Contributions to Zoology, 82 (3) 115-122 (2013)

Phenotypic variation in the Snowtrout *Schizothorax richardsonii* (Gray, 1832) (Actinopterygii: Cypriniformes: Cyprinidae) from the Indian Himalayas

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Key words: discriminant function analysis, India, shape, trans-Himalaya, truss box

Abstract

We investigated intraspecific variation of the Snowtrout, *Schizothorax richardsonii* on the basis of morphometric characters. Altogether, 217 specimens were collected from four rivers in the Western and Central Indian Himalaya. A truss network was constructed by interconnecting 14 landmarks to yield 31 distance variables that were extracted from digital images of specimens using tpsDig2 and PAST software. Transformed truss measurements were subjected to univariate analysis of variance, factor analysis and discriminant analysis. All variables exhibited significant differences between the populations. Altogether 86.6% of the specimens were classified into their original populations (82.9 % under a 'leave-one-out' procedure). With factor analysis measurements of the head region, the middle portion and the caudal region had high loadings on the on first and second axis. The results indicated that *S. richardsonii* has significant phenotypic heterogeneity between the Western and Central Indian Himalayas. We hypothesize that the marked interspecific variation in *S. richardsonii* is the result of local ecological conditions.

Contents

Introduction

The study of morphological characters, with the aim of defining or characterizing fish stock units, has for some time been a strong interest in ichthyology (Cadrin, 2000). In general, a 'fish stock' is a local population adapted to a particular environment, having genetic differences from other stocks (MacLean and Evans, 1981). Although genetic differences between stocks are a condition of this definition, phenotypic variations still continue to have an important role in stock identification among groups of fish (Costa *et al*., 2003). The usage of phenotypic characters is particularly important where the differences are attributed to environmental influences rather than to genetic differentiation (Mir *et al*., 2013a).

Various tools, such as meristics and morphometrics, traditional tags, parasites as natural tags, otolith chemistry, molecular genetics and electronic tags have been used for the purpose of stock identification, among which the study of morphometric traits is one of the frequently employed and cost-effective methods. Traditional multivariate morphometrics, accounting for variation in size and shape, have successfully discriminated between many fish stocks (Turan, 1999). However, traditional methods have been enhanced by image processing techniques, through better data collection, more effective descriptions of shape, and new analytical tools. The development of image analysis systems has facilitated progress and diversification of morphometric methods and expands the potential for using morphometry as a tool for stock identification (Cadrin and Friedland, 1999; Mir *et al*., 2013b). Truss network is much more powerful in identifying intraspecific groups with different life history stages according to shape variation than manual measurements (Strauss and Bookstein, 1982; Bookstein, 1991). The methodology is predicated on the measurement of across-body distances connecting two morphological landmarks from a sequential series of connected polygons. This type of landmark-based technique using geometric morphometrics imposes no restrictions on the direction of variation and localization of shape changes and is highly effective in capturing information about the shape of an organism (Cavalcanti *et al*., 1999).

The fishes of genus *Schizothorax* are the members of the family Cyprinidae, commonly known as snowtrouts, consist of 15 genera and over 100 species all over the world (Mirza, 1991). In India, these species are distributed in the cold waters from Jammu and Kashmir (Sunder and Bhagat, 1979), to Assam and Eastern Himalayas through Bhutan and Sikkim at an elevation of 1180-3000 m (Jhingran, 1982). So, far 28 species of snow trout have been reported in the Himalayan and Sub-Himalayan regions. Their inherent biological features, such as short growth period and slow growth to maturity, are the main constraints hindering their growth and population increase (Mir *et al*., 2012). This species of this genus are remarkably similar in general morphology and are often difficult to distinguish based on external morphological characters across the Indian Himalayas (Chandra *et al*., 2012). The taxonomy of these fishes has been studied from time to time (Negi and Negi, 2010; Mir *et al*., 2012) but a clear picture of their status has not been available till recently in a consolidated form (Vishwanath, 2010; Chandra *et al*., 2012).

Schizothorax richardsonii (Gray, 1832) is a coldwater fish, commonly known as Snowtrout, classified as vulnerable (VU) in India by the IUCN (2012). The distribution of this cyprinid species is confined to the Himalayan and Sub-Himalayan rivers and streams along Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Assam and Sikkim. Besides India, this species is distributed in Bhutan, Nepal, Pakistan and Afghanistan (Talwar and Jhingran, 1991). Although *S. richardsonii* is widely distributed along the Himalayan foothills and previous studies have indicated that it is abundantly and commonly found, but recent observations indicate drastic decline in the populations of many areas of its range due to introduction of exotic species, damming and overfishing (Negi and Negi, 2010). There is a strong belief that if alien species introductions are carried out throughout its range, this species may be completely displaced by exotic salmonids (Vishwanath *et al*., 2010). The phenomenon of slow growth, poor disease resistance and low survival rate are serious threats, which greatly affect the enhancement and stocking program. Therefore, the present study was considered as a first step towards the aim of exploring the stock structure of this species based on morphometric characters, using truss network system for its successful development and management across the Indian Himalaya.

Material and methods

Study area

The Himalaya is the youngest mountain chain on the planet and is believed to be still evolving, and thereby, is both geologically and geomorphologically unstable. Because of its extremely active geodynamic condition, even small tampering with the geoecological balance can initiate environmental changes that may eventually lead to alarming proportion (Bilham and Gaur, 2000; Valdiya, 2003). The Indian Himalayan region (IHR) stretches over 2500 km from Jammu and Kashmir in the West to Arunachal Pradesh in the East, between 21°57' – 37°5'N latitudes and 72°40' – 97°25'E longitudes. This great chain of mountains in Indian territory extends all along the northern border of the country from the eastern border of Pakistan in the West to the frontiers of Myanmar in the east covering partially/fully twelve states of India, *viz*., Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Sikkim, Arunachal Pradesh, Nagaland, Manipur, Mizoram, Tripura, Meghalaya and hills of Assam and West Bengal.

The Indian Himalayas are mainly drained by 19 rivers, including three major river systems; the Indus, Ganga and Brahmaputra. The Indus Basin system is the longest river system which originates from Western Indian Himalaya (160,000 km^2) and consist of five rivers. The Ganga basin system contributes nine rivers and originates from Central Indian Himalaya (150,000 km^2) and the Brahmaputra basin is the second longest river system which starts in Eastern Himalaya (150,000 km^2) having five rivers (Hora, 1954). This study includes four rivers two from Western Himalaya (Jhelum and Lidder) and two from Eastern Himalaya (Alaknanda and Mandakini). River Jhelum is a tributary of Indus basin and has a total length of about 813 km; it originates from Verinag Spring situated at the southeastern part of the valley of Kashmir in India. The Lidder River is the second largest tributary of river Jhelum covering 73 km distance in the Kashmir region of India and its source (Kolhoi Glacier) is located at a height of 4,653 masl (meters above sea level). These are the two least explored rivers of Western Himalaya and are an important fishery resource for the daily living of the local people. River Alaknanda is a tributary of the river Ganga basin of Central Himalaya in Uttarakhand. It is about 190 km long and originates from Satopanth Glacier in Garhwal Himalaya (Menon, 1954). River Mandakini is the least explored tributary of river Alaknanda. These two rivers are full of fish and play a critical role in the regional economy and food security of the people.

Sampling and digitization of samples

A total of 217 *Schizothorax richardsonii* specimens were collected from four different rivers across Indian Himalaya *viz*. Jhelum, Lidder, Alaknanda and Mandakini, by using different fishing gear (cast nets and gill nets) from January 2011 to November 2011 and analyzed for morphometric variations. The specimens of *S. richardsonii* were obtained before the breeding season and after the spawning period to avoid a bias toward size difference. The mesh size of the fishing gear (cast nets: 9m length, 9m breadth and 1/2cm mesh size and drag nets: 100m length, 20m breadth, 1⁄2cm mesh size) was designed for the large sized specimens to any avoid fingerling and fry capture. The GPS coordinates, altitude (masl), flow rate (ms^{-1}; meter per second), number of samples, min-max length and weight of *S. richardsonii* across the Indian Himalaya are presented in Table 1.

The sampled specimens were first cleaned in running water, drained and placed on a flat platform with graph paper as a background for calibrating the coordinates of digital images. The fins were erected to make the origin and insertion points visible. Each individual was labelled with a specific code to identify it. A Sony Cybershot DSC-W300 digital camera was used for capturing the digital images. To avoid errors in image capture all photos were taken by a single person from same angle and height at every shot. After image capture, each fish was dissected to identify the sex by macroscopic examination of the gonads. Gender was used as the class variable in ANOVA to test for significant differences in the morphometric characters, if any, between males and females of *S. richardsonii*.

Measurement of truss distances

The extraction of truss distances from the digital images of specimens was conducted using a linear combination of three software platforms, tpsUtil, tpsDig2 v2.1 (Rohlf, 2006) and Paleontological Statistics (PAST; Hammer *et al*., 2001). The truss protocol used for the *S. richardsonii* was based on fourteen landmarks (Fig. 1AB). A box truss of 31 lines connecting these landmarks was generated for each fish to represent the basic shape of the fish (Strauss and Bookstein, 1982; Mir *et al*., 2013b). All the measurements were transferred to a spreadsheet file (Excel 2007), and the X-Y coordinate data transformed into linear distances by computer (using the Pythagorean Theorem) for subsequent analysis.

Multivariate data analysis

Size dependent variation was corrected by adopting an allometric method as suggested by Elliott *et al*. (1995):

$$M_{adj} = M\ (L_s/L_0)^b$$

where M is original measurement, M_{adj} is the size adjusted measurement, L_0 is the standard length of the fish, L_s the overall mean standard length, and *b* was estimated for each character from the observed data as the slope of the regression of log *M* on log L_0 using all fish from every group. The results derived from the allometric method were confirmed by testing significance of the correlation between transformed variables and standard length (Turan, 1999). Univariate analysis of variance (ANOVA) was performed for the 31 morphometric characters to evaluate the significant difference

Table 1. GPS coordinates, altitude (masl; meters above sea level), flow rate (ms^{-1}; meters per second), number of samples, min-max. length and weight of *Schizothorax richardsonii* across the Indian Himalaya.

Parameters	rivers (sites)			
	Alaknanda (Pauri)	Mandakini (Kedarnath)	Jhelum (Srinagar)	Lidder (Pahalgam)
latitude °N	30°10'31"	30°43'59"	34°8'20"	34°0'45"
longitude °E	78°37'24"	79°4'6"	74°40'52"	75°18'56"
altitude (masl)	1814	3588	1584	2740
maximum flow rate (ms^{-1})	1.43	1.53	1.38	1.92
number of samples	69	41	58	49
min-max tl (cm)	10.5-48.6	12.3-51.5	9.8-45.3	10.2-40.5
min-max bw (g)	35.5-700	28.3-800.4	30.4-680.4	32.6-610.9

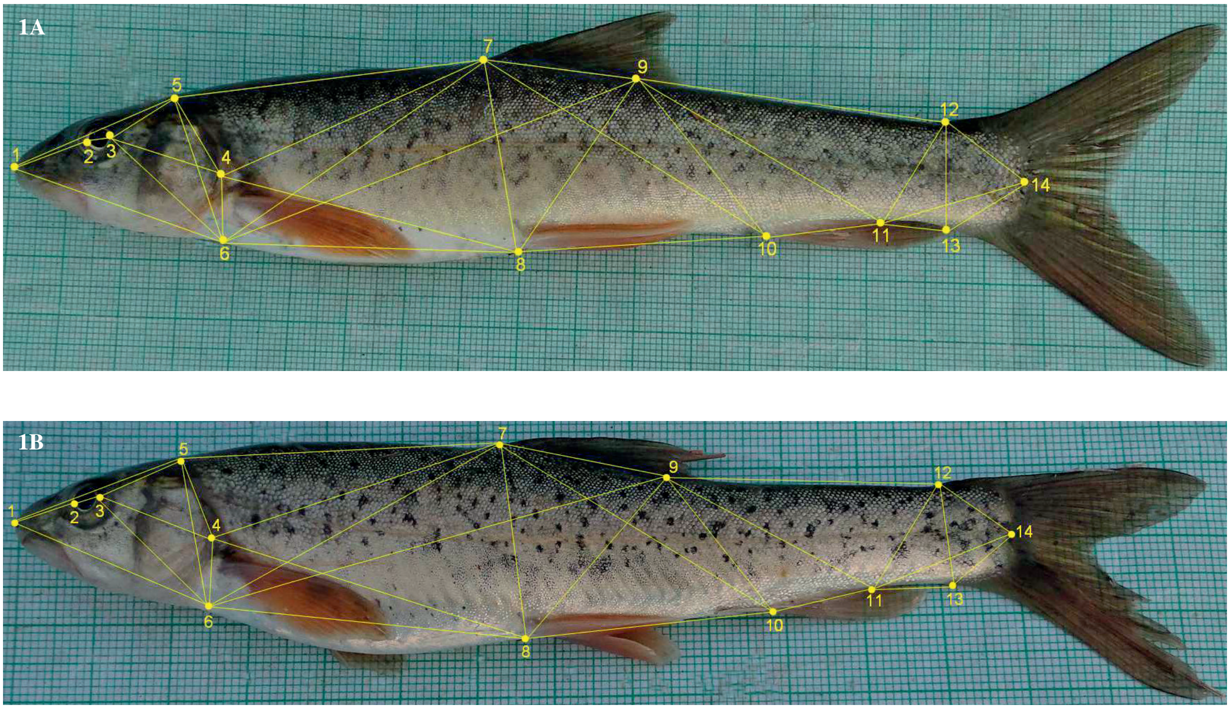

Fig. 1. Schematic image of *S. richardsonii* depicting the 14 landmarks and associated box truss used to infer morphological differences among populations. 1 Tip of snout; 2 end of eye towards mouth; 3 end of eye towards tail; 4 end of operculum; 5 forehead (end of frontal bone); 6 dorsal origin of pectoral fin; 7 origin of dorsal fin; 8 origin of pelvic fin; 9 termination of dorsal fin; 10 origin of anal fin; 11 termination of anal fin; 12 dorsal side of caudal peduncle, at the nadir; 13 ventral side of caudal peduncle, at the nadir; 14 end of lateral line (Adapted from truss box, after Strauss and Bookstein (1982) and Bookstein (1991)). Fig 1A (Western Himalaya), Fig 1B (Central Himalaya).

between the four locations. These 31 transformed truss measurements were subjected to FACTOR analysis, to explain these variables in terms of their common underlying dimensions. A maximum likelihood method was used to extract the factors. With the assistance of Scree plot, the cumulative variance explained by the factors and the meaningful biological groupings of the traits loading on each factor were taken into consideration to retain the number of factors for a rotation procedure. The retained factors were subjected to a Varimax rotation procedure and to identify the variables demonstrating high loadings for a given factor, the rotated factors were subjected to a scratching procedure, as described by Hatcher (2003). The Wilks' λ was used to compare the difference between all groups. The discriminant function analysis (DFA) was used to calculate the percentage of correctly classified (PCC) fish and a cross-validation using PCC was done to estimate the expected actual error rates of the classification functions. Statistical analyses for morphometric data were performed using the SPSS vers. 16.1.0 and Microsoft Excel 2007.

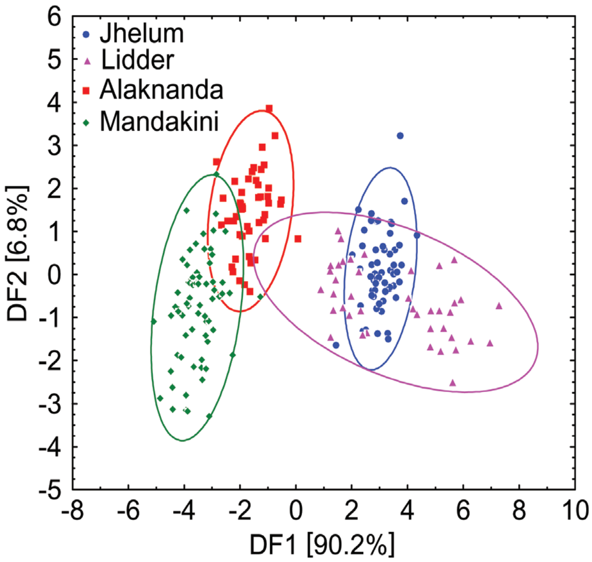

Fig. 2. Discriminant analysis plot for morphometric variables *Schizothorax richardsonii* collected from four rivers across from Indian Himalaya.

Table 2. Results of Wilks' lambda tests of the discriminant function analysis (function 1 through 3) of morphometric variables of *Schizothorax richardsonii* collected from four rivers across Indian Himalaya.

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1 through 3	.062	581.554	27	.000
2 through 3	.519	137.554	16	.000
3	.806	45.168	7	.000

Table 4. Percentage of specimens classified in each group and after cross validation for morphometric measurements of *Schizothorax richardsonii* from four rivers across Indian Himalaya (86.6% of original grouped cases correctly classified, 82.9% of cross-validated grouped cases correctly classified).

	Original group (%)				
Rivers	Jhelum	Lidder	Alaknanda	Mandakini	Total
Jhelum	81.0	19.0	.0	.0	100.0
Lidder	17.1	78.0	4.9	.0	100.0
Alaknanda	.0	.0	91.8	8.2	100.0
Mandakini	.0	.0	7.2	92.8	100.0
	Cross-validated (%)				
Jhelum	77.6	22.4	.0	.0	100.0
Lidder	19.5	73.2	7.3	.0	100.0
Alaknanda	.0	.0	89.8	10.2	100.0
Mandakini	.0	.0	11.6	88.4	100.0

Results

None of the size-adjusted truss measurements showed a significant correlation with the standard length of the fish, indicating that the variation in body length had been successfully removed by the allometric transformation. Among four selected rivers, means of all the truss measurements of *S. richardsonii* were found to be significantly ($P < 0.001$) different in univariate analysis of variance. The morphometric characters between two sexes did not differ significantly ($P > 0.05$) hence; the data for both sexes were pooled for all subsequent analysis.

A common problem with many fish morphology studies that use multivariate analysis is a potentially inadequate sample size. For decades, authors of theoretical works on DFA recommended that the ratio of the number of organisms measured (N), relative to the parameters included (P) in the analysis, should be at least 3-3.5 (Kocovsky *et al*., 2009). Small N values may fail to adequately capture covariance or morphological

Table 3. Contribution to discriminant functions (DFs) of morphometric variables of *Schizothorax richardsonii* collected from four rivers across Indian Himalaya. *indicates largest absolute correlation between each variable and any discriminant function.

Function			
Character	DF1 [90.2%]	DF2 [6.8%]	DF3 [3.0%]
1-6	.573(*)	-.069	-.184
10-11	.510(*)	.091	.063
6-9	.506(*)	.095	.022
11-14	.483(*)	.129	.466
12-14	.476(*)	-.322	.027
8-9	.470(*)	.063	.211
3-6	.444(*)	.177	-.337
13-14	.396(*)	-.102	-.065
3-5	.383(*)	-.329	.127
5-6	.360(*)	.126	-.325
11-12	.350(*)	.239	.190
6-7	.252(*)	-.101	-.186
2-3	.208(*)	-.128	.147
11-13	.188(*)	.020	.179
9-10	-.044	.629(*)	.215
9-11	.445	.552(*)	.176
7-10	.165	.540(*)	-.072
9-12	.391	.501(*)	.179
1-5	.289	-.393(*)	.288
4-6	.359	.365(*)	-.336
1-2	.053	-.349(*)	.273
7-9	.242	.278(*)	-.189
3-4	.159	-.162	.519(*)
4-8	.341	.359	-.501(*)
4-7	.367	-.046	-.454(*)
8-10	.384	.222	.452(*)
5-7	.329	-.067	-.396(*)
7-8	.180	.118	-.310(*)
4-5	.241	-.269	-.303(*)
6-8	.167	.118	-.230(*)
12-13	.023	.036	.162(*)

variation, which may lead to false conclusions regarding differences among groups (McGarigal *et al*., 2000). In the present investigation all 31 characters were retained and under these circumstance the N:P ratio was 7.0.

The Wilks λ tests of discriminant analysis indicated significant differences in morphometric characters of four populations, and all the functions were highly significant ($P<0.001$; Table 2).

Plotting DF1 and DF2 showed clear between-sample differentiation (Fig. 2). The first DF accounted for 90.2% and the second accounted for 6.8% of the between group variability, explaining 97% of the total between groups variability. All the samples from the rivers of Western and Central Indian Himalayan regions were clearly separated from each other in discriminant space. However, Alaknanda River showed slight overlapping with Mandakini River and river Jhelum showed intermingling with Lidder River.

Pooled within-group correlations between discriminating variables and DFs revealed that fourteen body measurements (1-6, 10-11, 6-9, 11-14, 12-14, 8-9, 3-6, 13-14, 3-5, 5-6, 11-12, 6-7, 2-3 and 11-13) covering whole organism contributed dominantly to first DF. The loadings on second DF (9-10, 9-11, 7-10, 9-12, 1-5, 4-6, 1-2 and 7-9) dominantly contributed to head, middle region and caudal peduncle of the fish. The third DF loadings (3-4, 4-8, 4-7, 8-10, 5-7, 7-8, 4-5, 6-8 and 12-13) were concentrated on opercular and predorsal region, implying that these characters are the most important in the description of population characteristics (Table 3). Factor loadings are correlations between the variables and the factors. In the present study the variables loaded on first, second and third factors were mostly positive indicating the positive correlation between the variables within a factor. This relationship is expected as the variables loading on first factor belonged to the middle portion of the body and these traits grow proportionately with one another. Another reason for positive loadings of variables may be, due to the rotation of the factors which helps to reduce the number of negative loadings to a minimum.

The classification of individuals into their original population varied between 78.0% and 92.8% by discriminant analysis and 86.6% of individuals could be classified into their original a priori grouping (Table 4). The proportion of correctly classified Mandakini River samples into their original population was the highest (92.8%). A cross-validation test using leave one out procedure was also performed by which 82.9% of the samples were correctly classified into their original populations. The slight intermingling was observed between rivers of Western Himalaya (Jhelum and Lidder; 19% misclassification) and between rivers of Central Himalaya (Alaknanda and Mandakini; 7.2% misclassification; Table 4).

Discussion

In general, fishes show higher degree of variation within and between populations than other vertebrates, and are more susceptible to environmentally induced morphological variation (Wimberger, 1992). Such variation in morphology is commonly due to the isolation of portions of a population within local habitat conditions. A sufficient degree of isolation may result in notable phenotypic and genetic differentiation among fish populations within a species, as a basis for separation and management of distinct populations (Turan, 2004). Such differentiation can occur through different processes. For example, reproductive isolation between different stocks of fishes may arise by homing to different spawning areas (Hourston, 1982) or by hydrographic features, which reduce or prevent migration between areas (Iles and Sinclair, 1982). Failure to recognize or to account for stock complexity in management units has led to an erosion of spawning components, resulting into a loss of genetic diversity, and other unknown ecological consequences (Begg *et al*., 1999).

The results obtained from the truss-based morphometrics indicated that the *S. richardsonii* showed significant phenotypic heterogeneity between the two geographically isolated Himalayan regions. Discriminant function analysis (DFA) could be useful method to distinguish different stocks of the same species (Karakousis *et al*., 1991). In the present investigation, 86.6% of individuals were correctly classified into their respective groups by DFA, indicating high variation between two stocks. Turan *et al*. (2004) studied Anchovy, *Engraulis encrasicolus* (Linnaeus, 1758) from the parts of Mediterranean Sea, finding significant morphometric heterogeneity among different populations by applying DFA and attributed it to migration of the fish. The DFA confirmed that the variation in morphological measurements was evident in the head region, eye diameter, body depth and caudal peduncle, between these morphologically distinct populations of snowtrout. Hossain *et al*. (2010) applied DFA and PCA on three populations of *Labeo calbasu* (Hamilton, 1822) from river Jamuna, Halda and hatchery and reported morphological discrimination among them due to the environmental factors and local migration of the fish. Similar observations were noticed by Khan *et al*. (2012) in case of *Channa punctatus* (Bloch, 1793) from three Indian rivers and lead the conclusion that environmental conditions play an important role in spatial distribution, movement and isolation of fish stocks. Mir *et al*. (2013a,b) observed similar inferences in *Schizopyge niger* (Heckel, 1838)) and *Labeo rohita* (Hamilton, 1822) from Indus basin and Ganga basin respectively, and attributed to changing physical and ecological conditions of water bodies.

The width of sampling location on the Jhelum River is more than 100 m with extensive human development. A large scale of diversions is present on this river which almost de-waters the natural channel towards upstream. The Pahalgam sampling station on the Lidder River is less than 50 m wide with less human interruption and minimally-regulated towards the upstream. This may be one of the reasons of stock divergence between these two rivers. River Lidder is largely fragmented due to presence of water impoundments and is broken into a large number of small streams for the irrigation purpose, which has resulted into the fishery stock divergence and ultimately to intraspecific morphological dichotomy. The variation among the stocks of four populations of two geographically isolated parts of Himalaya could also be a consequence of phenotypic plasticity in response to uncommon hydrological conditions like differences in alkalinity, current pattern, temperatures, turbidity, and land-use pattern among these rivers. The closeness between the stocks may be due to their similar habitat attributes and environmental impacts.

The causes of morphological differences among different populations are often quite difficult to explain. It has been suggested that the morphological characters of fish are determined by genetic, environment and the interaction between them (Poulet *et al*., 2004). The environmental factors prevailing during the early development stages, when the individual's phenotype is more amenable to environmental influence is of particular importance (Pinheiro *et al*., 2005). The phenotypic variability may not necessarily reflect population differentiation at the molecular level (Ihssen *et al*., 1981). Apparently, the fragmentation of river impoundments can lead to an enhancement of pre-existing genetic differences, providing a high interpopulation structuring (Esguicero and Arcifa, 2010). Thus, the possibility exists that the observed morphological variations in the present study might be due to genetic differences among the populations.

The truss system can be successfully used to investigate stock separation within a species, as reported for other species in freshwater and marine environments. In this study, the truss protocol revealed a clear separation of *S. richardsonii* stocks observed from two distinct geographic regions of Indian Himalaya, suggesting a need for separate management strategy to sustain the stock for future use. The observation given in the present study can further be confirmed based on molecular and biochemical methods. Application of molecular genetic markers such as microsatellite and mtDNA applications along with morphometric studies would be effective methods to further examine the genetic component of phenotypic discreteness between geographic regions and to facilitate the development of management recommendations. This additional examination would provide further confirmation of the stock structure resolved in this study with the truss analysis. However, further management measures have to be taken by the enforcement of mesh size regulation and imposition of a closed season during the breeding of some commercially important fish species in Himalayan regions to sustain this resource for the future use.

Acknowledgements

The authors are grateful to the fishermen for their help in fish capture. The authors would also like to thank Dr. Costa and an anonymous reviewer for their constructive comments regarding this work.

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Received: 25 January 2013
Revised and accepted: 7 June 2013
Published online: 13 September 2013
Editor: R. Vonk