DNA barcoding of fishes from River Song, Dehradun, Uttarakhand using mitochondrial cytochrome-c oxidase-I gene.

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Abstract

The entire Himalayan region is well known as a global hotspot for biodiversity. Many workers have documented different aspect of biodiversity. The Uttarakhand Himalaya region, a part of Himalayan system, is very rich in fresh water aquatic biodiversity because it has many fresh water streams and rivers within a short distance of 200 miles. All these fresh water bodies harbor diverse aquatic fauna with fishes being the most extensively studied. There are many fish species reported by many authors in Uttarakhand. Many of the fishes have similar morphological characters (morpho-metrics) and are difficult to identify. Attempts have been made to generate the DNA barcode of fishes but most of the attempt are limited to major rivers i.e. the Ganges and the Yamuna. Besides these two prominent river (the Ganges & the Yamuna), there are many supporting streams and small tributaries that are also inhabited by many species. No "long term" and "entire river scan" for fish species has ever been conducted in Uttarakhand. Our research aims to generate a molecular database (DNA Barcode) for entire fish species fauna in each small river/stream of Uttarakhand. Using a combination of morphometric & DNA bar-coding data will provide an efficient method for species level identification and contributes considerably to taxonomic and biodiversity research. The present study is first step towards establishing molecular taxonomy database of Uttarakhand based upon the bar coding pattern of cytochrome c oxidase I (COI) gene. We carried out an "entire river scan" of River Song (a tributary of river Ganges). 50 samples were collected from various sampling sites along the entire stretch of river and analyzed. Data was uploaded into the BOLD database and the analysis of data using this database suggests that the genetic variability (K2P distance) distribution of 50 individuals belonging with 15 species.The average intraspecific variation using K2P and NJ methods/models was assessed. The mean intraspecific distance was less than 1.45% for 32% of species. Mean K2P distance within species, genus and family was 0.1%, 16.3% and 21.70% respectively. Our results suggest that River Song has diverse fish fauna with substantial genetic diversity. Data from *Barillius* species suggests that this species has a very high variation. There is ahighly probability that as all the rivers are scanned new data on endemic and exotic fish fauna diversity will emerge which would be helpful in conservation efforts.

Keyword: *bar-coding*, *species identification*, *fresh water fishes*, *biodiversity*, *molecular taxonomy*

Introduction

Classifying animals and plants is one of the world's oldest professions. Accurate identification of species is crucial for research, other areas of biology and biodiversity conservation (Diwaker and Balakrishnan, 2007, Pfenninger, *et al.* 2007, Lumbsch *et al.* 2011). Estimating the number of species in a community is a classical problem in ecology, biogeography and conservation biology. Till recently only morpho-metrics/morphologywas the criteria for classification. But, it was discovered that some individuals that look similar but are genetically different and are a separate species altogether. Hence a new initiative was

launched i.e. to develop database that uses a combination of both approaches–collection and molecular identification. Thus specimen collections, their digitization and developing bioinformatics based databases are becoming vital and many barcoding initiatives are ongoing world over (Chakrabarty 2010, Pyke and Ehrlich 2010).

Use of molecular biological techniques started with protein electrophoresis on starch about 45 years ago (Manwell and Baker 1963), followed by the study of allozymes (Aron and Sole-Cava 1991, Gusma *et al.* 2000), restriction fragment length polymorphism (RFLP) (Moyse´s and Almeida-Toledo 2002), DNA arrays (Hajibabei *et al.* 2007), single nucleotide polymorphism (Shaffer and Thonsom, 2007), multiplex PCR (Mendonca *et al.* 2009), DNA sequences (Pook and McEwing 2005, Lemer *et al.* 2007) and many others. Parallel to all these

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studies Hebert *et al*. 2003 proposed the use of a small fragment of approximately 652 bases from the 5' end of the mitochondrial cytochrome c oxidase subunit I (COI) gene can be a universal standard to identify most species of animals based on which various studies were carried out world over (Hebert *et al.* 2003). After this discovery that, especially in animals, a specific region of COI gene is species specific, it has become a universal standard.

Uttarakhand Himalaya region, a part of Himalayan system, is very rich in fresh water aquatic biodiversity because it has many fresh water streams and rivers within a short distance of 200 miles. All these fresh water bodies harbor diverse aquatic fauna with fishes being the most extensively studied. There are many fish species reported by many authors in Uttarakhand. Many of the fishes have similar morphological characters (morpho-metrics) and are difficult to identify.This has already been reported in the case of *Schizothorax* (*richardsonii* and *plagistomas*). Attempts have been made to generate the DNA barcode of fishes but most of the attempt are limited to major rivers i.e. the Ganges and the Yamuna. Besides these two prominent river (the Ganges & the Yamuna), there are many other supporting streams and small tributaries that are also inhabited by many species. No "long term" and "entire river scan" for fish species has ever been conducted in Uttarakhand. Our research aims to generate a molecular database (DNA Barcode) for entire fish species fauna in each small river/stream of Uttarakhand and develop a molecular database. This data will also help in assessment of genetic diversity by adding some more experiment to current dataset.

Material and methods

Study site: The present study performed an "entire river scan" of River Song. Song River originates at the base of Himalaya near Mussoorie (around Uniyal Gaon) in Garhwal region of (30.18^0N; 78.14^0E and altitude 3794 feet – Fig. 1), Uttarakhand. After covering a distance of 140 kilometers (approximately), the Song River (a tributary of Ganges) merges into the Ganges River at Gohri Mafi, Dehradun (near Rishikesh - 30.01^0N; 78.14^0E and altitude (1024 feet).
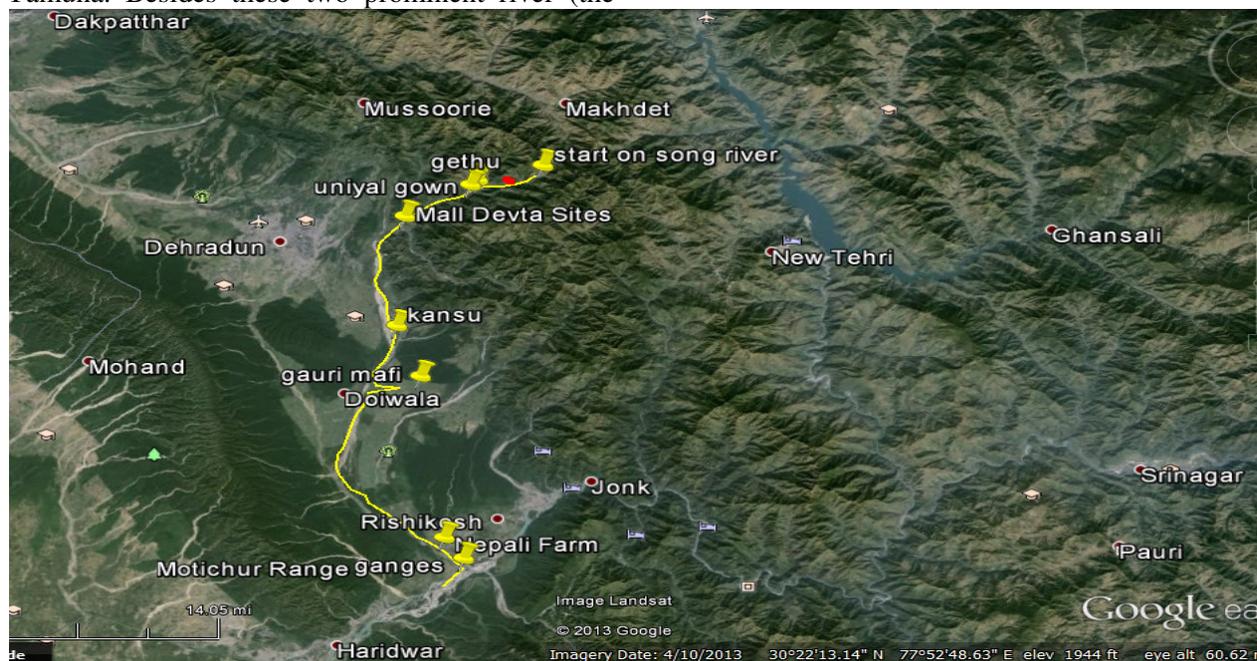

Figure 1: Location of sampling station.River song originates at Uniyal Gaon (Village) and after flowing through Mal Devta, Gauri Mafi, Kansu, Doiwala, it merges into the Ganges at Nepali farm (located near Rishikesh). Seven sampling station are shown in the map. (Made in Google earth)

50 fishes were collected from seven different sites in the song river basin (Figure 1). Fishes were sampled mainly from the medium course of the song river ranging from headwater to floodplains.Sampling stations were 20 km apart and a total of 7 such stations (one station was rendered inaccessible) were sampled with nets of different mesh size. Sampling was repetitive and during different seasons also - so as to get maximum fish catch (as per requirement of BOLD data). From the collected sample specimens, tissue samples were cut (a piece of pectoral fin) and preserved in 95% ethanol and the specimens were preserved in formalin for morphological analysis.

A voucher ID was assigned to each specimen. voucher specimens were accessioned in to collection of the Department of Zoology R.C.U Government PG College, Uttarkashi, Uttarakhand, India. Fishes were individually collected and photographed with their all taxonomical characters exposed (along with a measuring scale and a colour coding strip –Table 1).The original fish and the sampled tissue were tagged similarly and the sampled tissues were preserved at -20^0C freeze. The morphological data of these fishes was then uploaded to BOLD project entitled "The Song River fish barcoding, India" SRFBI (http://www.boldsystems.org).

Table1: Sample of data sheet for DNA barcoding as per BOLD database. Each sample was placed adjacent to a colour coded strip and a measuring scale and then photographed. Each sample was then preserved as per BOLD database requirements.

	Species	Sample location	Photo
1	*Barilius barna*	MALLDEVTA	
2	*Barilius bendelisis*	MALLDEVTA	
3	*Barilius tileo*	MALLDEVTA	
4	*Chagunius cnagunio*	NEPALI FARM	
5	*Garra gotyla gotyla*	GORIMAFI	
6	*Garra gotyla*	GORIMAFI	

DNA extraction, PCR amplification and sequencing: The DNA of the sampled tissues was then isolated by Wizard Genomic DNA Purification Kit (Qiagen Integrity Cat# A1120). The isolated DNAs was then checked on 1% agarose gel. Quantity of isolated DNA was checked on Nano-drop 1000 spectrophotometer (Applied bio-system, USA) at absorbance values of 260 and 280 nm.

The samples were then PCR amplified for COI gene using degenerate primers FF2d (forward): TTCTCCACCAACCACAARGAYATYGGFR1d (reverse): CACCTCAGGGTGTCCGAARAAYCARAA (Ivanova *et al* 2007). The thermal cycler program was run as mentioned in Table 2. After PCR the samples were stored at 4^0C.

Table 2: Thermal cycler program for PCR of COI gene in freshwater fishes

Process	Temperature	Time	Cycles
Initials denaturation	95^0C	5 MINUTE	1 CYCLE
Denaturation	95^0C	30 SECONDS	35 CYCLE
Annealing	55^0C	30 SECONDS	
Extension	72^0C	1 MINUTE	
Final Extension	72^0C	7 MINUTE	ONE CYCLE

After PCR, the samples were then run at 1.5% of agarose gel for their quality check (Fig. 2). The sample showing one clear band after PCR samples were sorted and purified with EXO1-SAP (Exonuclease1 and shrimp alkaline phosphatase: USB Corp – this step removes excess of dNTP's, Taq polymerase and buffer components from PCR samples) with the temperature conditions suggested by manufacturer. The purified PCR amplicons were then labeled with big dye terminator v3.1 (Applied Bio systems) by cycle sequencing with each side labelled separately. The cycle sequencing PCR reaction contained ready reaction mix (2.5x) 0.5µL, dilution buffer 1.75µL, template 200ug/µL and 1µL primer (0.8pMol/µL). The cycle sequenced amplicons were then purified with big dye (R) X terminator (TM)(Big dye terminator v 3.1 clean up applied bio systems, USA) each side labelled separately and was then sequenced on ABI 3130 DNA genetic analyzer with 2µL of purified sample and 4.75µL of MQ water. The cycle sequencing conditions was initial denaturation of 96^0C for 1min followed by 35 cycles of denaturation 96^0C for 10sec, annealing 50^0C for 5sec, extension 60^0C for 4min and then the samples were stored at storage temperature of 4^0C. The sequences were then obtained and analysed in the Sequence Scape software v2.7 for possibilities of

indels (Applied bio systems 3130 genetic analyzers). The names of fishes, collection codes, photographs, sequences, trace files were then uploaded to BOLDs project "The Song River fish bar-coding, India" SRFBI. The obtained sequences were also submitted to NCBI Gene Bank for accession numbers.
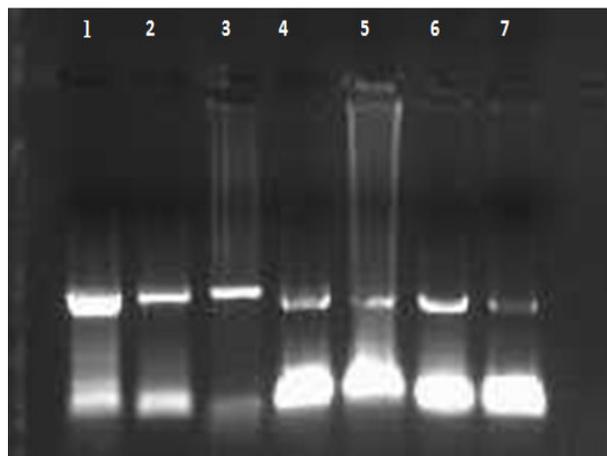

Figure 2: COI mitochondrial genes PCR amplified products. (PCR sample loaded in Gel. The sample id of each well is as follows: 1- *Barilius barna,* **2-** *Barilius bendelisis,* **3-** *Barilius tileo,* **4-** *Chagunius cnagunio,* **5-** *Garra gotyla gotyla,* **6-** *Garra gotyla* **and 7-** *Barilius barna.* **The first band is COI amplicon and the last band (running in front) is primer dimer.**

Data analysis

Song River data sequences were edited and aligned using codon code aligner (v5.6; codon code crop USA). All data (i.e. electro-pherograms, trace files, primer details, photograph and collection localities) for each specimen were deposited within the project song river fresh water fishes–the song basin population file on BOLD (http://www.boldsystems.org) and the assembled DNA sequence were also submitted to Gene bank (accession numbers included in appendix online tools implemented on BOLD were used to estimate pair wise sequence divergences using the kimura two-parameter (K2P)distance model, as well as to calculate nearest –neighbour distance (NND) values and neight bo-joining phonograms.

Results and Discussion

A total 15 species from fifty samples belonging to 02 families were sampled and barcoded under the "Song River Scan" program and uploaded into the "SRFBI" project on BOLD. The COI sequence obtained was high quality and the subsequent counting assemblies generated. A total of 32 COI barcode were obtained from fifty specimens in two families, constituting around 50% of all know fauna of the song river. All amplified sequences were large then 600bp and no insertions, deletions or stop codons were observed hence reducing the possibility of nuclear DNA. This also confirmed that the sequence originated from mitochondrial DNA. Accession numbers were granted by NCBI and were uploaded into the BOLD SYSTEM only one species failed to sequencing. Accession number to BOLD and Gene bank sequences for each specimen are provided (Table 3). All species were discriminated by the barcode sequences. A complete NJ tree of entire data is presented in figure 3. This was made by the entire dataset uploaded into BOLD Database. The average K2P distance of specimens within species was 0.61% as compared to within genus which was 16.3%. Within family, the mean distance was 21.79% for species with genera (Table 4). The overall variation was about 30 times (approximately) lower among the congeneric species as compared to within species. (Figure 5).

Table 3: NCBI Gene bank accession number for sequences generated during the study. Names and their corresponding accession number is mentioned in the table.

S No	Species name	Genbank Accession no
1	*Barilius barna*	JN965191
2	*Barilius barna*	JN965190
3	*Barilius bendelisis*	JN965192
4	*Barilius bendelisis*	JN965196
5	*Barilius bendelisis*	JN965195
6	*Barilius bendelisis*	JN965194
7	*Barilius bendelisis*	JN965204
8	*Barilius bendelisis*	JN965212
9	*Barilius bendelisis*	JN965193
10	*Barilius tileo*	JN965198
11	*Barilius tileo*	JQ692874
12	*Chagunius chagunio*	JN965199
13	*Garra gotyla*	JN965210
14	*Garra gotyla*	JN965211
15	*Garra gotyla*	KC473939
16	*Garra gotyla gotyla*	
17	*Macrognathus pancalus*	KC473940
18	*Puntius conchonius*	JN965201
19	*Puntius ticto*	JN965202
20	*Puntius ticto*	JN965203
21	*Schizothorax progastus*	JN965205
22	*Schizothorax progastus*	JQ692872
23	*Schizothorax progastus*	JQ692870
24	*Schizothorax progastus*	JQ692873
25	*Schizothorax sp.*	JQ692871
26	*Torchelynoides*	JN965207
27	*Tor chelynoides*	JN965206
28	*Tor putitora*	JN965209
29	*Tor putitora*	JN965197
30	*Tor sp.*	KC473941
31	*Tor tor*	KC473942
32	*Tor tor*	JN965208

Macrognathuspancalus|BS123
Garragotylagotyla|RS07
Bariliustileo|GM07
Bariliustileo|GM08
Bariliusbarna|KR04
Bariliusbarna|KR03
Bariliusbendelisis|GM01
Bariliusbendelisis|KR07
Bariliusbendelisis|KR06
Bariliusbendelisis|RS05
Bariliusbendelisis|KR01
Bariliusbendelisis|KR02
Bariliusbendelisis|GM02
Chaguniuschagunio|GM10
Puntiusticto|GM11
Puntiusticto|GM12
Puntiusconchonius|KR10
Garragotyla|BS55
Garragotyla|HD09
Garragotyla|HD10
Tortor|GM06
Tortor|BS153
Torputitora|UM05
Torputitora|GM05
Torchelynoides|RS04
Torchelynoides|UM04
Torsp.|BSS01
Schizothoraxsp.|HD07
Schizothoraxprogastus|HD08
Schizothoraxprogastus|RS01
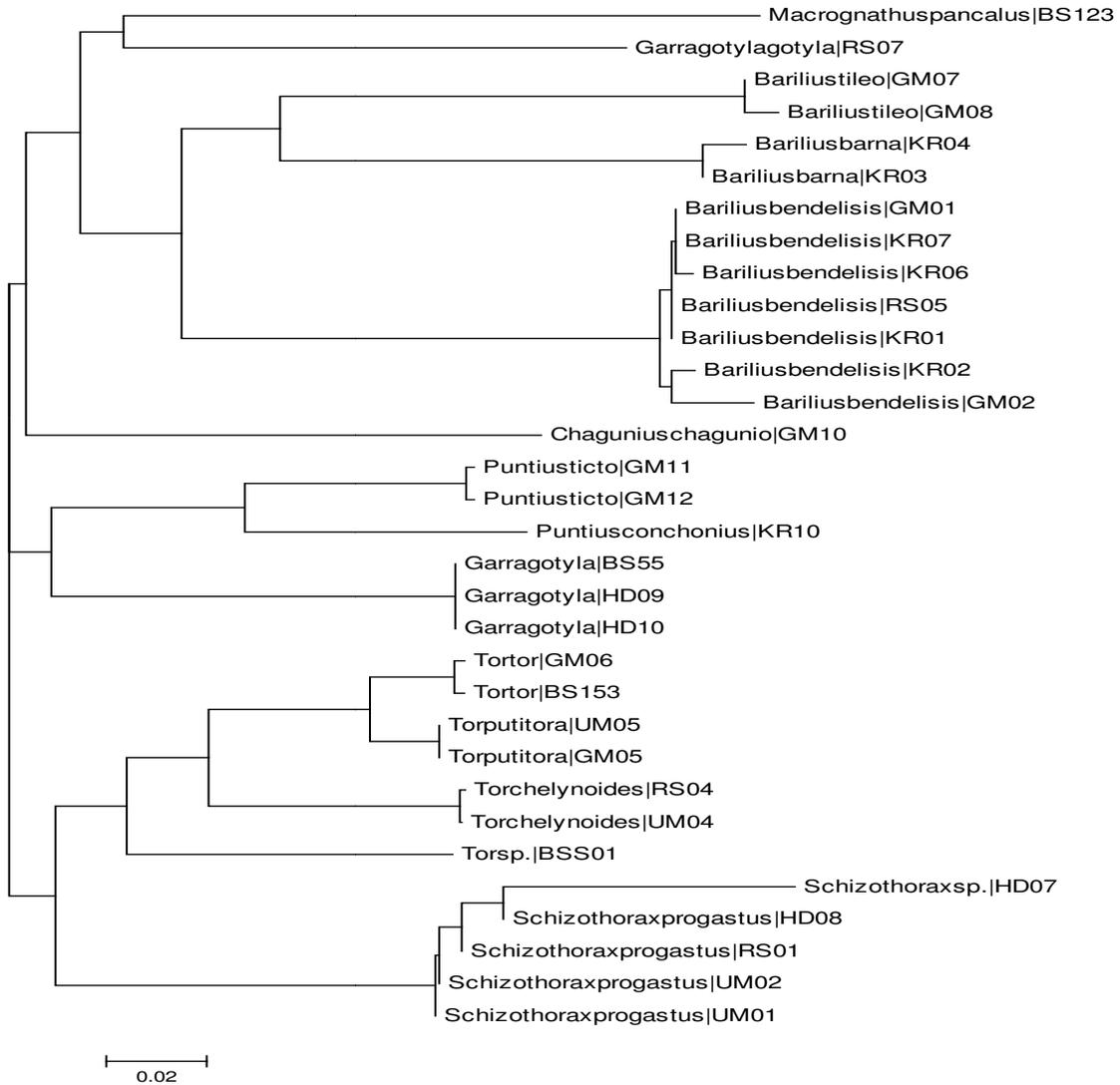Schizothoraxprogastus|UM02
Schizothoraxprogastus|UM01

0.02

Figure 3: Compact K2P distance (based on NJ tree method) showing 15 analyzed species from the River Song

Table4: Summary of the genetic variability (K2Pdistence) distribution at COI sequences for 50 individuals and 15 species.

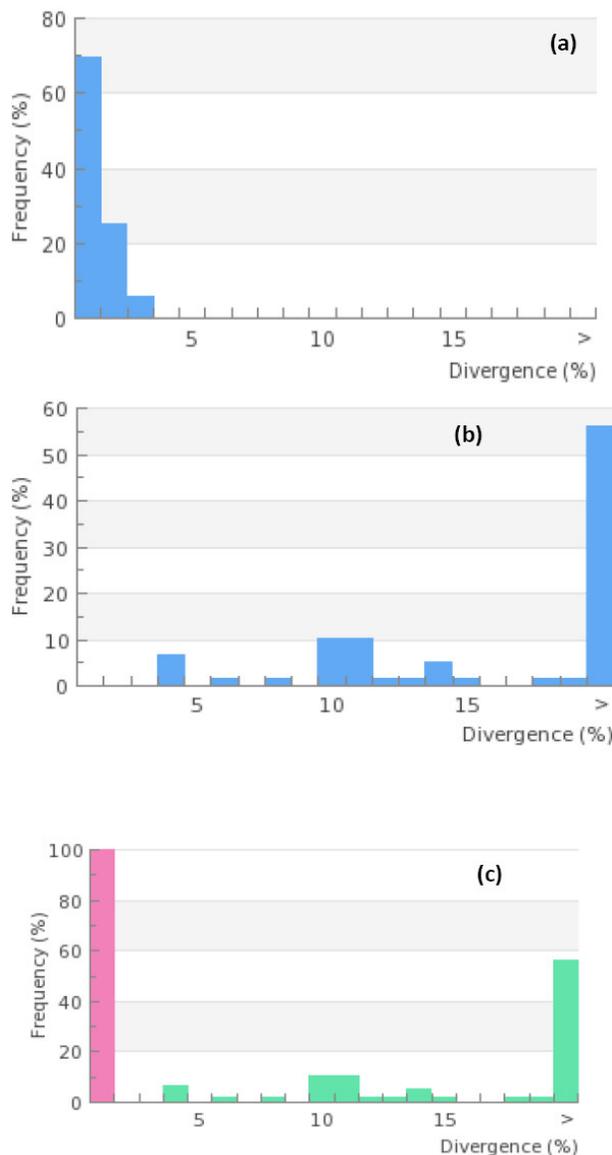|	n	Taxa	Comparisons	Min Dist. (%)	Mean Dist. (%)	Max Dist(%)	SE Dist(%)
Within Species	26	9	36	0	**0.61**	2.1	0.02
Within Genus	30	5	59	3.06	**16.3**	23.53	0.11
Within Family	31	1	370	14.87	**21.79**	33.78	0.01

Figure 5. Bold data generated under the project can be seen in following manner for species comparisons. Summary of the genetic variability (K2Pdistences) at COI sequence for 32 individuals and 50 specimens. Sequences divergence at each taxonomic level is summarized as (a) Within species (b) Within genus. (c)The within-species distribution is normalized to reduce bias in sampling at the species level. The table below summarizes this distribution, while the histogram plots the distribution of normalized divergence for species (first column on left) against the genus divergences (all the other columns).. (http://www.boldsystems.org/index.php/MAS_Analysis_DistSummary/process)

With the exception of two species (*Barilius bandilius* and *Barilius barna*), All species analyzed were monophyletic mean intra-specific distance was less than 1.45% for 32% of all species.Mean K2P distance within species genus family, 0.368%, 16.599%, 21.985%. In case of *Barillius* species, the data suggests that there is a higher variation in sequences of COI gene suggesting genetic variations (Fig. 6, 7). Usually individuals of a species are grouped in same place, e.g. in case of *Schizothorax* species (Fig.7). The data for Barillius species seems to differ from this norm (Fig.6).Cryptic species (look similar but are genetically different) pose a taxonomic challenge as these species cannot be identified easily morphologically. Best way to identify them is through the use of molecular techniques and the best technique available is based on the species specific sequence on particular region of COI gene in mitochondrial DNA. There are a lot fish species in Uttarakhand that inhabit the numerous small streams and rivers and not much is known about their molecular identity. Our study carried out a scan of one such river "River Song" and generated a molecular database base based on DNA barcode (each base in the species specific region of COI is given a colour and hence it is called a Bar Code). Our study identified 15 species from fifty samples belonging to 02 families and their mean divergence was calculated. The most surprising data set was obtained in case of *Barillius* species (especially *Barillius bendelisis*). This species is found in almost every small stream of Himalayan region and even in other regions of India. The *Barillius* species was collected from different location from Song River and even then the tends to show high degree of variability. The sequencing error part for this data was negated by the fact that other samples from other species did not show this variability. Similar data set was obtained for *Schizothorax* species (Fig. 6 & 7) and the data was analyzed and categorized into a similar group in the phylogenetic tree. However all the samples were assigned a different position in phylogentic tree in case of *Barillius* (Fig 6). *Barilius barna* and *Barilius bandilius* are morphologically similar and sometimes it is difficult to differentiate them on physical basis. They were clearly identified by the molecular dataset and both the species were categorized into two separate sections (Fig. 3).
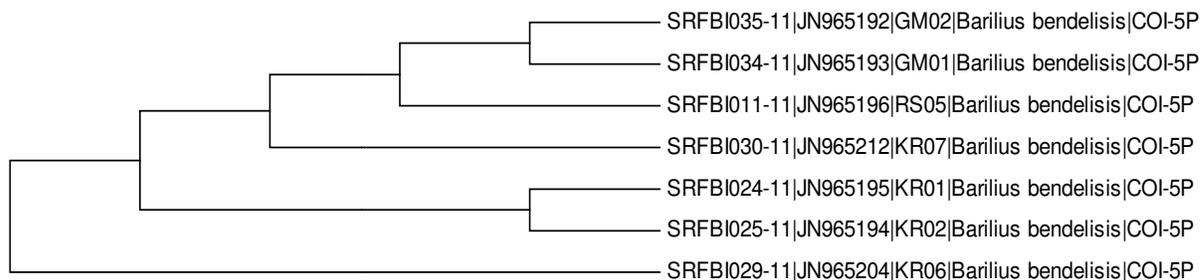
Phylogenic Tree Construction of *Barillus bendelisis* species of Song River

Figure 6: K2P distance (generated using NJ tree method) of *Barillus bendelisis* with high con-specific genetic divergence. Voucher numbers are indicated before species name. Phylogeny among *Barilius bendelisis* sample in voucher ID GM02 (first sample in figure) is suggestively different to *Barilius bendilius* voucher ID KR06 (last sample in figure).
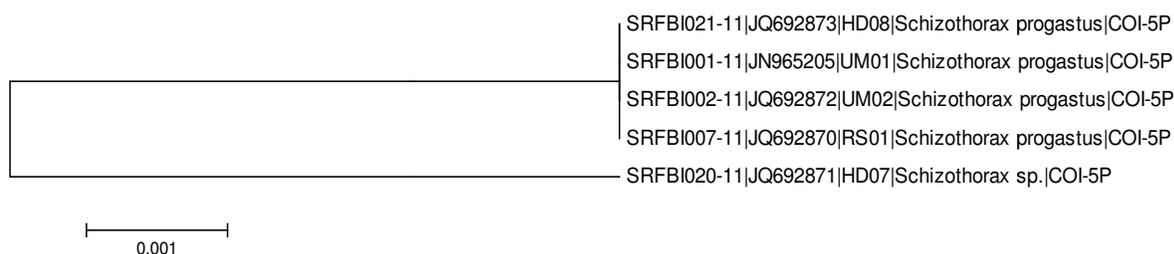

Figure 7: K2P distance (generated using NJ tree method) of *Schizothorax* species. Voucher numbers indicated before species name. *Schizothorax* species are mostly clustered together and do not show much variations among similar populations.

Overall the genetic mean distance between con-specific (0.61%) and con-generics (16.3%) for the song river fishes (Table-4 and Fig. 5)were higher than the previously reported in the marine (0.3 and 8.4% ; (Ward et al. 2009) and or fresh water ecosystem 0.45/5.1%;Hubert et al.(2008).The average K2P divergence within con-specific specimens was only 0.61%a value 30% (approximately)time lower than the found among congeneric species (16.3%).These values are consistent with those found in the literature. Ward et al. (2005) found average value of 0.39and 9.93% by cons specific and congeneric comparisons, respectively for marine fishes, discriminating all species.Hubert et al (2008) , studying freshwater fishes from Canada ,found average K2P values of 0.27% (con-specific) and 8.37% congeneric, discriminating 93% of the species.While Word et al.(2009) analyzed the values of K2P genetic divergence of 108 fish species available on BOLD and found the K2P valves of 0.3%(con-specific)

and 8.4%congeneric ,discriminating about 97.5%of the species .these values are very similar with those found in the present study.Thedifference between con-specific and congeneric average K2P divergence was more pronounced in our analysis 30% (approximately) fold difference compared with values found in other fish species .Analysis of 32 barcode sequence belonging to 50 aquatic species under phylum chordate showed that BOLD is well suited to the identification of biological organisms at different taxonomic level. Our analyses, based on the mitochondrial genes COI-1 are capable of discriminating cold water species with high accuracy. A total of 32 COI barcode of 656 bp were thus obtained for 15 species .nucleotide composition showed a CT bias within C=27.49 ,T=29.29 ,A=25.28, G=17.88 , sequence have been deposited in Gene Bank (Accession no in table 3). Accession numbers for the barcodes, and collection data, sequence, trace file and primers details were available in NCBI gene bank.In our present study 15 species were well resolved by

DNA barcoding using COI gene. Phylogenetic tree is also showing the species packing in resemblance with taxonomic hierarchies. It proves the authenticity of DNA barcoding using COI gene as shown in previous cases. All data generated under this research work can be accessed using internet resources using BOLD web page. (www.boldsystem.org). COI based species identification data shows that river is having15 species. A very high variation in sequences of COI from *Barillius* species was a surprise. It will be a matter of further studies to assess and investigate these variations.

Acknowledgement

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