth tip to anterior edge of orbit
Post orbital length	L_{PO}	posterior edge of orbit to posterior edge of operculum
Barbel length	L_{MB}	length of maxillary barbel on left side

Table 2: Description of morphometric characteristics studied.

from the tissue (fin clips) samples preserved in 95% ethanol, following the salting out procedure of Miller et al. [34], after removing ethanol by air drying and washing in Tris buffer (pH 8.0). The entire (approx. 950 bp) ATP synthase 8 (ATPase 8) and ATP synthase 6 (ATPase 6) genes were PCR amplified, using primers of Page et al. [35]. Amplifications were performed in 50 µl total reaction volume containing 5 µl 10X PCR buffer (SIGMA-ALDRICH, USA), with 15 mM $MgCl_2$, 2 µl each of 10 µM forward and reverse primers, 1 µl 10 mM dNTPs,1 µl (3 U) *Taq* DNA polymerase (SIGMA-ALDRICH, USA) and 2 µl (~ 40 ng) of template DNA. The thermal cycling conditions were as follows: an initial denaturation at 95°C for 3 min, denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min, repeated for 35 cycles, followed by a final extension for 7 min at 72°C. Following amplification, 2 µl of the PCR products were visualized on 1.5% agarose gel.

Sequencing and sequence analysis: All PCR products were purified using GenElute PCR Clean-Up Kit (SIGMA), and directly sequenced using the same forward and reverse primers using ABI Prism Big Dye Terminator v3.1 Cycle Sequencing kit, on an AB 3730 XL capillary sequencer (Applied Biosystems), following manufacturer's instructions. Sequences were edited and the contigs were assembled using BioEdit sequence alignment editor version 7.0.5.2 [36]. Multiple alignments of sequences were performed using ClustalW facility, available in the BioEdit program. Alignment was then manually checked and corrected. Nucleotide sequence characteristics after alignment were analyzed using the program DnaSP version 5.10 [37].

Population genetic analysis: Intra-population diversity was analyzed by estimating gene diversity (*h*) [37], and nucleotide diversity (π) [38,39]. Hierarchical genetic differentiation and the significance of group and population structure were tested using Analysis of Molecular Variance (AMOVA) [40], and *F*-statistics [41], respectively. Samples collected from the same site were treated as a single population sample, except for Periyar (PER). Specimens from Periyar consisted of two morphotypes (Figure 2); one with a black blotch on dorsal fin resembling *P. chalakkudiensis* [PER(c)], and the other without any black blotch on dorsal fin [PER(d)]. Based on the observations of initial analysis, these morphotypes were considered as different populations/groups in further analysis. This analysis was performed for three hierarchical groupings of the data. The first level compared the variation among individuals within each population. The second level examined genetic structure among populations of each group/species. The typical *P. chalakkudiensis* (CHD with a black blotch on dorsal fin, from Chalakkudy River) samples and its look alike samples from Periyar [PER(c)] and Pamba [PMB], were combined in Group A (*Pc*). All other samples of *P. denisonii* [CDR, KGD, VLP, CLR and PER(d)], were combined in Group B (*Pd*). Finally, variation among groups/species, *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 and migration rates/gene flow (*Nm*) were also calculated among the different populations. All population analyses were performed using Arlequin version 3.0 [42].

Phylogenetic analysis: Phylogenetic and molecular evolutionary analyses were conducted using MEGA 5 [43]. Sequence data was subsequently analyzed using Maximum Likelihood, Maximum Parsimony and Neighbour-Joining methods, with *Puntius conchonius* and *Puntius ticto* as outgroups. The robustness of the internal nodes of phylogenetic trees was verified using bootstraps of 1000 replicates. Pairwise sequence divergence among populations was calculated according to Kimura 2 parameter (K2P) model [44].

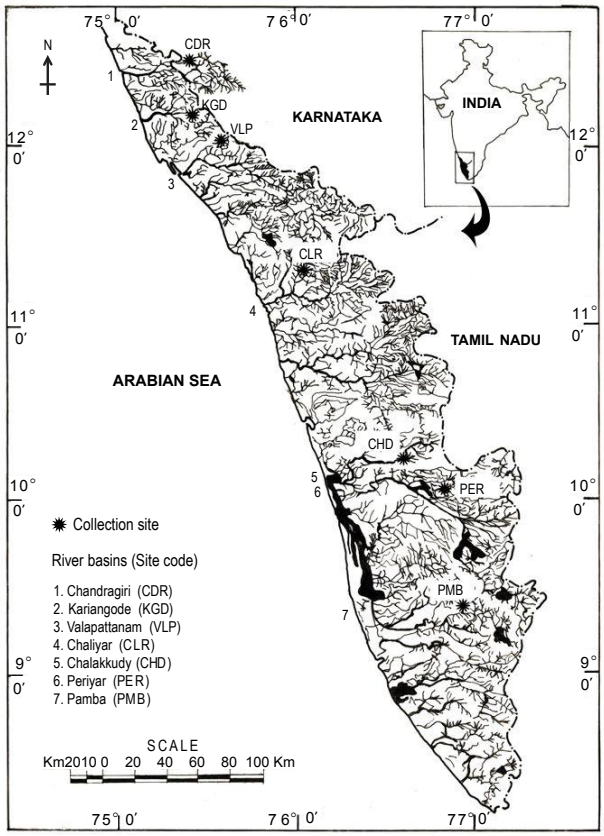

Figure 1: Map of Kerala (reproduced from the Water Atlas of Kerala), showing the distribution of the sampling sites.

Figure 2: Adult and juvenile specimens collected from different sites; a-Specimen from Valapattanam River (VLP) (total length 102 mm); b-Juvenile specimen from Valapattanam River (VLP) (total length 20 mm); c-Juvenile specimens from Valapattanam River (VLP) (total length ranges 20-40 mm); d-Specimen from Periyar River (PER), without a black blotch on dorsal fin (total length 150 mm); e-Typical *P. chalakkudiensis* specimen from Chalakkudy River (CHD) (total length 90 mm); f-Typical juvenile specimen of *P. chalakkudiensis* from Chalakkudy River (CHD) (total length 25 mm, formalin preserved); g-Juvenile specimens of *P. chalakkudiensis* from Periyar River (PER) (total length ranges 20-30 mm, formalin preserved); h-Specimen from Pamba River (PMB), with a typical black blotch on dorsal fin (total length 85 mm, formalin preserved).

Results

Basic ecological observations

Difference in size, body shape and color among the samples obtained from different sampling sites were readily noticeable (Figure 2). Especially the specimens from North Kerala and Karnataka (NK) region of the Western Ghats, which includes CDR, KGD, VLP and CLR population samples, were easily distinguishable from that of Central and South Kerala (SK) region specimens (includes CHD, PER and PMB population samples). Body of typical *P. chalakkudiensis* specimens was moderately deep (especially in larger specimens), with slightly rounded snout and comparatively more greenish dorsum than NK specimens. A black blotch on the dorsal fin was also prominent in these specimens. But, in *P. denisonii* specimens from North Kerala and Karnataka regions, body was comparatively slender and streamlined with a pointed snout. The body length of *P. denisonii* was relatively shorter (max. total length observed 135 mm) than that of *P. chalakkudiensis* (max. total length observed 176 mm), as observed in the present study. In addition, *P. chalakkudiensis* (Figure 2e) 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* (Figure 2a). Some specimens collected from Periyar River and the specimens from Pamba resembled *P. chalakkudiensis* in their color characteristics (Figure 2). Periyar River is found to be a typical habitat from where two types of specimens obtained; one of which resembled the typical *P. chalakkudiensis* in their morphology, and the second type (Figure 2c) showed more resemblance to *P. denisonii*, without a black blotch on dorsal fin.

Juveniles of both species exhibited different color patterns from that observed in adult specimens (Figure 2b, d, f, g and h). Shape and color patterns 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 present in early juveniles, whereas three prominent black vertical bands were observed. The early juveniles of *P. denisonii* (Figure 2b and d) from NK region possesses comparatively narrow and diffuse vertical bands on the sides of the body, whereas that of *P. chalakkudiensis* (Figure 2f and h) possess broader and more prominent bands. However, this pattern disappears in sub-adults and adults in both species. The identity of juveniles was confirmed by sequencing ATPase 8 and 6 genes from one representative sample, each from both species, and by comparing it with that of the adult specimens.

Structure and arrangement of pharyngeal teeth and gill rakers

Structure and arrangement of pharyngeal teeth and gill rakers were examined to find out any variation between the two species *viz. P. denisonii* (from Valapattanam) and *P. chalakkudiensis* (from Chalakkudy). Microscopic examination of pharyngeal teeth revealed the presence of an additional fifth pair of teeth in the inner most rows on pharyngeal bones, in addition to the formula given by Day [4], for *P. denisonii.* The pharyngeal teeth was found to be arranged as 5,3,2-2,3,5 in both species, as observed in the present study. The pharyngeal bones of both left and right sides possess three rows, with two teeth on the outer row, three on the middle and five on the inner. Out of the 5 teeth in the innermost row, four were of similar size, and the fifth was comparatively smaller (Figure 3a). The gill rakers on the first gill arch in *P. denisonii* were slender and villiform in structure (Figure 3c), whereas in *P. chalakkudiensis,* they were comparatively stout with blunt tips (Figure 3b). The second type of specimens from Periyar (PER[d]), without a black blotch on dorsal fin possesses gill rakers, similar to that observed from NK specimens (Figure 3d).

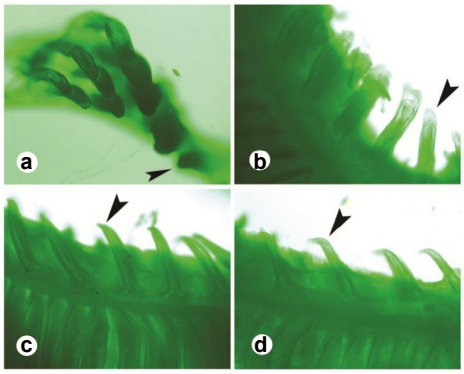

Figure 3: Microscopic images (4X magnification) of pharyngeal teeth and gill rakers of outer gill arch; a-Pharyngeal teeth arranged on one side of the pharyngeal bone of *P. denisonii* specimen from Valapattanam river (arrow shows the smallest fifth teeth); b-Gill rakers of outer gill arch of typical *P. chalakkudiensis* specimen from Chalakkudy river; c-Gill rakers of outer gill arch of *P. denisonii* specimen from Valapattanam river; d-Microscopic image of gill rakers of outer gill arch of specimen without a black blotch on dorsal fin from Periyar river (arrow head on 'b' shows the blunt tip and 'c'and 'd' shows slender and villiform structure of gill rakers).

Morphometric traits

The Coefficient of Variation (CV) for all morphometric traits was generally lower within each population of *P. denisonii* (1.01-9.49%; Table 3) and *P. chalakkudiensis* (1.46-8.75%; Table 4). The multivariate generalized coefficient of variation (CVp) in each population was also relatively low. Specimens from Periyar showed the highest CVp (5.51%) among all the populations, even with a relatively low value; indicating minimal or very low intra-population variation. Univariate analysis of variance carried out among populations showed that fish samples from different sites differed significantly (at $p<0.05$ and $p<0.01$ levels of significance), in 18 and 19 out of the 20 morphometric characters examined in *P. denisonii* (Table 3) and *P. chalakkudiensis* (Table 4), respectively. This indicated heterogeneity in fish morphology among riverine populations of both the species. There was no significant differences ($P>0.05$) observed in FBDO and L_O, and MBD among populations of *P. denisonii* and *P. chalakkudiensis,* respectively.

Specimens of *P. chalakkudiensis* collected from its type locality (Chalakkudy river; CHD), and the specimens which resembled the species collected from Periyar (PER) and Pamba (PMB) river systems, were grouped together and compared with other population samples of *P. denisonii* from NK region (CDR, KGD, VLP and CLR). 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 (excluding standard length) studied (Table 5). *P. denisonii* samples from four different sites of NK region shared several of the morphometric characters that are significantly different from those in *P. chalakkudiensis,* with high F values. For example, the specimens from NK region have shorter HD, MBD, FBAN, CPD and L_{PD}. Moreover, larger mean L_{PRV}, L_{PRA}, L_{PTFPLF}, MW, L_{PO}, L_{MB} and L_H were identified in *P. denisonii* specimens from this region; whereas, *P. chalakkudiensis* specimens could be differentiated by larger mean L_{CP}, CPD, HW, L_O and L_{PRO} (Table 5).

Principal Component Analysis (PCA) was carried out factoring the correlation matrix of the morphometric data between the two

	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 ± 7.2°(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} ± 0.8°(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} ± 1.7°(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} ± 2.3°(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} ± 2.5°(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} ± 1.1°(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 3: 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* from North Kerala and Karnataka region of Western Ghats.

	Chalakkudy (n=30)		Periyar (n=30)		Pamba (n=30)		
	Mean ± SD (min. - max.)	CV	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)	-	69.16 ± 12.58 (53.60-98.97)	-	-
L_H	22.21^{a} ± 1.27 (20.30-24.47)	5.72	23.30^{b} ± 1.56 (19.99-24.88)	6.70	23.96^{b} ± 0.67 (22.55-24.94)	2.80	15.50**
MBD	26.92^{a} ± 0.91 (25.48-28.63)	3.40	26.98^{a} ± 0.81 (25.87-28.86)	3.00	26.59^{a} ± 1.22 (24.93-29.30)	4.59	1.32 NS
L_{PRD}	46.11^{a} ± 1.33 (44.51-48.45)	2.88	46.57^{a} ± 0.97 (44.93-48.55)	2.08	48.06^{b} ± 1.07 (45.94-50.81)	2.23	24.26**
L_{PD}	41.48^{a} ± 1.19 (39.66-43.30)	2.86	39.49^{b} ± 1.78 (37.51-43.96)	4.51	37.76^{c} ± 1.06 (36.48-39.70)	2.81	54.59**
L_{PRV}	48.89^{a} ± 1.07 (47.01-50.52)	2.20	50.61^{b} ± 1.1°(47.67-52.26)	2.17	51.92^{c} ± 1.08 (49.88-53.56)	2.09	58.81**
L_{PRA}	73.04^{a} ± 1.58 (70.17-75.48)	2.16	74.75^{b} ± 1.39 (72.41-77.25)	1.87	74.47^{b} ± 1.08 (71.23-75.93)	1.46	13.49**
L_{PTFPLF}	26.11^{a} ± 1.57 (24.12-28.71)	6.00	27.31^{b} ± 1.2°(25.96-31.03)	4.38	27.32^{b} ± 0.98 (25.32-29.89)	3.59	9.04**
L_{PLFAF}	24.77^{ab} ± 0.82 (23.32-25.87)	3.31	25.33^{a} ± 1.28 (22.80-29.00)	5.06	24.23^{b} ± 0.87 (22.83-25.89)	3.58	8.92**
L_{BC}	49.14^{a} ± 2.18 (45.43-53.10)	4.44	48.60^{a} ± 1.84 (44.72-53.23)	3.80	47.11^{b} ± 1.36 (44.53-50.68)	2.89	9.94**
FBDO	15.97^{a} ± 0.83 (14.75-17.54)	5.20	16.05^{ab} ± 0.83 (14.83-17.50)	5.17	16.48^{b} ± 0.54 (15.75-17.38)	3.28	4.11*
FBAN	09.41^{ab} ± 0.67 (08.37-10.48)	7.17	09.60^{a} ± 0.82 (08.59-11.72)	8.51	08.99^{b} ± 0.51 (07.81-09.68)	5.65	6.26**
L_{CP}	19.02^{a} ± 1.01 (17.03-20.65)	5.33	17.69^{b} ± 1.03 (16.30-20.28)	5.82	17.11^{b} ± 0.69 (16.42-19.27)	4.01	33.96**
CPD	12.22^{a} ± 0.48 (11.45-13.14)	3.89	11.91^{b} ± 0.53 (10.47-13.04)	4.49	11.82^{b} ± 0.41 (11.15-12.54)	3.45	5.83**
HD	69.68^{a} ± 2.73 (65.63-74.34)	3.92	67.00^{b} ± 2.93 (62.25-75.67)	4.37	65.31^{b} ± 2.68 (60.37-70.02)	4.10	18.91**
HW	58.36^{a} ± 1.82 (55.54-60.73)	3.12	55.82^{b} ± 1.15 (53.49-58.79)	2.06	56.46^{b} ± 1.68 (53.72-60.08)	2.98	21.11**
MW	25.48^{ab} ± 0.69 (24.01-26.48)	2.69	24.86^{a} ± 1.27 (21.44-26.74)	5.12	25.85^{b} ± 1.55 (23.12-28.23)	5.99	5.11**
L_O	30.68^{a} ± 1.98 (27.34-33.60)	6.45	33.22^{b} ± 1.47 (29.14-35.20)	4.42	33.81^{b} ± 1.15 (31.41-35.11)	3.40	33.58**
L_{PRO}	37.38^{a} ± 1.99 (34.18-40.46)	5.33	32.26^{b} ± 1.74 (28.10-36.18)	5.38	31.87^{b} ± 1.45 (29.01-34.56)	4.54	93.66**
L_{PO}	34.33^{a} ± 1.0°(32.19-35.69)	2.92	36.79^{b} ± 2.67 (33.91-42.37)	7.27	37.69^{b} ± 1.45 (35.00-39.87)	3.85	26.54**
L_{MB}	26.68^{a} ± 2.33 (22.94-32.38)	8.75	29.84^{b} ± 1.74 (27.25-33.09)	5.81	27.56^{a} ± 1.38 (25.07-30.02)	5.03	23.20**
CVp		**5.36**		**5.51**		**4.91**	

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 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 three populations of *P. chalakkudiensis* and similarly looking specimens, from South Kerala region of Western Ghats.

species, and also among populations of *P. denisonii* (CDR, KGD, VLP and CLR) and *P. chalakkudiensis* (CHD, PER and PMB), respectively. PCA of the 18 significant variables between *P. chalakkudiensis* and *P. denisonii* yielded 8 principal components, accounting for 82.23% of the total variation in the original variables. The variance explained by the first two components was found to be 48.63%. The first component

	P. denisonii (n=120)			*P. chalakkudiensis* (n=90)			
	Mean ± SD (min.-max.)	**CV**		**Mean ± SD (min.-max.)**	**CV**		**F value**
L_S	81.08 ± 8.43 (64.55-101.30)	-		82.73 ± 19.4°(53.60-129.75)	-		-
L_H	23.96 ± 0.93 (22.01-25.93)	3.88		23.16 ± 1.41 (19.99-24.94)	6.09		24.76**
MBD	24.42 ± 1.23 (21.96-26.41)	5.04		26.83 ± 1.0°(24.93-29.30)	3.73		229.33**
L_{PRD}	47.12 ± 1.03 (44.96-49.51)	2.19		46.91 ± 1.4°(44.51-50.81)	2.98		1.59 NS
L_{PD}	37.22 ± 1.09 (35.15-39.96)	2.93		39.58 ± 2.05 (36.48-43.96)	5.17		115.49**
L_{PRV}	52.99 ± 2.01 (49.19-58.95)	3.79		50.47 ± 1.65 (47.01-53.56)	3.26		93.74**
L_{PRA}	75.55 ± 1.69 (72.31-78.88)	2.24		74.09 ± 1.55 (70.17-77.25)	2.09		41.49**
L_{PTFPLF}	28.1°± 1.38 (25.13-30.77)	4.91		26.91 ± 1.38 (24.12-31.03)	5.13		37.87**
L_{PLFAF}	24.59 ± 1.14 (21.89-26.62)	4.64		24.78 ± 1.1°(22.80-29.00)	4.43		1.4°NS
L_{BC}	48.98 ± 2.34 (44.72-53.25)	4.78		48.28 ± 2.0°(44.53-53.23)	4.15		5.19*
FBDO	16.41 ± 0.63 (15.18-18.45)	3.84		16.17 ± 0.77 (14.75-17.54)	4.78		6.13*
FBAN	08.03 ± 0.51 (06.83-08.88)	6.35		09.33 ± 0.72 (07.81-11.72)	7.69		233.43**
L_{CP}	15.95 ± 1.43 (13.13-18.67)	8.97		17.94 ± 1.22 (16.30-20.65)	6.80		112.15**
CPD	10.99 ± 0.51 (10.27-12.65)	4.64		11.98 ± 0.5°(10.47-13.14)	4.18		196.12**
HD	61.31 ± 2.74 (55.55-67.85)	4.47		67.33 ± 3.29 (60.37-75.67)	4.89		208.39**
HW	55.8°± 2.45 (50.77-61.95)	4.39		56.88 ± 1.9°(53.49-60.73)	3.34		12.11**
MW	26.22 ± 2.01 (22.60-31.23)	7.67		25.4°± 1.28 (21.44-28.23)	5.03		11.43**
L_O	31.45 ± 1.6°(28.56-34.22)	5.09		32.57 ± 2.07 (27.34-35.20)	6.34		19.65**
L_{PRO}	32.59 ± 1.67 (28.97-35.88)	5.12		33.84 ± 3.05 (28.10-40.46)	9.03		14.37**
L_{PO}	38.39 ± 1.82 (34.28-41.94)	4.74		36.27 ± 2.32 (32.19-42.37)	6.40		55.22**
L_{MB}	32.06 ± 3.44 (22.56-38.31)	10.73		28.03 ± 2.28 (22.94-33.09)	8.12		93.00**
CVp		**5.79**			**5.85**		

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 *P. denisonii* and *P. chalakkudiensis*.

was mainly defined by measurements of L_{PRD}, CPD, L_{CP}, MBD, L_{PD} and FBAN. The second component was mainly correlated with L_{PTFPLF}, L_{PRA}, HD and L_{PRV}. These observations indicated that the above morphometric characters contributed maximum in differentiating *P. denisonii* and *P. chalakkudiensis*. The scatter diagram based on PCA clearly distinguishes the two major groups, which are evidently distinct as two species (Figure 4a), (Supplementary data (Table 9)).

Out of 20 morphometric measurements (excluding L_S) taken, 18 measurements found to be significant ($p<0.05$) among the populations of *P. denisonii,* and 19 measurements found to be significant ($p<0.05$) among the populations of *P. chalakkudiensis*. These significant morphometric variables were used to carry out PCA with the population samples of both species. Eight principal components accounted to describe 80.39% of the total variation in *P. denisonii* and 81.99% in *P. chalakkudiensis* in the original variables. Among *P. denisonii* population data, the first two components explained 46.33% variance, in which the first component was mainly defined by measurements of FBDO, L_{PTFPLF}, HD, L_{PRA} and L_{PO}. The second component was mainly correlated with the measurements of L_{MB}, L_{CP}, L_{PLFAF}, L_H and FBAN. This indicated that the above morphometric characters contributed maximum in differentiating *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 (Figure 4b) (Supplementary Data (Table 10)). The first two principal components explained about 43.54% variance of total variation in the original variables in *P. chalakkudiensis*. The first component was mainly defined by L_{PD}, L_{CP}, L_{PRV}, L_O and L_{PRO}; and the second component was mainly correlated with length of L_{PLFAF}, L_{PTFPLF}, L_{MB}, L_{BC} and L_{PO}. 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 among populations of *P. chalakkudiensis* (Figure 4c) (Supplementary Data (Table 11)).

Sequence characteristics, genetic divergence and population variability

An 832 bp DNA sequence comprising partial regions of ATP synthase 8 and 6 genes were studied from 74 samples, including *P. denisonii* and *P. chalakkudiensis* from seven different geographic locations. Out of the 832 characters obtained, 676 (81.25%) were constant and 156 (18.75%) were variable, in which 144 (17.31%) were informative for parsimony among all population samples, including *P. denisonii* and *P. chalakkudiensis*. Sequence comparison revealed 32 different haplotypes, defined by 156 divergent nucleotide sites. DNA sequences of all different haplotypes are deposited in GenBank (accessions GQ247534 to GQ247548 and JF927912 to JF927928). Among the 7 different river systems analyzed, the Group A (*Pc*), samples were characterized by the presence of a single haplotype of high frequency, accompanied by several other closely related haplotypes of lower frequency. The CHD and PER(c) samples were represented by the high frequency haplotype, and the accompanying low frequency haplotypes were distributed again in PER(c) and PMB populations (Table 6). The sample population of Chalakkudy river (CHD) of *P. chalakkudiensis* was found to be the least diverse based on haplotype diversity (0.0) observed. The Periyar river system showed the maximum number of haplotypes (9 out of 17 samples sequenced), which included both morphotypes resembling the morphologic features of *P. denisonii* and *P. chalakkudiensis*. Haplotype diversity (h), within the geographic populations, was high in the case of Group B (*Pd*) samples [ranged from 0.2500 in KGD to 0.9643 in PER(d)], than that observed within Group A (*Pc*) samples (ranged from 0.0 in CHD to 0.8462 in PMB); whereas the nucleotide diversity (π) was generally low and ranged from 0.0 (CHD) to 0.0056 [PER(d)]. Except CHD and PER(c), none of the population samples shared common haplotypes, indicating significant genetic separation among different populations. The only single haplotype (H17) observed within CHD was found to be shared with PER(c) (Table 6).

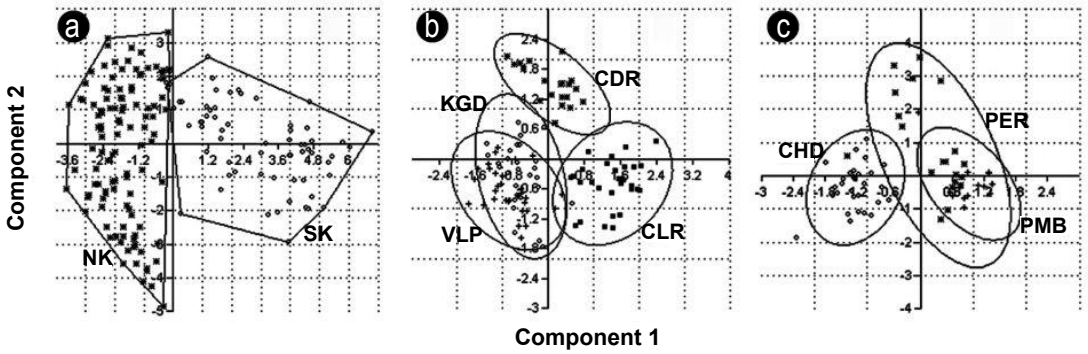

Figure 4: Scatter plots of principal component 1 and principal component 2 from PCA of significant morphometric variables from *P. denisonii* and *P. chalakkudiensis* specimen from different isolated water sheds; a-With the specimens of *P. denisonii* from North Kerala and Karnataka region (NK includes CDR, KGD, VLP and CLR), and that of Central and South Kerala region (SK includes CHD, PER and PMB; *P. chalakkudiensis* and its look alkies') of the Western Ghats were analyzed as two groups; b-With four different populations from North Kerala and Karnataka (CDR, KGD, VLP and CLR) region analyzed separately; c-With three different populations from Central and South Kerala (CHD, PER and PMB) region analyzed separately.

	P. denisonii					*P. chalakkudiensis*		
	CDR	**KGD**	**VLP**	**CLR**	**PER(d)**	**PER(c)**	**CHD**	**PMB**
CDR	***0.17%***	0.9686(+)	0.9390(+)	0.9761(+)	0.9577(+)	0.9901(+)	0.9913(+)	0.9843(+)
KGD	0.0349 (0.0081)	***0.03%***	0.7647(+)	0.9910(+)	0.9644(+)	0.9972(+)	0.9986(+)	0.9890(+)
VLP	0.0299 (0.0162)	0.0039 (0.0770)	***0.21%***	0.9756(+)	0.9537(+)	0.9891(+)	0.9905(+)	0.9829(+)
CLR	0.0537 (0.0061)	0.0617 (0.0023)	0.0578 (0.0063)	***0.07%***	0.9611(+)	0.9950(+)	0.9963(+)	0.9874(+)
PER(d)	0.0834 (0.0111)	0.0862 (0.0092)	0.0859 (0.0121)	0.0795 (0.0101)	***0.56%***	0.9686(+)	0.9702(+)	0.9658(+)
PER(c)	0.1146 (0.0025)	0.1128 (0.0007)	0.113°(0.0028)	0.109°(0.0013)	0.0924 (0.0081)	***0.03%***	0.0000(-)	0.7510(+)
CHD	0.1145 (0.0022)	0.1128 (0.0003)	0.1129 (0.0024)	0.1089 (0.0009)	0.0923 (0.0307)	0.0000	***0.0%***	0.7822(+)
PMB	0.1193 (0.0040)	0.1175 (0.0028)	0.1177 (0.0044)	0.1136 (0.0032)	0.0969 (0.0088)	0.0034 (0.0829)	0.0037 (0.0696)	***0.17%***

(+), significant at $p<0.05$ after sequencial Bonferroni agjustment; (-), not significant at this level; (c), PER specimen with a black blotch on dorsal fin; (d), PER specimen without a black blotch on dorsal fin

Table 6: Mean pairwise distances, gene flow (*Nm*) and Pairwise F_{ST} among populations of *P. denisonii and P. chalakkudiensis*, based on *ATPase 8/6* gene sequences. Pairwise F_{ST}, and its significance in parenthesis, are given above diagonal; genetic distance based on K2P and *Nm* in parenthesis is given below diagonal; mean K2P distance within each population is given in % across the table.

The mean K2P distances and pairwise F_{ST} values observed between populations across *P. denisonii* and *P. chalakkudiensis* were found to be markedly higher than that observed within each population (Table 7). Mean K2P distances observed between the Group A (*Pc*) and Group B (*Pd*) population samples ranged from 0.0969 (between PER(d) and PMB) to 0.1193 (between CDR and PMB)). Distance observed among samples of Group B (*Pd*) was comparatively higher, whereas that among samples of Group A (*Pc*) were markedly smaller (Table 7). The PER(d) population was found to be equally separated from all other populations, with mean K2P distances values ranging from 0.0795 (with CLR) to 0.0969 (with PMB). The pattern of genetic diversity observed was moderate to high, and nucleotide diversity was low in general within the populations of *P. denisonii* and *P. chalakkudiensis* (Table 6).

Analysis of molecular variance (Table 8), for the two species as groups, indicated a total variance of 65.31% (Φ_{CT}=0.653), which was significant ($P<0.05$) enough, and attributed to the differences between them. Grouping individuals into 3 or 4 groups based on morphologic features and K2P distances observed further emphasized significant ($P<0.005$; 0.001) genetic divergence among the geographically isolated populations of *P. denisonii*.

Phylogenetic relationships

Phylogenies constructed using the Maximum Likelihood, Maximum parsimony (Figures 5a and b), and Neighbor Joining (not presented in figure) methods showed similar topologies, and consistently indicated clear population structuring across geographically distinct riverine populations of *P. denisonii*. All specimens of Group B (*Pd*) formed four well differentiated monophyletic sister groups (clades), as clade 1 (formed by CDR population samples), clade 2 (formed by VLP and KGD samples), clade 3 (formed by CLR samples) and clade 4 (formed by PER(d) samples, the specimens without a black blotch on dorsal fin obtained from the river Periyar)-all with high bootstrap support; whereas in the case of Group A (*Pc*), all specimens of *P. chalakkudiensis* from its type locality, Chalakkudy river (CHD) and from the other 2 different river systems–Periyar [PER(c)] and Pamba (PMB) were found to be clustered together into an unresolved sister clade, indicating least genetic divergence among the riverine populations of the species.

Discussion

Biometric and genetic divergence in *P. denisonii* complex

The present study reveals clear distinction between the two closely related species, *P. denisonii* and *P. chalakkudiensis*. The presence of high level of genetic divergence among geographically separated populations of *P. denisonii* suggests the possibility of cryptic species within them. Day [4] described the species *P. denisonii* 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

Population	n_t	n_h	Haplotypes at each sampling site (n_h)	Nucleotide diversity (π)	Haplotype diversity (*h*)
CDR	10	5	H01(2), H02(1), H03(5), H04(1), H05(1)	0.0017	0.7556
KGD	8	2	H06(7), H07(1)	0.0003	0.2500
VLP	8	6	H08(1), H09(3), H10(1), H11(1), H12(1), H13(1)	0.0021	0.8929
CLR	9	3	H14(7), H15(1), H16(1)	0.0007	0.4167
PER(d)	8	7	H19(1), H20(2), H21(1), H22(1), H23(1), H24(1), H25(1)	0.0056	0.9643
PER(c)	9	2	H17(8), H18(1)	0.0003	0.2222
CHD	9	1	H17(9)	0.0000	0.0000
PMB	13	7	H26(2), H27(5), H28(1), H29(1), H30(1), H31(2), H32(1)	0.0017	0.8462

*, *P*<0.05; **, *P* <0.01; ***, *P*<0.005; n_t-Number of individuals analyzed per site; n_h-Number of haplotypes per site

Table 7: Intrapopulation nucleotide (π) and haplotype (*h*) diversities per population of *P. denisonii* and *P. chalakkudiensis* based on *ATPase 8/6* gene sequences.

Grouping tested	Source of variation	*df*	% total variance	Φ	*P*-value
Group 1 [CDR+KGD+VLP+CLR+PER(d)] Group 2 [CHD+PER(c)+PMB]	Among groups	1	65.31	Φ_{CT}=0.653	<0.05
	Among populations within groups	6	33.25	Φ_{SC}=0.958	<0.001
	Within populations	66	1.44	Φ_{ST}=0.986	<0.001
Group 1 [CDR+KGD+VLP] Group 2 [CLR+PER(d)] Group 3 [CHD+PER(c)+PMB]	Among groups	2	72.54	Φ_{CT}=0.725	<0.005
	Among populations within groups	5	25.81	Φ_{SC}=0. 940	<0.001
	Within populations	66	1.65	Φ_{ST}=0.983	<0.001
Group 1 [CDR+KGD+VLP] Group 2 [CLR] Group 3 [PER(d)] Group 4 [CHD+PER(c)+PMB]	Among groups	3	85.16	Φ_{CT}=0.852	<0.001
	Among populations within groups	4	13.20	Φ_{SC}=0.890	<0.001
	Within populations	66	1.64	Φ_{ST}=0.984	<0.001

Table 8: Results of the hierarchical analysis of molecular variance (AMOVA) of populations of *P. denisonii* and *P. chalakkudiensis*, based on mitochondrial ATPase 8/6 region. Significance values were obtained from 100° random permutations of the DNA sequence using Arlequin 3.0.

"Fishes of Malabar" and "The Fishes of India; being a Natural History of the fishes known to inhabit the seas and freshwaters of India, Burma and Ceylon", he described the species as *Puntius denisonii* and *Barbus denisonii,* respectively. Presently the valid generic name is *Puntius*. According to Menon et al. [1], *P. chalakkudiensis* can be distinguished from *P. denisonii* and all other *Puntius* species by its inferior mouth and a distinct black blotch on the dorsal fin. In the present study, a series of phenotypic characters [18 out of 20 morphometric characters–other than L_S–examined (Table 5)] showed significant differences revealed by the univariate Analysis of Variance (ANOVA), to differentiate *P. denisonii* and *P. chalakkudiensis*. The multivariate analysis PCA using significant morphometric variables clearly indicated the separation of two species, without any inter mixing (Figure 4a). Structural variation observed on examination of gill rakers on the first gill arch was distinct between the two species (Figure 3). The color pattern observed in the adult specimens of *P. denisonii*, *P. chalakkudiensis* and their juveniles were also different (Figure 2), which emphasized the clear differentiation of *P. chalakkudiensis* from *P. denisonii*. Breeding season of *P. denisonii* was reported during the month of October to March [45], however, a slight variation in the peak breeding months among different watershed populations within the species was observed.

Molecular analysis based on ATPase 8 and 6 genes showed unambiguous genetic divergence between the two species. Both species were genetically distinct, as the amount of pairwise sequence divergence within them was much less than that between the two (Table 7). The degree of genetic divergence between the two species was comparable to that existing between many of the teleost species [46], including several cyprinid species from the Western Ghats [47]. In the phylogenetic analysis based on both ML, MP (Figure 5) and NJ methods, all the haplotypes with a black blotch on dorsal fin (CHD, PER(c) and PMB population samples), which is a typical characteristic of *P. chalakkudiensis*, formed a single monophyletic clade, well separated from the other sister clades. Based on the morphologic and genetic pattern observed in the present study, the specimens with a black blotch on dorsal fin obtained from Chalakkudy, Periyar and Pamba river systems (CHD, PER(c) and PMB samples, respectively), could be considered as *P. chalakkudiensis*. Thus, the distribution of *P. chalakkudiensis* should be extended to Periyar and Pamba River systems, in addition to the previously reported type locality, the Chalakkudy River [1] and Achankovil [48]. The rest of the haplotypes of *P. denisonii,* without a black blotch on dorsal fin, obtained from four different river systems of north Kerala (CDR, KGD, VLP and CHR samples) and from Periyar river [PER(d)], were further separated into four distinct well separated monophyletic clades (C1, C2, C3 and C4), with high level of genetic (Figure 5; Table 7), and morphologic divergence (Figure 4b; Table 3), indicating the presence of four independent evolutionary lineages within the species. An unrecognized diversity and possibility of at least two morphologically cryptic lineages within *P. denisonii,* obtained from international aquarium trade, has been reported recently [46].

The species *P. denisonii* and *P. chalakkudiensis* have been reported from different west flowing rivers of Kerala [1-3,48], including the river systems under the present study. Earlier reports regarding the distribution of these species were purely based on the morphological examinations alone. The Periyar river system was found to be the most diverse, based on the pattern of haplotype diversity observed (Table 6), and more over with the presence of two morphotypes [PER(d) and PER(c)], which resemble the characteristics of *P. denisonii* and *P. chalakkudiensis,* respectively. Even though the specimens without a black blotch [PER(d)], obtained from the same habitat, resembled *P. denisonii* in many features, especially the shape of the head, position of mouth and a more pointed snout, the colour pattern shows more similarity with *P. chalakkudiensis* (Figure 2c). The pattern of clustering with phylogenetic analysis (Figure 5) and the mean pairwise distances value (0.0924) observed between the two morphotypes [PER(d) and PER(c)], obtained from the Periyar river system, clearly indicates them as two genetically distinct groups, as members of the two different species. The molecular data revealed the PER(d) population samples are almost equally well separated from the North Kerala specimens of *P. denisonii* (mean pairwise distance value ranged from 0.0795 with CLR to 0.0862 with KGD), and the typical *P. chalakkudiensis* (mean pairwise GD 0.0923; Table 7 and Figure 5). The mean K2P distance observed

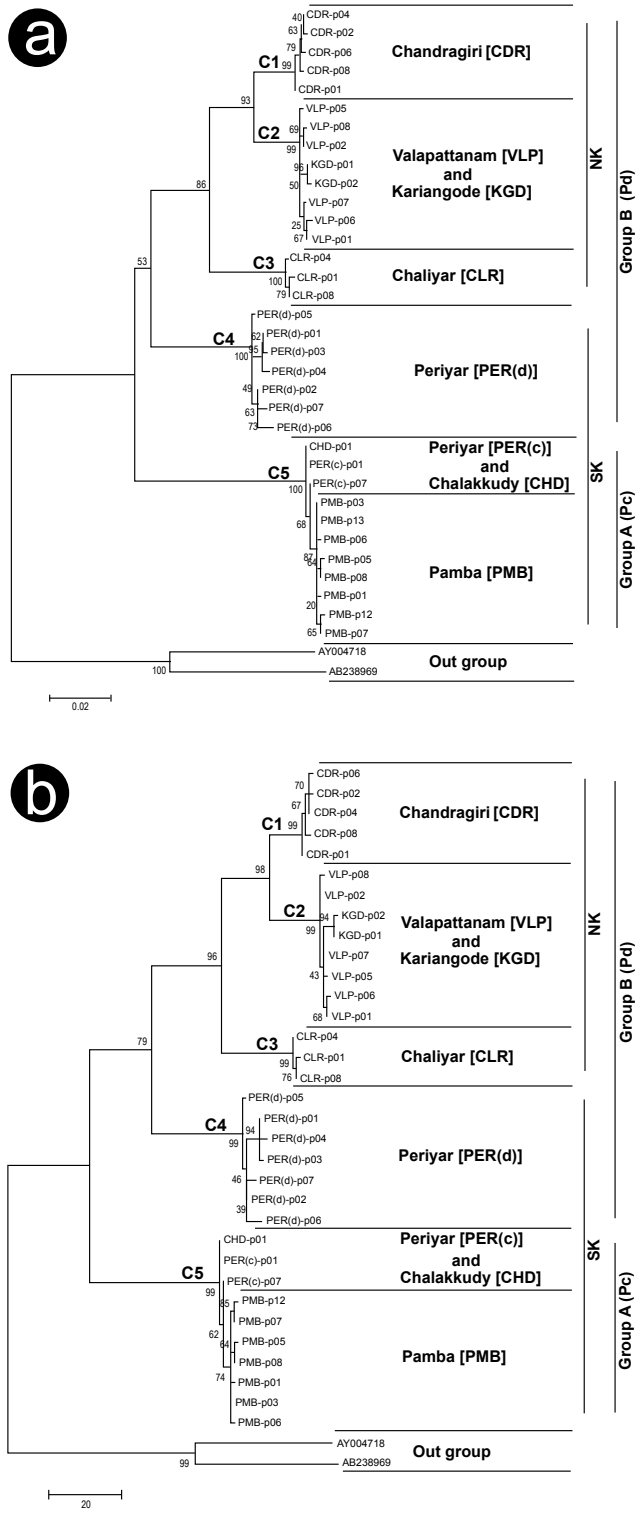

Figure 5: (a) Maximum Likelihood and **(b)** Maximum Parsimony trees of the populations of *P. denisonii* and *P. chalakkudiensis* inferred from haplotype (representative haplotypes alone showed here) sequence variation of the mitochondrial DNA ATPase 8/6 genes. Numbers at nodes indicate the bootstrap values. AY004718 (*Puntius conchonius*) and AB238969 (*Puntius ticto*) are from Genbank, and are included as outgroup species.

between the samples of *P. denisonii* and *P. chalakkudiensis* was found to be significantly higher ranging from 0.0923 (between PER(d) and CHD) to 0.1193 (between CDR and PMB). Even though, the type locality from where Francis Day described *P. denisonii* was mentioned as Mundakayam, Travancore hill region of the Western Ghats [4], any specific stream or river system has not been mentioned anywhere. The sampling survey carried out in and around Mundakayam during the present study could obtain specimens of Denison's barb from the river Pamba. The sampling site at Pamba River was about 15 km away from Mundakayam town, and the specimens obtained resembled the typical *P. chalakkudiensis*. But, these specimens obtained near the type locality in the present study could not be considered as the topotypes of typical *P. denisonii*. Because, on examination it is observed that the type series of *P. denisonii* deposited in the Natural History Museum, London, is devoid of a clear black blotch on the dorsal fin, and with terminal mouth (personal communication, Dr. K. Rema Devi, Zoological Survey of India and Dr. Rajeev Raghavan, Conservation Research Group, Kerala, India). However, though we could not obtain a single specimen without a black blotch on dorsal fin (typical *P. denisonii*) from Pamba, the chances for the occurrence of such specimens in those habitats towards South Kerala region could not be eliminated.

The present mtDNA locus ATPase 8 and 6 genes studied showed almost similar genetic divergence (K2P) and phylogenetic patterns observed with the DNA barcoding locus COI, within the species *P. denisonii* [49]. However, the pattern of monophyletic association exhibited among different lineages within the species *P. denisonii,* with high branch support, and with an average of 10X greater divergence between than within each lineage (Figure 5, Table 7) satisfies the basic concept of COI based DNA barcoding, for species identification and discovery [50-52], in identifying new species. The high level of genetic and morphologic diversity observed among the Denison's barb, distributed along the isolated habitats of the Western Ghats, make it a species complex, with a scope of at least four cryptic species within them, being discussed further. The population samples such as CDR, CLR and PER(d) were found to be well separated from VLP and KGD samples of *P. denisonii* (Figure 5, Table 7). A thorough study with detailed sampling from all the distributed areas of these species, and comparison with available data on type specimen, is required to get a conclusive picture regarding the taxonomic positions of the species.

Population structure

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 [53,54]. 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*) are distributed. Generally, neighboring 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 population samples of *P. denisonii* between drainages were consistent with these expectations.

Univariate and multivariate analyses of morphometric traits clearly demonstrated an intraspecific morphological variation among the samples of *P. denisonii* and *P. chalakkudiensis,* from different River systems of the Western Ghats of India (Tables 3 and 4, Figure 4). Measurements of head were the most discriminating variable in this study. Accordingly, the samples from Chaliyar River possess the deepest head and lowest L_{PO}, whereas the Chandragiri population possesses the highest mean L_{H} (Table 3). But, in the case of *P. chalakkudiensis,* the

variation among population samples was mainly related to measures of L_{PRO}, HW, L_{PRV}, L_O, L_{PD}, and L_{PO}. The coefficient of variation observed in the present study was comparatively low across all the populations studied, ranging from 4.80% (CDR) to 5.51% (PER). In fishes, the coefficients of variation within populations are usually far greater than 10% [55]. The lower coefficient of variation indicates minimal or very low intra-population variation. Similar results were obtained by Mamuris et al. [56], in the seven populations of red mullet (*Mullus barbatus*) and by Quilang et al. [27], in four populations of Silver perch (*Leiopotherapon plumbeus*).

Environmental factors, in particular the temperature that prevails during some of the sensitive developmental stages, can have great influence on the morphological characters [57,58]. 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*, especially between CHD and PER(c). 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 conducted. Many teleost populations exhibit morphological changes depending on the environment, and are still genetically homogenous, as reported in Brazilian populations of yellowtail snapper (*Ocyurus chrysurus*, *Lutjanidae*) [59], and in three different riverine populations of *Liza abu,* a mugilid species from Turkey [60]. 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 morphological heterogeneity observed in *P. denisonii* populations is highly consistent with the genetic data, and most probably does not result from phenotypic plasticity. The present study has thus, shown the efficiency of using multivariate techniques in analyzing the population structure, in both species. However, the true reasons for the observed morphologic variability needs further studies, extended to other watersheds also, if the natural distribution of the species exists there.

Analysis of molecular variance, *F*-statistics and phylogenetic analysis indicated marked genetic structure in *P. denisonii* populations at the inter-regional scale. The extent of genetic differentiation observed among the populations of *P. denisonii* is related to the degree of geographic separation between river systems, from where the samples were collected. The ANOVA analyses showed that populations from different regions were differentiated from each other (Table 8). 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 7) values derived 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. 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 [61]. The degree of differentiation between populations is obviously associated with highly rugged geographical pattern of the Western Ghats, leading to the formation of fragmented populations of the species with limited dispersal abilities. The reconstructed phylogenetic trees, as seen from this study, also supported the high differentiation among geographical regions. In the present study, the *Nm* values observed were considerably low; indicating that gene flow among spatially distant populations of both the species is very restricted (Table 7). In fact, a value of *Nm* much lower than 1 is interpreted as evidence of gene flow, insufficient in preventing differentiation, as a result of genetic drift [62,63]. None of the haplotypes were found to be shared between any population samples analyzed in the present study, other than Periyar and Chalakkudy samples of *P. chalakkudiensis*. This could represent occasional or rare migrants between the later. The overall level of genetic exchange may be below that required to homogenize populations, or there may be a barrier to gene flow between these populations. It may also be noted that phylogenetic analysis revealed separate clades of haplotypes, representing each population. This suggests some degree of reproductive isolation of *P. denisonii* populations, leading to geographical structuring with limited or no gene flow between the populations.

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' [64]. 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 [65]. Such patterns of diversity strongly suggest a historical influence on the genetic structure of populations, as estimated by analysis of haplotype frequencies in *P. denisonii* population samples (Table 6). Similar 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 [66].

Both *P. denisonii* and *P. chalakkudiensis* have shown a marked decline in abundance and population in recent years [67-69]. 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 could have 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 might be one of the reasons behind the reduced number of haplotypes found in *P. chalakkudiensis* population, and higher number of unique haplotypes found in *P. denisonii* populations.

Phylogenetic relationships

The results of phylogenetic analyses in the present study: 1) establish the validity of *P. chalakkudiensis* and *P. denisonii* as distinct species; 2) estimate the validity of subpopulations within *P. denisonii*; 3) clarify the relationships of populations of *P. denisonii,* with that of *P. chalakkudiensis,* and 4) indicate the presence of different evolutionary lineages forming cryptic species within *P. denisonii*.

A monophyletic pattern was observed with all the analyses. All haplotypes of the two species separated clearly as two major distinct groups, thus exhibiting phylogenetic separation between *P. chalakkudiensis* (Group A) and *P. denisonii* (Group B). A distinct pattern could be visualized within group B, as four well separated clades (C1, C2, C3 and C4) reflecting a clear region-wise population sub-structuring. Even though clade C2 is formed by VLP and KGD populations, some degree of isolation could be observed, without any shared haplotypes among them. Overall, there was no evidence of homogeneity among these populations; 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 CHD and PER(c) populations of *P. chalakkudiensis*; PMB was found to be separated from them without any shared haplotypes.

Implications for fisheries management and conservation

Puntius denisonii and *P. chalakkudiensis* are exploited in large numbers from the Western Ghats, to meet the demand from domestic and international aquarium trade [7], and as such the issue of population structure/gene flow is critical to their conservation and management. The result of the present study strongly indicates that, although both species appear similar in some respects, management strategies should be developed for each species separately. Findings of the present study have direct relevance to the definition of conservation units for these species [70]. Under this criterion, the five riverine populations of *P. denisonii*, *viz.* Chandragiri, Kariangode, Valapattanam, Chaliyar and Periyar (d), and two populations of *P. chalakkudiensis* (Chalakkudy and Periyar (c) together forms one, and Pamba), can be proposed as distinct MUs, which require separate monitoring and management. In future, a conservation policy based on these MUs is advisable. The genetic data further suggest the presence of at least four evolutionarily significant units, ESUs [70], within *P. denisonii*: (1) Chandragiri (2) Valapattanam and Kariangode (3) Chaliyar and (4) Periyar(d). These groups have been isolated and represent evolutionarily independent lineages in all phylogenetic analysis, and the gene flow between the groups is restricted.

In contrast, the analysis for *P. chalakkudiensis* shows a very different picture. The lack of genetic divergence found in this species, especially between the Chalakkudy and Periyar populations, is consistent with a "one stock" management; whereas with Pamba population, it exhibits a high level of genetic divergence, indicating a population expansion after a bottleneck. Secondly, the extremely low level of genetic variation observed within 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 could compromise the 'genetic health' of the population, and thus may affect its long term viability. Efforts should be directed to ensure that the current level of genetic variation for this species is not eroded further. Hence, conservation action should concentrate on both species separately, especially as the species *P. chalakkudiensis* appears to have a smaller population size than *P. denisonii*. Habitat protection for natural breeding would be a solution to sustain the subpopulations within each region, in which these species are found.

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