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Morphometric and isozyme confirmation for species level divergence between *Puntius dorsalis* (Pisces: Cyprinidae) and its presumed red-fin variety in Sri Lanka

K. B. Suneetha Gunawickrama* and H. G. B. N. Damayanthi
Department of Zoology, Faculty of Science, University of Ruhuna, Matara, Sri Lanka
*Correspondence: suneetha@zoo.ruh.ac.lk

Abstract. Isozyme and morphological variation provide evidence for species-level divergence between *Puntius dorsalis* and the red-fin variety, presumed to be conspecific to *P. dorsalis*, thus warrant the recognition of the *P. dorsalis* like red-fin variety in Sri Lanka as a separate phylogenetic taxon, hitherto undescribed. *Idh*, *Mdh*-1 and *Pgm* isozymes display fixed allelic differences between the two varieties indicating reproductive isolation and species-level divergence. In addition to the presence of 3½ transverse scale rows above lateral line (vs. 4½ in *P. dorsalis*) which is a distinctive autaphomorphy, the snout is significantly longer in the red-fin variety (30.3%HL vs. 22.6%HL of *P. dorsalis*).

Keywords: biological species, fixed alleles, phenotypic divergence, *Puntius dorsalis*, reproductive isolation.

1 Introduction

Puntius dorsalis (Jerdon, 1849), the long-snouted barb is a widely distributed indigenous species in Sri Lanka (Pethiyagoda, 1991) and adjacent countries, while it was originally described from India (Talwar & Jhingran, 1991; Jayaram, 1999). The descriptions of *Puntius dorsalis* from several sources include a distinctive meristic feature of 4½ + 2½ transverse rows of scales respectively above and below the lateral line (Deraniyagala, 1952; Talwar & Jhingran, 1991; Jayaram, 1999). However, in Pethiyagoda (1991), the illustrated *Puntius dorsalis* specimen show 3½ + 2½ transverse rows of scales suggesting an involvement of another closely related morph or taxon. Although formal records of the distribution are lacking, a different variety of the *Puntius* genus, called 'red-fin dorsalis', presumed to be a color morph of *P. dorsalis* is also found in Sri Lanka (hereafter called the 'red-fin' or RF variety). Its fins are red in color at both juvenile and adult stages in life (pers. observ). Lack of a black blotch at the dorsal fin base of adults also distinguishes it from the formally identified *P. dorsalis*. RF variety seems to be relatively rare. As a foremost biodiversity issue, it is of high conservation importance to investigate whether RF variety is actually conspecific thus a true color morph of *P. dorsalis*. Among many other genetic markers, isozymes have been used to distinguish separate species through fixed-allelic differences that are indicative of reproductive isolation between sympatric species (Shaklee *et al*., 1982; Taylor & Bentzen, 1993; Turner, 1999). The present study focuses on revealing the existing variation between *P. dorsalis* and the red-fin variety in Sri Lanka using selected isozymes and morphological characteristics, and thereby aims to contribute towards unveiling the taxonomic status of the red fin variety.

2 Materials and methods

Adult fish specimens were collected by gillnets during Jan 2008. *Puntius dorsalis* were collected from 'River Nilwala' at Godapitiya/ Akuressa area in (Matara district) Southern Sri Lanka (31 specimens for morphological analysis and 30 specimens for isozyme analysis). The red-fin variety has a restricted distribution, and was found in a wet zone hill stream 'Digili Ela' at 'Dediyagala', which is a tributary that join the main body of the River Nilwala (23 specimens for morphological data and 30 specimens for isozyme analysis) (Figure 1). Samples for isozyme analysis were stored at -80°C. As no prominent external features are available for sex determination, sex of individual fish was determined by dissection and observation of gonads.

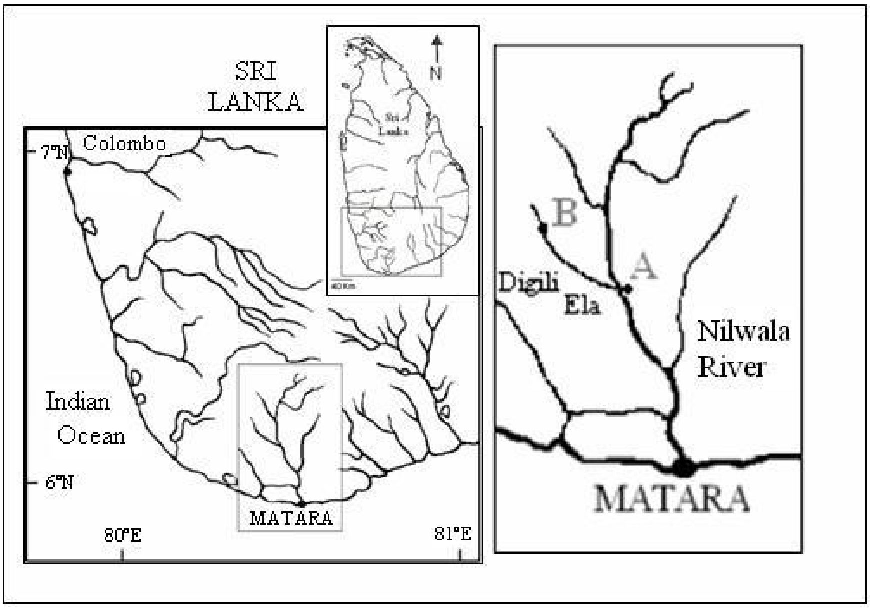

Figure 1 Map of Sri Lanka, with inserts showing the main drainage Nilwala River, and the collection localities of the specimens (A: Godapitiya at Akuressa, B: Dediyagala/ Digili Ela).

Individually imaged fish (with an mm scale) was used to collect data on standard length (L_S) and 14 other morphometric characters of fish, to the nearest 0.1 mm keeping character homology between specimens. All length measurements (prefix L) are taken parallel to the antero-posterior body axis between the identified points (or between vertical lines drawn across those points) as follows: standard length (L_S) is the distance between anterior tip (ST: snout tip) and caudal finbase (CFB: end of perforated series of lateral line scales), head length (L_H) is from ST to posterior opercular margin, pre-orbital length (L_{PrO}) from ST to the anterior orbital margin, caudal peduncle length (L_{CP}) from the base of the last anal fin ray to the CFB, orbital length (L_O) is the diameter of the orbit between fleshy margins, post-orbital length (L_{PsO}) is between posterior orbital and opercular margins, pre-dorsal length (L_{PrD}) and pre-anal length (L_{PA}) are measured from the ST to the origin of each fin, dorsal finbase length (L_{DFB}) and anal finbase length (L_{AFB}) are between the externally visible origins of the first spine and the last ray of the respective fin bases, pectoral fin length (L_{PCF}) is from the base of the first ray and the distal tip of the longest ray, head depth (HD) is perpendicular to the body axis between dorsal and ventral margins of the head at the laterally visible intersection of the isthmus, maximum body depth (MBD) is measured perpendicularly at dorsal fin origin, post dorsal diagonal (PDD) is point-to-point distance between ST and dorsal fin origin, transverse length (TR) is between the origins of dorsal and anal fins diagonally.

Size-correlation detected in the morphometric data were removed by Reist (1985) method using Elliott *et al.* (1995) formula as described in Gunawickrama (2007). Sexual dimorphism in the morphometrics was checked by t-test using size-corrected data. Mean values of standardized morphometric data of the two groups were compared by t-test (Zar 1984), discriminant function analysis (DFA) and principal component analysis (PCA). All morphological data analyses were carried out with STATISTICA V7.0 (Statsoft, USA). Data on meristic characters including number of un-branched and branched rays of the dorsal, pectoral, pelvic and anal fins, number of pre-dorsal scales, number of pre-pelvic (anterior to the pelvic fin origin), number of scale rows along the transverse line (along the dorsal fin/ anal fin diagonal) and number of scales along the lateral line were also collected.

Aqueous crude homogenates of a piece of muscle tissue (approx. 1-2 g) from individual fish (n = 30 each for *P. dorsalis* and red fin variety) were subjected to horizontal starch gel (11.5% W/V) electrophoresis (run at 220V, 100mA over 2-3 hours) as described by Murphy *et al.* (1996). Preliminary analyses were performed with two separate continuous buffer systems, namely, Histidine-tri-sodium citrate (pH 7.0) and Tris-Citrate II (pH 8.0, Selander et al. 1971), but the latter was found to be adequate to resolve the detected loci. The activities for seven enzymes were visualized by separate histo-chemical staining procedures described in Murphy *et al.* (1996); Glycerol-3-phosphate dehydrogenase (GPD, EC 1.1.1.8), Isocitrate dehydrogenase (IDH, EC 1.1.1.42), L-Lactate dehydrogenase (LDH, EC 1.1.1.27), Malate dehydrogenase (MDH, EC 1.1.1.37), Malic enzyme (ME, EC 1.1.1.40), Glucose-6-phosphate isomerase (GPI or PGI, EC 5.3.1.9) and Phosphoglucomutase (PGM, EC 5.4.2.2).

3 Results

A few discernible external characters exist between *P. dorsalis* and the red-finned (RF) variety. All the fins of the RF variety are red in color (in life) even in immature specimens, and it lacks any prominent markings on the body (Figure 2, A1). In *P. dorsalis*, the most prominent marking was the black "blotch" on the posterior part of the basal sheath of the dorsal fin irrespective of the body size, in both live and preserved fish (Figure 2B, B1). It is prominent even in fish >120mm SL (personal observation; not included in the present data). In addition, a dark lateral stripe from opercle to caudal base, and a round, rather diffused black blotch with diameter *c*. 1/3 of the peduncle depth appear mostly in the preserved specimens of *P. dorsalis* irrespective of size, but may not be visible in life. Both have single pair of maxillary barbels. The overall external appearance seems coarser and scalier in the former. The overlapping edges of consecutive scales are mottled with darker pigmentation in *P. dorsalis*, giving particularly the upper body scales (above lateral line) characteristically rhomboid appearances. Both have complete lateral lines (LtL), but slightly more elevated positioning of the LtL in the RF variety. The forth un-branched ray (= 4th spine) of the dorsal fin is strong and smooth in both. Among the meristic characters, the RF variety has 3½ +1+ 2½ rows of transverse scales (Vs. 4½ +1+ 2½ in *P. dorsalis*) (Figures 2 A, B). The rest of the meristic counts some of which have zero variation are not different between the two groups. The counts are; Dorsal IV 8, Pectoral I 11-13, Pelvic I 7, Anal III 5 where numbers of un-branched (roman) and branched rays (Arabic) respectively; pre-dorsal scales 8; pre-pelvic scales 10-11; LtL scales 23-25.

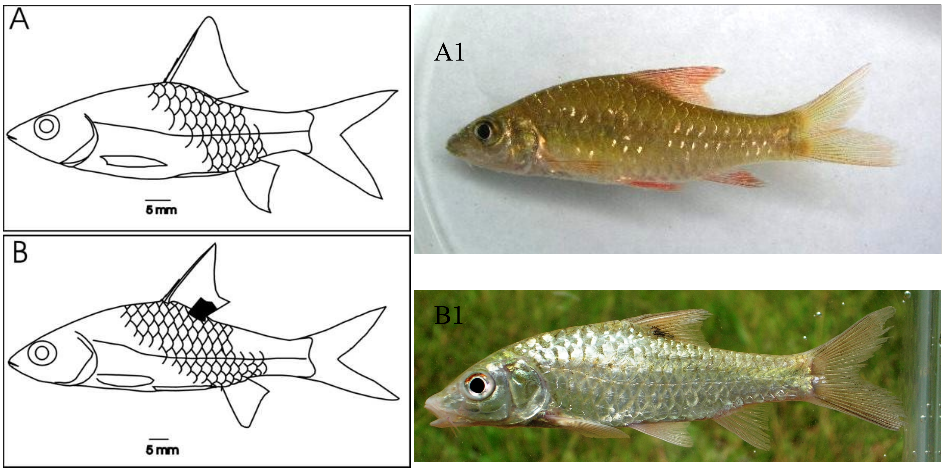

Figure 2. Schematic outline diagrams showing the differences in scalation along the transverse line (between the origins of the dorsal and anal fins), and photographs in life of the red fin *Puntius* variety (A and A1), and *Puntius dorsalis* (B and B1).

Because all the morphometric characters showed significant correlation to size even when taken as % relative standard length (%L_S), all test results presented hereafter were based on the size-corrected standardized characters (%L_S or % L_H relative values are only to give informative descriptive statistics). External sexual differentiation was not possible in either variety, but slight yet significant sexual dimorphism was seen only in the RF variety (t-test) in three morphometrics. RF Females (n = 10) had slightly longer measures than in males (n = 12) of the body depth (MBD: 34.6% vs. 33.5 %L_S; p = 0.04), transverse line depth (TRL: 42.4% vs. 41.0 %L_S; p = 0.01) and pectoral fin length (PECFL: 23.8% vs. 22.2 %L_S; p = 0.03). Inter-variety differences were therefore tested (t-test) for separate sexes. In females, MBD and PECFL was greater in the RF variety than in *P. dorsalis* (34.6 %L_S Vs. 32.7%L_S, and 23.8%L_S Vs. 21.9%L_S respectively) while in males, no differences were found between varieties.

Table 1. Observed morphometric data (mean ± SD) for the Red fin *Puntius* variety and *Puntius dorsalis* in Sri Lanka given as % L_S, (and % L_H within brackets).

	Red fin *Puntius* variety (n = 23)		***P. dorsalis*** (n = 31)	
	Mean	**range**	**Mean**	**range**
Standard length [a]	65.6 ± 5.4	61.1 – 77.6	79.0 ± 10.1	65.9 – 98.3
Caudal peduncle length [c]	18.9 ± 0.9	17.7 – 21.2	20.1 ± 1.3	16.9 – 22.2
Pre-dorsal length [b]	50.1 ± 1.2	48.0 – 52.0	48.5 ± 1.1	46.3 – 50.9
Head length [b]	28.6 ± 1.0	26.7 – 30.4	25.8 ± 1.7	22.7 – 29.4
Orbital length	8.4 ± 0.5	7.0 – 9.2	7.6 ± 0.7	6.3 – 8.9
	(29.2 ± 3.0)	(24.8 – 32.5)	(29.4 ± 1.8)	(26.2 – 33.3)
Post-orbital length [c]	11.6 ± 0.7	10.0 – 12.6	12.4 ± 0.6	11.0 – 13.5
	(40.5 ± 3.1)	(33.9 – 45.5)	(48.0 ± 2.5)	(42.9 – 54.3)
Pre-orbital length [b]	8.7 ± 1.1	6.2 – 10.8	5.9 ± 1.0	4.3 – 8.3
	(30.3 ± 3.3)	(23.2 – 36.6)	(22.6 ± 2.6)	(18.1 – 28.4)
Head depth [c]	21.4 ± 1.1	18.6 – 22.9	21.9 ± 0.9	20.4 – 24.6
Maximum body depth	34.0 ± 1.3 [d]	31.4 – 36.5	32.6 ± 1.2	30.0 – 34.6
Depth of transverse line	41.6 ± 1.4 [d]	39.6 – 44.1	41.2 ± 1.1	38.6 – 43.5
Pre-anal length [c]	72.1 ± 1.2	70.3 – 74.1	70.6 ± 1.3	67.2 – 73.0
Pre-dorsal diagonal [c]	54.8 ± 1.1	53.0 – 56.7	53.4 ± 1.0	51.0 – 55.1
Dorsal fin base length	17.0 ± 0.9	15.4 – 18.5	16.1 ± 1.2	14.2 – 19.3
Anal fin base length	9.2 ± 0.7	8.0 – 10.7	9.3 ± 0.9	7.8 – 11.7
Pectoral fin length	22.9 ± 1.9 [d]	19.8 – 26.6	22.0 ± 1.9	18.3 – 25.0

[a] p<0.001, t-test for mean size comparison between varieties
[b] p<0.001 and [c] p<0.05, t-test for between variety comparison on size-corrected data
[d] p<0.05, t-test for within variety sexual dimorphism on size-corrected data

The two varieties significantly differ in eight other morphometric characters as well, i.e. L_{PrO}, L_H, L_{PrD} (t-test, $p<0.001$) and L_{CP}, L_{PsO}, HD, L_{PA}, and PDD (t-test, $p<0.05$) (Table 1). Of these, pre-orbital length (snout) of the RF variety is about 7.7 %L_H (or 2.8 %L_S) longer than that of *P. dorsalis*, while the head length is 2.8 %L_S longer in the RF. Although significant, the numerical differences in the rest of the characters between varieties are less than 1.6%L_S. For the discriminant function analysis, the 'number of transverse scales ('T-Scales') could not be included as a variable due to within group zero variation, and thus. DFA based on 11 variables (that do not show sex-based differences) identified a model that contains only two significant morphometric contributors. The L_{PrO} (partial Wilk's λ = 0.673) is the mot important contributor for the discrimination between the two varieties while the other contributor was L_{DFB} but with a partial Wilk's λ of 0.916 indicating much lower discriminatory power. The rest of the characters were either not significant contributors or those not in the model. The plot of the principal components using the two significant contributors (L_{PrO} and L_{DFB}) and the 'T-Scales' depicted 100% separation of the two varieties along the factor 1 axis (Figure 3).

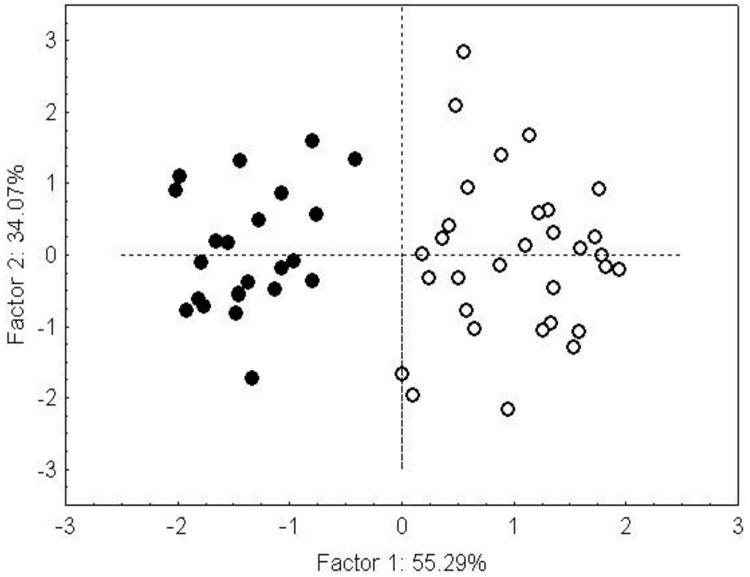

Figure 3 Plot of principal component analysis using two size-corrected morphometrics (pre-orbital length and dorsal fin-base length) and number of entire transverse scales above lateral line of the red fin *Puntius* variety (•) and *Puntius dorsalis* (o) (loadings for Factor 1: T-scales 0.917 and PrOL -0.867, Factor 2: FBD 0.956).

Isozyme analysis resulted in a total number of 14 putative isozyme loci coding for seven enzymes (*Gpd*, *Idh*, all five *Ldh* isozymes, two *Mdh* isozymes, two *Me* isozymes, two *Pgi* isozymes and *Pgm*). All putative loci except *Pgi*-1 and *Pgi*- 2 are monomorphic. Three putative loci are taxonomically informative (most common allelic product has differential migration between the two varieties), namely *Idh*, *Mdh*-1 and *Pgm* which showed clearly resolvable fix allelic differences between the two varieties, and therefore were diagnostic of the RF variety and *P. dorsalis* (Figure 4). For *Idh*, more cathodic isozyme representing a slow moving allelic product (homozygous) in the RF variety than in *P. dorsalis* is resolved. For *Mdh*-1 and *Pgm*, isozymes with more anodic mobility representing a faster moving allele product each (homozygous) are resolved in the red variety compared to that in *P. dorsalis*. Isozymes suggesting two *Pgi* putative loci are resolved, and both are polymorphic at 1% criterion (the frequency of the most common allele is <0.99), however, the number of individuals of each variety was not sufficient to report the genotypic distributions and statistical differentiation. Of the rest of the resolvable loci, *Me*, *Ldh* and *Gpd* are all monomorphic, and the same putative allele representing isozymes with the same electrophoretic mobility is expressed in both varieties.

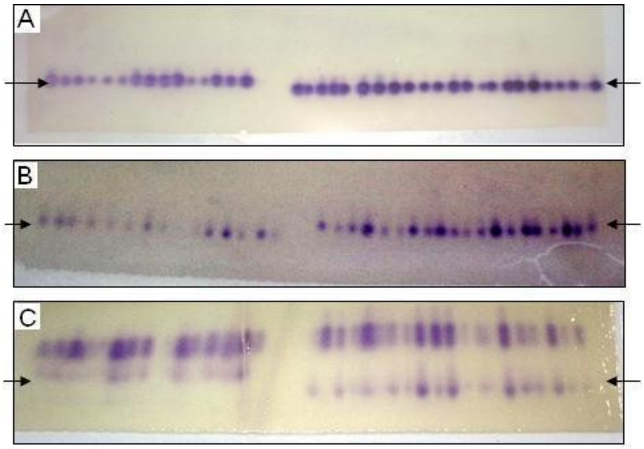

Figure 4 Photographs of the starch gels showing the fixed allelic differences between red fin *Puntius* variety (n = 15, left) and *Puntius dorsalis* (n = 22, right) at three putative isozyme loci PGM (A), IDH (B) and MDH-1 (c). Left and right arrows indicate level-differences at respective loci between the two varieties.

4 Discussion

Results of the present genetic and morphological analysis clearly reveal that a marked level of divergence exists between the 'red-fin' *Puntius* variety and the common *P. dorsalis* in Sri Lanka. Currently used dichotomous taxonomic keys based on external body shape and other characters for cyprinids in Sri Lanka (Deraniyagala 1952, Munro 1955) place the RF variety closer to *P. dorsalis* than any other congener. However, the absence of 4½ transverse scales above lateral line (LtL) in the RF variety fails to identify it as *P. dorsalis*. Red-fin variety has one less transverse scale rows in the upper body (above LtL) compared to *P. dorsalis*, which is an autaphomorphy that has so far not been reported in literature, and thus the identification of the redfin variety as *P. dorsalis* is apparently doubtful. The present data does not support the status of redfin variety as a color morph of *P. dorsalis*. In addition, the redfin variety does not share the body coloration and markings of *P. dorsalis* described in Deraniyagala (1952) at any stage of its life. Observed phenotypic distinctiveness is likely to represent a deep evolutionary split between them.

The maximum body size we can report of the RF variety is about 80 mm SL (not in present data), indicating that it is a relatively smaller fish compared to *P. dorsalis*, which is reported to grow up to 250 mm SL in Sri Lanka (Deraniyagala, 1952). The RF specimens as small as 50 mm SL collected in the present study are found to be reproductively mature. As indicated by their habitat differences and different adult size distributions, they probably have different life histories as well. No distinctive body colorations are present between juveniles and larger RF fish, either in living or preserved status.

It is generally accepted that sympatric morphs having fixed allelic differences at a given locus provides an unambiguous genetic evidence for complete reproductive isolation between them (Shaklee *et al*., 1982; Taylor & Bentzen, 1993; Turner, 1999). Fix allelic differences at a given nuclear locus are also indicative of diagnosable lineages, which may in turn represent minimal terminals for a phylogenetic analysis (Nixon & Wheeler, 1990; Davis & Nixon, 1992). Among molecular markers, fixed allelic differences in isozyme loci are particularly useful indicators of a considerable divergence between lineages and taxa (Shaklee *et al.*, 1982; Avice, 1994; Ward & Grewe, 1995). Analysis of isozymes/ allozymes has been used for discrimination of species and clarification of taxonomic status in many taxa (e.g. Rossi *et al.*, 1998; Gusmão *et al.*, 2000; Manchenko *et al.*, 2000) particularly for cryptic siblings of fish (Shaklee *et al.*, 1982; Smith & Robertson, 1981). The detected fix allelic differences provide evidence that the RF variety is considerably diverged from the formally described species *P. dorsalis*, suggesting reproductive isolation and deep lineage splitting. The present genetic and morphological evidence stipulating species status for the red-fin variety assures the agreement with the criteria of various species concepts including phylogenetic (PSC) and biological species concepts (Turner, 1999). The examination of fresh collections made in the present work further substantiates the need of taxonomic clarification of the traditionally presumed 'red-fin *dorsalis*'. The RF variety share superficial similarities more with *P. dorsalis* than with any other congeneric species or members of the subfamily Cyprininae. This study provides clear evidence that the number of transverse rows of scales in this taxon is an autapomorphy (i.e. a character shared by all members of the group but not in the other closely related taxon), which is one row of scales (above lateral line) less than in *P. dorsalis*. The above autopomorphy apparently exists without any significant impact on the body depth of the red fin variety. Therefore, the wider, coarser appearance of the scales (along dorso-ventral line), and the slightly elevated positioning of the lateral line in the RF compared to *P. dorsalis* are explicable. Moreover, conspicuously longer 'snout' characterizes the red-fin, and this is likely to represent some selective advantage as well. Morphometric distinctiveness of the red-fin variety is further substantiated by the PCA based on just two morphometrics and one meristic character. Overall, the present evidence proposes that the morphological distinctiveness of the two taxa studied have a genetic basis. A complete taxonomic treatment for the redfin variety is a requisite, while a phylogenetic analysis involving all *Puntius* congeners may give insight on the evolutionary relationship and phylogenetic position of the new species.

Identification and description of species is a foremost step in conservation planning and appropriate decision-making especially with respect to vulnerable taxa. According to recent literature, the survey of freshwater fish fauna in Sri Lanka and adjacent countries in the region is far from complete (Bhat, 2003; Pethiyagoda, 2006; De Silva *et al.*, 2007). Application of molecular techniques in clarification of problematic taxa and identification of new species should be a forward step in completion of checklists of species. Relevant work with respect to freshwater fish fauna of Sri Lanka and adjacent countries is relatively rare.

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