

Intra- Specific Morphometric and Genetic Comparisons in Hillstream Loach, *Nemacheilus montana* (McClelland 1838) from Western Himalaya, India

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ABSTRACT: Intra- specific variation between the *Nemacheilus montana*, a hill stream fish species was investigated with morphometric and mitochondrial cytochrome b sequence data. An analysis was conducted to compare specimens from two lesser Himalayan hill stream tributaries of River Ganga: Khanda and Saung in the Western Himalayan region of India. The results of principal component analysis on sixteen morphometric variables and fourteen derived variables per individual were found to be considerably different for the two stream samples. The meristic characters also varied significantly. The cyto-b sequence analysis showed no congruence with the morphometric data suggesting phenotypic plasticity in the two populations.

KEYWORDS: Morphology, meristic, loaches, inter-population, cytochrome b

I. INTRODUCTION

Nemacheilus montana McClelland (1838) earlier described as *Nemacheilus montanus* belongs to family Balitoridae. The fishes of this family have a preference for water with strong current, and many species only occur in torrential mountain streams, therefore they are known as hill stream loaches. A few species of this group of fishes are also occasionally trade as aquarium fish [1].

The multivariate analysis has been used as a powerful method for the determination of morphological variation between the populations of species [2, 3, 4, and 5]. The mitochondrial Cyt-b gene contains both slowly and rapidly evolving codon positions, as well as more conservative and more variable regions or domains overall. Therefore, the cyt-b gene has been used for a diversity of systematic questions, from phylogeny [6 and 7] to the population and recent divergence levels [8 and 9].

This paper investigated the morphometric and mitochondrial DNA variability of *Nemacheilus montana* from two hill streams of Western Himalayan in India, and delineates the principal morphological characters that elucidate the differentiation.

II. MATERIAL AND METHODS

SAMPLE COLLECTION

Specimens of *N. montana* (Fig.1) were collected from two hill stream tributaries of river Ganga basin from Western Himalayan region of India.

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Figure 1 *Nemacheilus montana* collected from (a) Khanda stream and (b) Saung stream

The sampling locations included Khanda (S1) and Saung (S2) (Fig. 2). Altogether fifty two specimens were collected for detail morphometrics (Table 1). For the molecular analysis, the muscle tissues were dissected from thirty seven specimens and preserved in a 95% ethanol.

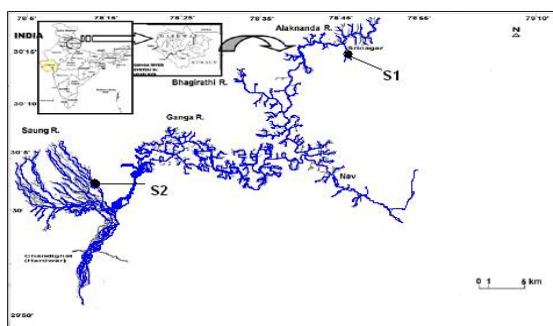


Figure 2 The drainage map showing sample sites from Khanda (S1) and Saung (S2) streams in Western Himalaya.

Stream (site code)	Location of samples	Samples size	
		Morphometric study	Molecular analysis
Khanda(S1)	30°12'89" N; 78°46'34" E	28	17
Saung (S2)	30°20'63" N; 78°08'15" E	24	20
Total		52	37

Table 1 Details of samples taken for studies.

MORPHOMETRIC AND MERISTIC CHARACTERISTICS

In total, twenty one morphometric characteristics and eighteen length ratios were analyzed from each fish specimen. Measurements were taken using electronic digital calipers to the nearest 0.01mm. The meristic characteristics included number of bands on body and dorsal fin rays.

DNA EXTRACTION

High molecular weight DNA was isolated from fin and muscle tissues. Approximately 50mg of tissue was taken using sterile scalpel blades and forceps. The total genomic DNA was isolated by using a standard phenol-chloroform procedure of [10] followed by digestion with proteinase-K at 37° C for overnight, alcohol precipitation and wash with 70% ethanol. The final pellet of DNA suspended with 50 µl (microliters) of T.E. Buffer. The concentration of isolated DNA was estimated using a UV spectrophotometer. The DNA was diluted to get a final concentration of 100 ng/µl.

DNA AMPLIFICATION AND SEQUENCING

A 1127 base pair region of mitochondrial cytochrome b regions was amplified by PCR using an upstream primer L14724 (5'-TGACTTGAARAACCAACCGTTG-3') and a downstream primer H15915 (5'-CTCCGATCTCCGGATTACAAGAC -3'). Amplification was performed in a 50µl volume, each containing 5µl 10X PCR buffer, 2 µl (50 mM) MgCl₂, 0.25µl (0.05 mM) of each dNTP, 0.4µl (0.6 U) of Taq DNA polymerase (Bangalore Genei, Bangalore), 0.5 µl (0.01 mM) each of forward and reverse primer and 2µl genomic DNA. Amplification was carried out by a thermocycler (MJ PTC-200, Watertown, MA) at 95°C for 4 min for initial strand separation, followed by 40 cycles of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C, and a final 10 min extension at 72°C. Negative (without DNA template) and positive (lambda DNA) controls were also included in all experiments. The linear PCR product DNA samples were then sequenced by an automatic DNA sequencer (ABI 3730 DNA sequencer, Genewiz, NJ, USA).

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Statistical and DNA sequence analysis

A t-test was carried out to test the significance of morphological differences among two stream samples for all morphometric measurements and length ratios. Prior to t-test, an F-test was also performed to check the variance between the two stream samples. Welch test for unequal variances was used for the samples having low P-value ($P < 0.05$) in F-test.

To reduce the dataset and to eliminate the redundancy among variables, a data discarding process was done following t-test. Only those morphometric measurements and length ratios that were significantly different were retained for the further analysis

A multivariate technique, principal component analysis (PCA), using a correlation matrix was used to identify a combination of characters in our selected morphometric data set which accounted for the major variation between the two populations. In order to reduce the effect of size, all the morphometric measurements were log transformed prior to PCA analysis. This was necessary as, there were significant linear correlations between all morphometric characters and standard length of fish samples collected from two streams. Size-dependent variation was removed using an allometric formula [11]

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

Where M_{trans} is the transformed measurement, M is the original measurement, b is the within-group slope regression of the M versus SL , L_0 is the standard length of the fish, and L_s is the overall mean of the standard length.

The two meristic characteristic i.e., number of bands on body and dorsal fin rays were compared between populations using non-parametric Mann–Whitney U tests.

All the morphometric analyses were carried out using the software's Statistical Package for Social Sciences [12] and Minitab [13].

In order to analyze the molecular data, both light and heavy strand sequences of mitochondrial cytochrome *b* region were obtained for all the samples. A consensus sequence from combined light and heavy strands was made for all samples and then aligned by using ClustalW [14] with default parameters. Gene diversity analyses were performed with the software package DnaSP 4.2 [15]. For each data set, gene diversity statistics was estimated at nucleotide and haplotype levels. The neutrality was tested with the statistics D of [16] and D of [17], respectively.

III. RESULTS

MORPHOMETRICS

The mean, standard deviation, and significance value of each morphometric characteristic and derived measurements i.e., length ratios of *N. montana* are shown in Table 2a & 2b. Following t-test, all the morphometric characters and length ratios except PoDH, IOW, AFL, LDCB and PpL, SL/ PrDL, SL/Distance from pectoral fin to pelvic fin origin, SL/PFL and SL/AFL showed statistically significant difference between the two stream samples. The meristic characters of *N. montana* and the U-value are given in Table 3. The median values of both meristic counts were found higher in the Khanda stream samples. Results of Mann–Whitney U test showed that the number of bands on body and dorsal fin rays varied significantly between populations of Khanda and Saung streams.

In PCA analysis, the first principal component (PC1) with 44.79 % cumulative variance resulted in both positive and negative factor loadings (Table 4). B.D, LCPD, ratio of SL/DFL and from SL/DFBL, was among the characteristics most highly correlated with PC-1. The second and third principal component accounted for 16.77 % and 9.69 % of the total variance respectively. Principal component-2 described the variance, with SL, PIFL, PrDL and distance from Pectoral fin origin to Pelvic fin base being highly correlated with PC-2. Altogether the first three principal components accounted for 71.246 % of total variance in the two populations. Individual fish from each population are plotted in fig 3. Some level of separation along PC 1 is evident as shown in fig 3.

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Characters	S1	S2	Total	t-values
Standard length (S.L)	52.17 ± 2.68	56.32 ± 6.32	54.09 ± 5.11	2.98 (0.0056)**
Head length (H.L)	12.55 ± 1.17	11.20 ± 1.36	11.93 ± 1.42	3.82 (0.0004)**
Body depth (B.D)	9.93 ± 1.42	6.96 ± 1.07	8.56 ± 1.95	8.36 (0.0001)**
Snout length (SntL)	5.87 ± 1.01	3.88 ± 0.73	4.95 ± 1.34	7.98 (0.0001)**
Postorbital distance of Head (PoDH)	6.09 ± 1.24	5.69 ± 0.79	5.90 ± 1.06	1.42 (0.1600)
Eye diameter (ED)	2.06 ± 0.33	1.52 ± 0.12	1.81 ± 0.37	7.89 (0.0001)**
Interorbital length (IOW)	2.39 ± 0.49	2.31 ± 0.42	2.35 ± 0.45	0.63 (0.5295)
Dorsal fin length (DFL)	14.04 ± 1.74	10.27 ± 1.31	12.30 ± 2.44	8.69 (0.0001)**
Dorsal fin base length (DFBL)	9.85 ± 1.30	7.34 ± 1.21	8.69 ± 1.77	7.10 (0.0001)**
Pectoral fin length (PFL)	11.79 ± 1.10	10.62 ± 1.63	11.25 ± 1.48	3.07 (0.0034)**
Pelvic fin length (PIFL)	8.87 ± 0.80	9.43 ± 1.79	9.12 ± 1.36	1.42 (0.1655)
Anal Fin length (AFL)	9.33 ± 0.83	9.48 ± 1.22	9.40 ± 1.02	0.51 (0.6101)
Caudal Fin length (CFL)	12.90 ± 0.99	11.38 ± 1.72	12.20 ± 1.56	3.81 (0.0005)**
Pre dorsal length (PrDL)	25.17 ± 1.64	27.19 ± 3.31	26.10 ± 2.72	2.70 (0.0107)**
Length of origin of Dorsal to Caudal base (LDCB)	27.45 ± 1.56	27.70 ± 3.28	27.56 ± 2.48	0.34 (0.7322)
Pre-pelvic length (PpL)	28.62 ± 0.98	28.23 ± 3.26	28.44 ± 2.31	0.56 (0.5800)
Pre-anal length (PaL)	42.61 ± 1.67	40.99 ± 5.13	41.86 ± 3.74	1.48 (0.1500)
Distance from pectoral to pelvic	16.75 ± 1.47	18.78 ± 2.53	17.69 ± 2.26	3.46 (0.0014)**
Distance from pelvic to anal	14.97 ± 1.15	13.47 ± 2.18	14.28 ± 1.85	3.00 (0.0049)**
Least height of Caudal Peduncle (LCPD)	6.71 ± 1.09	4.82 ± 0.70	5.83 ± 1.32	7.50 (0.0001)**
Caudal peduncle length (CPL)	6.40 ± 1.06	8.69 ± 1.05	7.45 ± 1.56	7.74 (0.0001)**

** P < 0.01; P values are given in parentheses.

Table 2(a) Mean morphometric characters (mm)(± standard deviation) and t-values of *N. montana*.

Morphological descriptors	S1	S2	Total	t-values
SL/HL	4.18 ± 0.37	5.07 ± 0.72	4.59 ± 0.71	5.45 (0.0001)**
SL/SntL	9.08 ± 1.34	14.8 ± 2.19	11.72 ± 3.37	11.11 (0.0001)**
SL/ PrDL	2.07 ± 0.07	2.08 ± 0.23	2.08 ± 0.16	0.19 (0.8441)
SL/Pre-pelvic distance	1.90 ± 0.19	28.23 ± 3.26	1.90 ± 0.19	3.59 (0.0014)**
SL/Pre-anal distance	1.29 ± 0.14	40.99 ± 5.13	1.29 ± 0.14	4.54 (0.0001)**
SL/Distance from pectoral fin to pelvic fin origin	3.13 ± 0.22	3.03 ± 0.41	3.08 ± 0.32	1.04 (0.3018)
SL/PFL	4.46 ± 0.46	5.40 ± 0.92	4.89 ± 0.85	4.52 (0.0001)**
SL/PIFL	5.91 ± 0.45	6.10 ± 0.95	6.00 ± 0.72	0.91 (0.3693)
SL/Height of dorsal	3.77 ± 0.58	5.53 ± 0.70	4.58 ± 1.08	9.84 (0.0001)**
SL/AFL	5.63 ± 0.58	6.01 ± 0.90	5.80 ± 0.76	1.76 (0.0858)
SL/DFBL	5.38 ± 0.78	7.79 ± 1.12	6.49 ± 1.53	9.06 (0.0001)**
SL/CFL	4.06 ± 0.38	5.01 ± 0.71	4.50 ± 0.73	5.81 (0.0001)**
SL/CPL	8.35 ± 1.31	6.53 ± 0.85	7.51 ± 1.44	5.95 (0.0001)**
HL/SntL	2.17 ± 0.30	2.94 ± 0.46	2.53 ± 0.54	6.89 (0.0001)**
HL/ED	6.18 ± 0.90	7.40 ± 0.99	6.74 ± 1.12	4.59 (0.0001)**
SntL/ED	2.89 ± 0.63	2.56 ± 0.53	2.74 ± 0.60	2.01 (0.0488)*
IOW /ED	1.18 ± 0.30	1.52 ± 0.29	1.34 ± 0.34	4.07 (0.0002)**
CPL/LCPD	0.98 ± 0.28	1.83 ± 0.31	1.37 ± 0.51	10.33 (0.0001)

* P < 0.05; ** P < 0.01; P values are given in parentheses.

Table 2(b) Relative values of individual characters (mean ± standard deviation) and t-values of *N. montana*.

Meristic characters	S1		S2		Total	U
	$\bar{x} \pm s$	Median	$\bar{x} \pm s$	Median		
No. of bands on body	11.83 ± 1.87	12	10.88 ± 1.63	11	11.38 ± 1.81	850.5 (0.047)
No. of dorsal fin rays	8.76 ± 0.52	9	8.21 ± 0.89	8	8.51 ± 0.76	853.5 (0.041)

\bar{x} = mean; s = standard deviation; U = Mann-Whitney U value. P values ($P < 0.05$) are given in parentheses.

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Table 3 The result of Mann–Whitney test between the two populations of *N. montana* for two meristic measurements.

Morphological descriptors	component 1	component 2	component 3
Standard Length (SL)	0.432	0.735	0.264
Head Length (HL)	-0.677	0.491	-0.039
Body Depth (BD)	-0.871	0.175	0.117
Snout Length (SntL)	-0.725	0.308	0.516
Eye Diameter (ED)	-0.794	-0.077	0.005
Dorsal fin length (DFL)	-0.851	0.126	0.144
Dorsal fin base length (DFBL)	-0.814	0.331	0.015
Pectoral fin length (PFL)	-0.639	0.417	-0.284
Pelvic fin length (PIFL)	0.128	0.786	-0.202
Caudal Fin Length (CFL)	-0.561	0.392	-0.075
Pre- Dorsal Length (PrDL)	0.300	0.829	-0.188
Pre-Anal length (PrAL)	-0.370	0.663	-0.332
Distance from Pectoral fin origin to Pelvic fin base	0.279	0.744	-0.148
Distance from Pelvic fin origin to Anal fin base	-0.502	0.633	0.069
Least Height of Caudal Peduncle (LCPD)	-0.880	0.164	0.047
Caudal Peduncle Length (CPL)	0.682	0.536	-0.223
SL/HL	0.796	0.073	0.196
SL/SntL	0.840	-0.019	-0.405
SL/Pre-pelvic distance	0.633	0.167	0.566
SL/Pre-anal distance	0.723	0.096	0.534
SL/PFL	0.777	0.066	0.430
SL/DFL	0.890	0.172	-0.013
SL/DFBL	0.888	-0.037	0.179
SL/CFL	0.726	0.075	0.267
SL/CPL	-0.558	-0.249	0.333
HL/SntL	0.573	-0.080	-0.703
HL/ED	0.464	0.414	-0.018
SntL/ED	-0.169	0.449	0.685
Inter orbital width/ED	0.531	0.307	0.064
CPL/LCPD	0.904	0.181	-0.087
Eigen Value	13.437	5.031	2.906
% of variance	44.789	16.769	9.688
Cumulative % variance	44.789	61.558	71.246

Table 4 Principal component analysis of selected transformed morphometric characteristics and length ratios of *N. montana* in Khanda stream, S1 (n=28) and Saung stream, S2 (n=24).

MITOCHONDRIAL DNA SEQUENCING

In total, 10 variable sites were found among the 37 individuals sequenced. All of the variable sites were transition substitution (A↔G at 149,177,479,506,681 and C↔T at 254,266,459,830,995). Altogether, the two stream samples were defined by 8 different haplotypes (H1-H8). Details are given in Table 5. H3 and H8 were found to be least prevalent (5.5%) while H6 was most prevalent (24.5 %). No haplotypes were shared by populations, with the exception of H2. Sequences of these haplotypes have been submitted to GeneBank (accession numbers: Khanda stream, FJ711402 to 1418 & Saung stream, FJ711419 to 1438). The population specific diversity indices were presented in Table 6. Nucleotide diversity in the both streams samples (between Khanda and Saung streams) was low ($\pi_T = 0.0018 \pm 0.00016$). However, of the two populations, the Khanda stream population had slightly higher nucleotide diversity ($\pi_S = 0.0017 \pm 0.00016$) than the Saung stream population ($\pi_S = 0.0014 \pm 0.00024$). Both the populations were represented by moderate haplotype diversity (H_d) i.e., 0.743 & 0.742 for Khanda and Saung streams respectively. Tajima's D results were found to be significantly negative at the $P > 0.10$ level.

Haplotypes	Frequency of haplotypes	Stream
H1	5	S1
H2	8	S1,S2
H3	2	S1
H4	3	S1
H5	4	S2
H6	9	S2
H7	4	S2
H8	2	S2

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Table 5 Frequencies of mitochondrial DNA haplotypes (cyt-b region) observed in *N. montana* from two different streams.

Parameters	Khanda stream	Saung stream	Total
Sample size	17	20	37
No. of segregating sites (S)	5	5	10
Haplotype diversity (Hd) ± standard deviation	0.743 ± 0.064	0.74211 ± 0.072	0.863 ± 0.026
Average number of nucleotide differences (Kt)	1.92647	1.67368	2.07207
Nucleotide diversity (π) ± standard deviation	0.00171 ± 0.00016	0.00149 ± 0.00024	0.00184 ± 0.00016
Tajima' (1989) D statistic	0.97632*	-0.03304*	-0.40896*
Fu and Li's (1993) D statistic	1.20628*	0.54727*	1.40352

* not significant values

Table 6 Summary of genetic diversity indices of *N. montana*.

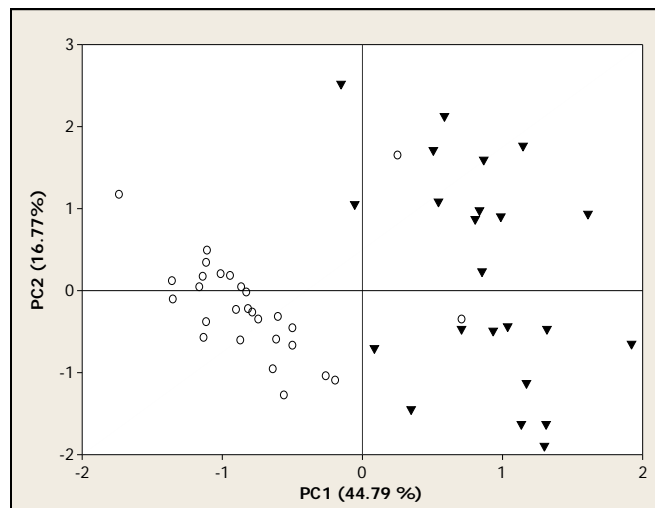


Figure 3: Scatter plot of Principal Components Analysis of *N. montana*. The samples from S1 (n=28) are shown as open circles (○) while the samples from S2 (n=24) are shown as solid diamond (▼).

IV. DISCUSSION AND CONCLUSION

The morphological analysis of the two populations of *N. montana* in the hill streams of Western Himalayan region of India revealed two phenotypically distinct populations with no genetic differentiation.

Examination of the loading of the morphometric characters and length ratios along PCA axes and the magnitude of their correlations revealed that the observed differences among two streams samples were mainly (90%) attributable to measurements of Body depth, Pelvic fin length, Pre-dorsal length, distance from Pectoral fin origin to Pelvic fin base, Least Height of Caudal peduncle, and length ratios of SL/SntL, SL/DFL & SL/DFBL, indicating these regions to be important in the description of population characteristics. The morphological findings presented here were further explored using results of mitochondrial cyt-b sequence analysis. Because morphological analysis is a highly indirect method for assessing population genetic variation as phenotype can be strongly influenced by environmental factors; thus, morphology may not necessarily reflect population differentiation at the molecular level [18]. While the moderate haplotype diversity may be inferred as the mixing among several genetically different populations, low nucleotide diversity indicates less genetic divergence among the two studied populations. The low values of nucleotide diversity are in accordance with the previously observed data in the range from 0.0015 to 0.0047 which meant lower genetic diversity [19]. The Tajima's D results were found to be significantly negative at the $P > 0.10$ level, which pointed to an excess of rare haplotypes.

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The low congruence between morphological and molecular results reported here establishes that the morphological differences between populations have little genetic basis and are more due to environmental influences.

The species of genus *Nemacheilus* live in an environment of rapid running water where they have to adjust themselves to any fluctuations in the microclimate or ecological niche. The results can be interpreted as phenotypic plasticity in the body of the two streams samples to adapt themselves to the ecological conditions of that particular stream. It may be due to change in altitude, temperature, water quality or the course of water flow etc. It is well known that morphological characteristics can show high plasticity in response to differences in environmental and habitat conditions, such as temperature, turbidity, food availability, and water depth and flow [20, 21 and 22] (Allendorf 1988, Swain et al. 1991, Wimberger 1992). Such plasticity is thought to be an important adaptive strategy for populations experiencing variable environmental conditions (Scheiner 1993) and it is likely that phenotypic plasticity plays an important role in diversification [23].

This study results are preliminary finding of significant morphological differentiation among the two populations of *N. montana*, more experimental data on streams ecology and micro-habitat characteristics are required to further establish the role that environmental conditions play in maintaining morphological diversity in these two populations. Changes in morphology of *N. montana* could also be interpreted as alteration in habitat quality, thereby indicating that intervention may be necessary to maintain habitat viability.

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