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Mitochondrial DNA Variation and Population Genetic Structure of Snow trout from Kumaun and Garhwal Himalayan Regions of India

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Abstract: *Snow trout (Schizothorax richardsonii) is one of the economically important coldwater fish, widely used as a food fish in the Himalayan region of India. The present study examined the genetic variations between five geographically isolated populations of S. richardsonii using ATPase 6/8 and COI gene sequences of mitochondrial DNA. Analysis of 25 sequences of ATPase 6/8 gene (842 bp) and 22 sequences of COI gene (847 bp) revealed 16 and 8 haplotypes respectively. The estimated haplotype and nucleotide diversity was high in GR (Gola river, Ranibagh) population (h = 1.00000, π = 0.00475 for ATPase6/8 and h = 0.83333, π = 0.00354 for COI). Results of AMOVA indicated that majority of the genetic variations in both genes are due to variation among populations (65.41 % for ATPase6/8 and 55.57 % for COI gene). The pairwise F_{ST} comparison and Neighbour-joining tree revealed low genetic divergence of KU (Kosi river, Uttarwahini) and KR (Kosi river, Ramnagar) population from other populations. The findings of this study showed that variation levels detected by ATPase6/8 and COI gene in population of S. richardsonii were very close to each other and demonstrate utility of ATPase6/8 and COI gene sequences for the population study of S. richardsonii for conservation, breeding and management progammes.*

Keywords: ATPase6*/8*, *COI*, Genetic variations, Mt-DNA, *Schizothorax richardsonii* (Snow trout).

Introduction

The *Schizothorax richardsonii* (Grey) commonly known as snow trout belongs to the subfamily Schizothoracinae under the family cyprinidae, constitutes an important food fish of coldwater region and are found distributed in the fast flowing streams and lakes all along the Himalayan and sub-Himalayan regions of Indian subcontinent, Afghanistan and China (Tilak, 1987; Sharma, 1988). Due to over exploitation and use of destructive fishing methods prevalent in the hill regions, the population of snow trout has shown a sharp decline in catches and thus has been listed as vulnerable by IUCN (Vishwanath, 2010).

The assessment of population genetic diversity and structure is important for maintaining productive fisheries and sustainable harvesting of populations (Park and Moron, 1994; Begg *et al.*, 1999). Genetic diversity can reflect changes in population size and therefore a population decline can be revealed by decreased genetic diversity (Frankham, 1996)**.** Moreover, the estimates of gene flow can give a measure of the movement of individuals among locations (Glenn *et al*., 1999). Mitochondrial DNA is widely used to determine the variation at interspecific and intraspecific levels (Avise *et al*., 1986; Avise, 1998). Since mtDNA substitution rates are homogenous across lineages, the rate of divergence can also be estimated on genetic distance data (Bermingham and Avise, 1986). Other advantages to mtDNA analysis include its higher rate of evolution in comparison to nuclear DNA, and it is strictly maternally inherited with no homologous recombination (Zardoya *et al.*, 1996; Bermingham *et al.*, 1997)**.** *COI,* ATPase8 and *ATPase6* genes of mtDNA are generally

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variable in vertebrates and have high evolutionary rate (Zardoya *et al.*, 1996; Bermingham *et al*., 1997; Simon *et al.,* 1994). *COI, ATPase 8* and *ATPase 6* regions of mitochondrial DNA have been successfully analyzed for both phylogeny as well as phylogeography in several fish species (Chow and Ushiama, 2004; Xin-Hong *et al.,* 2004; Yu *et al*., 2005; Dammannagoda *et al.,* 2008; Vergara-Chen *et al.,* 2009; Chandra *et al.,* 2012; Habib *et al*., 2012).

In the present investigation we studied the sequence variability of *ATPase 6/8* and *COI* genes in *Schizothorax richardsonii* populations from Kumaon and Garhwal regions of central Himalayan region of India and also tried to assess the phylogenetic relationship between the populations. Comparison has also been made between *ATPase 6 and 8 and COI* genes for the sequence variability.

Materials and Methods

Sample Collection and DNA isolation

A total of 25 specimens were collected from five different locations from Kumaun and Garwhal regions *viz*., River Bhilanaga (Bairangna), River Kosi (Ratighat, and), River Gola (Ranibagh), River (Champawat) from October 2010 to March 2011 using cast net (Bairangna-BB; Ratighat-KR; Uttarwahani-KU; Ranibagh-GR; Champawat-LC) (Table1; Fig. 1). Fin samples were collected and preserved in 70% ethanol and stored for future analysis. Genomic DNA was extracted from 50mg fin tissue sample by the phenol–chloroform procedure (Sambrook and Russell, 2001). Analysis on agarose gels and spectrophotometric methods were used to determine DNA quality and quantity.

PCR amplification and Sequencing

ATPase6/8 gene and *COI* gene were amplified using PCR (Eppendorf, Mastercycler gradient). The *ATPase6/8* gene was amplified using the primers A6/8 F′ (5′-AAAGCRTYRGCCTTTTAAGC-3′) and A6/8 R′ (R′-GTTAGTGGTCAKGGGCTTG GRTC-3′) (Sivasundar *et al.,* 2001). The *COI g*ene was amplified using a set of primer COIHA (5′- CCT-GAGAATAAGGGGAATCAG-3′) and COILB (5′-GCATTCCCACGAATAAATA-3′) (Bielawski and Gold, 2002)**.** Amplification was carried out in thermal cycler (Eppendorf, Mastercycler Gradient) in 50 µl of PCR mix, containing 5 µl of 10X PCR buffer (100 mM Tris, pH 9.0, 500 mM Kcl, 15 mM $MgCl_2$, 0.1% Gelatin) (B-Genei), and 1 unit of Taq DNA polymerase (B-Genei), 2.5 mM of each dNTPs (dATPs, dCTPs, dGTP, dTTPs) (B-Genei, India), 10 pmol of each primer, 50ng of genomic DNA The thermal profile used to amplify *ATPase6/8* and *COI* gene consisted of an initial denaturation of 95^{o}C for 3 min; followed by 34 cycle of 94^{o}C for 30sec, 55^{0} C for 45 sec (*ATPase6/8*) or 52^{0} C for 50 sec (*COI*), 72^{o}C for 1 min 30 sec and a final extension at 72^{o}C for 7 min, then hold at 4^{o}C. PCR products were stored at 4^{o}C. For each sample, 3 µL of PCR product were electrophoresed through 1.2 % agarose gels following ethidium bromide staining, and visualized under UV illumination in the Gel-Doc system (Alpha Imager 3400, Alpha Innotech Corporation, USA). PCR products were column purified and sequenced in both directions using an ABI automated DNA sequencer (Applied Biosystem, USA) with Big Dye Terminator cycle sequencing kit v3.1 with

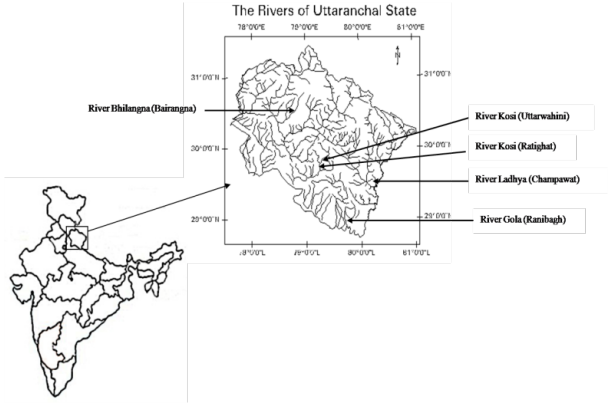

Fig. 1 Locations of sampling station in Kumaon and Garhwal regions of India.

same primers used for amplification of the target genes.

Sequence analysis

Multiple sequence alignments for *ATPase6/8* and *COI* gene sequences were performed using Clustal X (Thompson *et al.,* 1997) and MEGA v.4.0 (Tamura *et al.,* 2007). The parameters of genetic variability such as, nucleotide diversity (π) and haplotype diversity (h) were calculated using software DNAsp v.4.5 (Rozas *et al.,* 2003). Sequence composition, number of haplotypes, molecular diversity indices, genetic differentiation (F_{ST} values) and correlation between genetic and geographic distance (Mantel test) were calculated using Arlequin v.3.11 (Excoffier *et al.,* 2007). The phylogenetic relationship among five populations of *S. richardsonii* based on Neighbour-joining (NJ) tree and mean genetic distances between the populations were assessed with MEGA v.4.0 software. Bootstraps support was calculated using 1000 replication.

Results and Discussion

The studies on population genetic structure of a species are powerful tools for understanding genetic variation which will lead to develop strategies for conservation and management of natural small fish populations. The mtDNA was the first widely used DNA marker and has provided many insights into the demography of natural populations (Oleksiak, 2010). In the present study moderate to high diversity using *ATPase 6/8* and *COI* gene in five populations of *S. richardsonii* has shown the utility of mtDNA markers to study genetic variation and population differentiation of natural populations.

Sequence variation

A total of 25 sequences of *ATPase 6/8* gene (842 bp) and 22 sequences of *COI* gene (847 bp) were analyzed to determine genetic variation between five populations of *S. richardsonii.* All the sequences were submitted to NCBI GenBank (Table 1). In *ATPase 6/8* gene a total 20 variable sites were detected and out of that 18 sites were parsimony informative while 2 sites were singleton, defining 16 haplotypes in all five populations (designated as hap01-hap16, Table 2). Most of nucleotide variation resulted from transition (75%) followed by transversion (25%) and overall transition/transversion bias was R = 1.622. The average nucleotide frequency for all the samples of *S. richardsonii* were A = 30.6%, T = 28.8%, C = 26.9%, G = 13.6%. Nucleotide sequences of *ATPase6/8* gene were A+T rich (59.4%). It was found that no haplotypes were shared by all populations. However, only one haplotype (hap13) was shared by more than one population (KR and KU with observed frequency 80% and 60% respectively) (Table 2). In *COI* mitochondrial gene a total of 9 variable sites were detected, out of which 5 sites were parsimony informative while 4 sites were singleton, defining 8 haplotypes (designated as hap01-hap08, Table 3). Most of nucleotide variation resulted from transition (88.88%) followed by transversion (11.11%) and overall transition/transversion bias was R = 21.45. The average nucleotide frequency for all the samples of *S. richardsonii* were A = 26.1%, T = 29.4%, C = 25.8%, G = 18.7%. Nucleotide sequences of *COI g*ene were A+T rich (55.5%). It was found that no haplotype was shared by all populations. However, hap01 was shared between BB and LC (Observed frequency 40% and 100% respectively) and hap02 was shared between KR, GR and KU with observed frequency 80%, 25% and 50% respectively (Table 3). Nucleotide Sequences of *ATPase6/8* and *COI* gene in *S. richardsonii* were A+T rich, which was also reported in many fish species (Jones and Avise, 1998). Rate of transition is higher than transversion in both the gene. A high transition bias is well known in vertebrate mtDNA and also reported in other fishes (Meyer, 1993).

Population variability

The genetic diversity information in *S. richardsonii* for *ATPase 6/8* and *COI* region are given in Table 1. In ATPase6/8 gene the

Table1 Measure of sequence variation within populations of *Schizothorax richardsonii from central Himalayan region.*

Collection sites	Population code	Gene	*N*	*S*	*h*	*π*	T_s	T_v	Accession no.
River Bhilangna (Bairangna)	BB	*COI*	5.00	1.00	0.60000	0.00071	1.00	0.00	JX485937-JX485941
		ATPase6/8	5.00	7.00	0.90000	0.00403	5.00	0.00	JX485904-JX485908
River Kosi (Ratighat)	KR	*COI*	5.00	1.00	0.40000	0.00047	0.00	1.00	JX485942-JX485946
		ATPase6/8	5.00	1.00	0.40000	0.00047	1.00	0.00	JX485919-JX485923
River Ladhiya (Champawat)	LC	*COI*	4.00	0.00	0.00000	0.00000	0.00	0.00	JX485929-JX485932
		ATPase6/8	5.00	3.00	0.60000	0.00237	1.00	0.00	JX485909-JX485913
River Gola (Ranibagh)	GR	*COI*	4.00	6.00	0.83333	0.00354	6.00	0.00	JX485947-JX485950
		ATPase6/8	5.00	7.00	1.00000	0.00475	2.00	5.00	JX485914-JX485918
River Kosi (Uttarwahini)	KU	*COI*	4.00	4.00	0.83333	0.00236	4.00	0.00	JX485933-JX485936
		ATPase6/8	5.00	3.00	0.83333	0.00166	1.00	0.00	JX485924-JX485928

Table 2 The variable site and haplotype frequency of ATPase6/8 gene fragment of five populations of *Schizothorax richardsonii* from central Himalayan region.

	Variable site																								
	0	2	3	3	4	4	5	5	5	6	6	7	7	8	8	8	8	8	8	8	Populations				
	2	0	1	2	1	3	0	3	8	6	9	6	8	1	2	2	2	2	3	3					
Haplotype	**4**	**7**	**8**	**6**	**3**	**1**	**0**	**9**	**1**	**9**	**5**	**4**	**5**	**8**	**4**	**5**	**8**	**9**	**0**	**1**	**BB**	**KR**	**LC**	**GR**	**KR**
Hap01	T	A	T	T	G	C	G	A	A	T	A	A	A	C	A	T	A	C	A	A	1	0	0	0	0
Hap02	–	–	–	–	–	–	–	–	–	–		–	–	T	–	–	–	–	–	–	1	0	0	0	0
Hap03	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	-	2	0	0	0	0
Hap04	–	–	–	C	–	–	–	G		C		–	–	–	–	–	–	–	–	–	1	0	0	0	0
Hap05	–	G	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	-	0	0	2	0	0
Hap06	–	G	C	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	G	0	0	1	0	0
Hap07	–	G	C	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0	2	0	0
Hap08	–	–	–	C	A	–	–	G	–	–	–	–	G	–	–	–	–	–	–	–	0	0	0	1	0
Hap09	–	–	–	C	A	–	–	G	–	–	–	–	G	–	–	–	–	–	–	G	0	0	0	1	0
Hap10	C	–	–	C	A	–	–	G	–	–	–	–	G	–	T	A	T	A	C	–	0	0	0	1	0
Hap11	–	–	–	C	A	–	–	G	–	–	–	–	G	–	T	A	T	A	C	–	0	0	0	1	0
Hap12	C	–	–	C	A	–	–	G	–	–	–	–	G	–	T	A	–	–	–	–	0	0	0	1	0
Hap13	–	–	–	C	–	–	A	–	–	–	–	G	–	–	–	–	–	–	–	–	0	4	0	0	3
Hap14	–	–	–	C	–	–	A	–	–	–	–	G	–	–	–	–	–	–	–	G	0	1	0	0	0
Hap15	–	–	–	C	–	–	A	–	G	–	–	G	–	–	–	–	–	–	–	-	0	0	0	0	1
Hap16	–	–	–	C	–	–	A	–	G	–	–	G	–	–	–	–	–	–	–	–	0	0	0	0	1

Table 3 The variable site and haplotype frequency of *CO1* gene fragment of five populations from central Himalayan region.

	Variable site													
	1	2	3	5	7	7	7	8	8	Populations				
	5	7	6	4	2	8	9	0	1					
Haplotype	**0**	**9**	**4**	**9**	**1**	**3**	**5**	**5**	**9**	**BB**	**KR**	**LC**	**GR**	**KU**
Hap01	C	A	T	T	C	G	C	C	T	2	0	4	0	0
Hap02	–	–	–	C	–	–	–	T	–	0	4	0	1	2
Hap03	–	–	–	C	–	–	T	T	–	0	0	0	0	1
Hap04	–	–	–	–	T	–	–	–	–	0	0	0	0	1
Hap05	T	–	–	–	–	–	–	–	–	3	0	0	0	0
Hap06	–	–	–	C	–	–	–	T	G	0	1	0	0	0
Hap07	T	G	C	–	–	–	–	–	–	0	0	0	2	0
Hap08	T	G	C	–	–	A	–	–	–	0	0	0	1	0

haplotype diversity (*h*) within the populations was high ranged from 0.40000 (KR) to 1.00000 (GR). Nucleotide diversity (π) was generally low ranged from 0.00047 (KR) to 0.00475 (GR). In *COI* gene also, haplotype diversity (*h*), within the populations was high, ranged from 0.00000 (LC) to 0.83333 (GR; KU) and nucleotide diversity (π) was found low, ranged from 0.00000 (LC) to 0.00354 (GR). The overall nucleotide diversity (π) was found low in both *COI* gene (π = 0.00270) and *ATPase 6/8* gene (π = 0.005866) while haplotype diversity (*h*) was found high (*h* = 0.827 for *COI* and *h* = 0.9371 for *ATPase 6/8*). Mitochondrial nucleotide and haplotype diversities can provide some information on population history of the *S. richardsonii.* High haplotype diversity and low nucleotide diversity were found in all the population examined, which can be attributed to a recent population expansion after a low effective population size (Luhariya *et al.*, 2012). The overall haplotype and nucleotide diversity was low in *COI* gene as compared to *ATPase 6/8* gene. This low nucleotide and haplotype diversity in *COI* gene may be due to presence of small number of variable site; hence in the present study *ATPase 6/8* gene is more informative for population genetic study than *COI* gene. The high genetic variability of GR population is due to the presence of large number of haplotypes and polymorphic sites as compared to other populations.

Geographic differentiation and gene flow among populations and their evolutionary relationship

The hierarchical analysis of molecular variance (AMOVA) within 5 populations revealed a significant subdivision between populations in the total sample (F_{ST} = 0.65411for *ATPase 6/8* and F_{ST} = 0.55573 for *COI*) (Table 4). The mean genetic divergence among population was higher in *ATPase6/8* than COI. It further revealed that majority of the genetic variation in *ATPase 6/8* and *COI* gene was contributed due to variation among population (65.41% for *ATPase6/8* and 55.57% for *COI* gene), while within population variation was low (34.59% for *ATPase6/8;* 44.43% for

Table 4 Analysis of variance (AMOVA) of *CO1* and *ATPase6/8* gene fragment of *Schizothorax richardsonii* populations from central Himalayan region.

Source of variation	Degree of freedom		Variance components		Percentage of variation	
	COI	ATPase6/8	COI	ATPase6/8	COI	ATPase6/8
Among population	4.00	4.00	0.69902va	2.11800va	55.57	65.41
Within population	17.00	20.00	0.55882vb	1.12000vb	44.43	34.59
Total	21.00	24.00	1.25785	3.23800	100.00	100.00

F_{ST} = 0.55573 (*COI*), 0.65411 (*ATPase6/8*).

Table 5 Pairwise Fixation indices (FST) for five populations of Schizothorax richardsonii.

	BB	KR	LC	GR	KU
BB	–	0.59746 (0.00000±0.0000)	0.44672 (0.00000±0.0000)	0.54433 (0.00000±0.0000)	0.52756 (0.01802±0.0121)
KR	0.82143 (0.00000±0.0000)	–	0.81928 (0.00000±0.0000	0.72906 (0.02703±0.0194)	0.06250 (0.35135±0.0459)
LC	0.45205 (0.22523±0.0311)	0.89691 (0.00000±0.0000)	–	0.71264 (0.00901±0.0091)	0.75852 (0.00901±0.0091)
GR	0.37294 (0.00000±0.0000)	0.62963 (0.00000±0.0000)	0.50000 (0.11712±0.0305)	–	0.69178 (0.00000±0.0000)
KU	0.52756 (0.00000±0.0000)	0.04040 (0.00000±0.0000)	0.50000 (0.12613±0.0337)	0.41176 (0.00000±0.0000)	–

*The pairwise values calculated by mtDNA *COI* and *ATPase6/8* gene are below and above the diagonal respectively p values given in brackets. Significance level = 0.0500.

COI gene). The genetic structure was analyzed with BB, KR, KU, GR and LC populations. The pairwise F_{ST} values ranged from 0.06250 (KR/KU) to 0.81928 (KR/LC) for ATPase6/8, and 0.04040 (KR/KU) to 0.89691 (KR/LC) for COI gene (Table 5). A Mantel test was carried out and the correlation coefficient for genetic and geographic distance was 0.174 for COI ($p \geq 0.362$) and 0.160 for *ATPase* 6/8 ($p \geq 0.650$), which was not significant based on 1000 permutation. The low within population variation and high among populations variation in *S. richardsonii* were found in agreement with Vrijenhoek (1998) who reported 67% variation among population and 32.4%, within population in a non migratory fish. Hence the genetic divergence level between populations, observed in this study was lower than non migratory fish. This observation can be attributed to the fact that *S. richardsonii* are known as short distance migrants which migrate between stream for food and spawning.

The pairwise gene flow comparison of population samples using *COI* and *ATPase 6/8* gene sequences is given in Table 6. In *COI* gene, estimated gene flow ranged from 0.0287 (between LC-KR populations) to 5.9381 (between KU-KR populations). In *ATPase 6/8* gene, estimated gene flow ranged from 0.0551 (between LC-KR populations) to 3.7500 (between KU-KR populations). According to Slatkin (1987), it was hard to prevent genetic divergence caused by genetic drift if the gene flow [$N_m = (1 - F_{ST})/4F_{ST}$] value was less than one. Analysis of *COI* and *ATPase6/8* gene revealed that level of gene

Table 6 Gene flow estimation for five populations of *Schizothorax richardsonii.*

	BB	KR	LC	GR	KU
BB	–	0.1684	0.3096	0.2092	0.2238
KR	0.2500	–	0.0551	0.0929	3.7500
LC	0.3030	0.0287	–	0.1008	0.0795
GR	0.4917	0.1470	0.2500	–	0.1113
KU	0.2238	5.9381	0.2500		–

*The gene flow values calculated by mtDNA *COI* and *ATPase6/8* gene are below and above the diagonal respectively. $N_m = (1 - F_{ST})$.

Table 7 Mean genetic distance (p-distance) for populations of *S. richardsonii.*

Populations	BB	KR	LC	GR	KU
BB	–				
KR	0.004	–			
LC	0.002	0.005	–		
GR	0.005	0.007	0.007	–	
KU	0.004	0.000	0.005	0.007	–

*The mean genetic distance (p-distance) values calculated by combined mtDNA *COI* and *ATPase6/8* gene sequences.

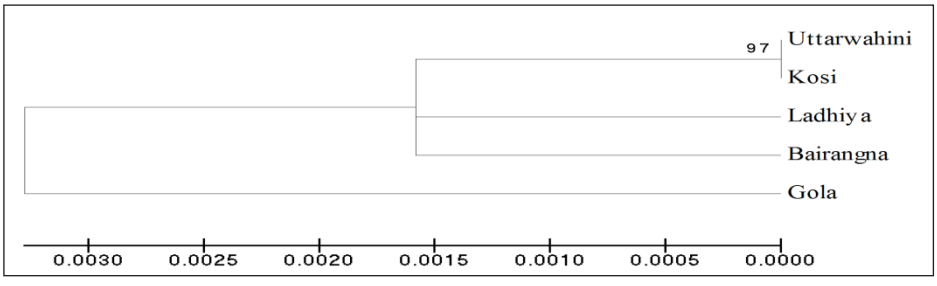

Fig. 2 Neighbour-joining tree of *S. richardsonii* inferred from COI and ATPase6/8 combined data.

flow in *S. richardsonii* population was below one except KU/KR population. This indicated that there might be continuous gene flow between KU and KR population. F_{ST} value using both the genes also revealed the non-significant genetic divergence between populations of KU and KR.

The nucleotide sequences of *ATPase6/8* and *COI* gene were aligned in order to determine the phylogenetic relationships among the 5 populations of *S. richardsonii*. Neighbour-joining tree of *S. richardsonii* using *ATPase6/8* and *COI* gene combined data showed that the sequences were clustered into two major groups. First Group was divided into two clades; one clade consisted of KU and KR populations where as BB and LR populations clustered into another clade. The second group consisted of only GR population (Fig. 2). Mean genetic distance (*P* distance) over all the 7 populations was 0.005 and between populations it ranged from 0.000 to 0.007 (Table 7). Neighbour-joining tree inferred from *ATPase6/8* and *COI* combined data suggested that KU and KR population were closely related to each other than other populations. Haplotype sharing between KU and KR population also indicated extensive gene flow between these two populations, suggesting that KU and KR lineages could have evolved from common ancestor.

$$(\log_a + \log_b + \log_c + \cdots + \log_x)$$

$$g = \text{anti}^{-} \log n$$

Results obtained in this study demonstrated that mitochondrial gene is a potential marker for studying variation within and among populations of *S. richardsonii.* In the present study we were able to provide some useful insights into phylogenetic relationship and genetic identity of *S. richardsonii* from the different drainage of central Himalayan. The findings also showed that variation levels detected by *ATPase6/8* and *COI* gene in population of *S. richardsonii* were very close to each other and demonstrated the utility of *ATPase6/8* and

COI gene sequences for a population study of *S. richardsonii* for conservation, breeding and management progammes.

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