Decoding Evolution of Native Fishes in Garhwal Himalaya using Molecular Markers and DNA Barcoding

Madhu Thapliyal, Bipin Sati, Ashish Thapliyal, K. K. Joshi

Abstract: ***As we are moving forward into the modern era of science, several new technologies have revolutionized various branches of science. Techniques of biodiversity conservation, fish biology etc. has also adapted to modern techniques. For a long time, most of the researches in taxonomy, including fisheries science were based on morphology and traditional methods. After the decade of 90's, slowly severalmolecular markers like RFLP, RAPD, SNP's etc. made inroad into taxonomy and fisheries. Molecular markers have several applications in the field of livestock improvement and understanding population dynamics to name a few. Since the 2004, a specific molecular marker, generally known as DNA Bar-coding for species identification, came up. This molecular marker is a part of mitochondrial genome that encodes for Cytochrome C Oxidase Unit I (also called as COX or COI). It is advantageous because it has been tested across several animal species and it can differentiate species very well. This marker has also been used as a forensic tool to identify the species. In the current paper, we have used this molecular marker to decode evolution of native fishes of Garhwal Himalayan region. Over 350 barcodes were developed and these barcodes were used to for phylogenetic analysis.***

Key words- Molecular Markers, DNA Bar-coding, Evolution, Himalaya, Breeding, livestock

I. INTRODUCTION

Himalaya has diverse and extensive network of fresh water rivers, streams, lakes etc. 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. It is suggested that the native fish species of Himalayan region might be one of the earliest inhabitant of these fresh water systems and hence they are a good model to study evolution unfolding. These fishes have been well documented. However, prior to the year 2000, most of the studies were based on morphological characters and books like "Day Fauna" were served as the "KEY" for identification of fishes. All these so called "KEYS" were extensive illustrations of each species. About 2500 species of fishes have been reported in India and approximately 930 of these are fresh water fishes. The Himalayan region of India harbor's about 225 of these fresh water fishes. Various researchers have reported up to 50 different fish species from Garhwal Himalayan region.

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In Uttarakhand, most of the fresh water fishery resourcesare contributed by the River Yamuna or River Ganges. Fishes of Rivers Ganges (and is tributaries) has been well documented by fishes of River Yamuna in Garhwal Himalaya have not been well known except some contributions. There are numerous morphological based studies but there are only few report investigating fish species using molecular markers. Molecular markers are also being used used for assessing biodiversity using environmental DNA and meta-genomics (Krehenwinkel et. al., 2019; Adams et. al., 2019; Xing et. al. 2020). In about last three decades, the scenario of most of the Himalayan region as changed due to fast changing ecology of upland waters. Impact of anthropogenic activity on genome of fish species is among the interest in Himalayan region. The genetic variation in Himalayan region due to the regular floods in rivers and Dam constructions still not reported. Attempts have also been made to generate the DNA barcode & Population genetics of fishes, but most of the attempts are limited to major rivers i.e. the Ganges and the Yamuna (Thapliyal et al., 2013).

The molecular markers based on DNA are helpful to provide evolutionary relationship among different populations and cryptic species identification. The current paper is an attempt to investigate these changes using molecular markers specially focusing on DNA barcoding.

II. WHY MOLECULAR MARKERS

During several studies on morphological characters, research encountered a dilemma. There were several individuals which looked alike or had only small variations. A good example is that of *Schizothorax* species. It needs an expert to identify (ID) the two species of *Schizothorax* namely *S. progastus* and *S. richardsonii* and even after identification there could be queries about the ID. This happens in several species that they look alike but they are actually different species genetically. The latest example is of Giraffe (Petzold&Hassanin, 2020). To solve this issue, molecular markers emerged and as they are specific sequences of DNA, these studies when coupled with morphometric studies were considered better option for species level identification. Introduction of molecular biology techniques in fisheries had a huge impact on the entire fishery research. Through application of these techniques we can figure out the variations in specific regions of genome. We can also develop a marker for desired characters and identify species based on DNA Barcode which is somewhat similar or just like a product barcode.

III. MOLECULAR MARKERS

Molecular marker is a specific sequence located on a specific gene. During the process of new progeny formation, inheritance of specific character occurs and these molecular markers stay together with the desired character. Hence the name molecular markers as the desired character can be followed by just following the molecular marker sequence. The interest in the DNA sequence based molecular markers had started as soon as the DNA model was presented by Watson and Crick and this model was awarded a Nobel Prize. This was followed by a rapid development of new technologies and methods like Polymerase Chain Reaction and DNA Sequencing. Once the sequencing of genome started, it opened an entire new ear of molecular markers and even a change in single nucleotide in a gene could be followed – technically called as Single Nucleotide Polymorphsim.

IV. WHY MITOCHONDRIAL MARKER:

Besides the nuclear DNA, the eukaryotic mitochondria also has an extra DNA. This mitochondrial genome codes for 37 genes (two rRNAs, 22 tRNAs and 13 polypeptides).Mitochondrial DNA is of interest because of its unique features. These features are: a) This DNA is maternally inherited, b) it is a haploid molecule, c) there is no recombination process, d) there is no repair mechanism during DNA replication process e) there are several mitochondria in a cell and so it can be isolated and targeted easily, f) there are no introns in mitochondrial genome and g) mitochondrial genome is not too big and the optimum size makes it a favorite h) The COI marker can be used as a universal marker across entire animal species.The COI marker was first reported by Dr. Paul Hebert from University of Guelph, Canada as a molecular marker that can be used effectively to develop molecular database based catalogue of various animals inhabiting different regions (Hebert et al., 2003; Hebert et al., 2004). The sequences of these markers can also be compared using different available software's of sequence alignment and analysis.

V. STUDIES IN GARHWAL HIMALAYAN REGION OF UTTARAKHAND :-

As Garhwal Himalayan region is the origin point of River Ganges and River Yamuna, it is important to understand the evolutionary context of fauna inhabiting these river. Attempts have been made to generate the DNA barcode &study population genetics of fishes in these rivers. Besides these river, there are several small tributaries that also inhabited by many species. In the entire study – one of the molecular marker called as COI – Cytochrome C Oxidase Unit I has been used. A specific region of this gene is sequenced and the bases are represented as colour codes and hence the name DNA Barcode (Fig. 1).

Example: GenBank Accession numbers ***NCBI ID*** **JN965201**

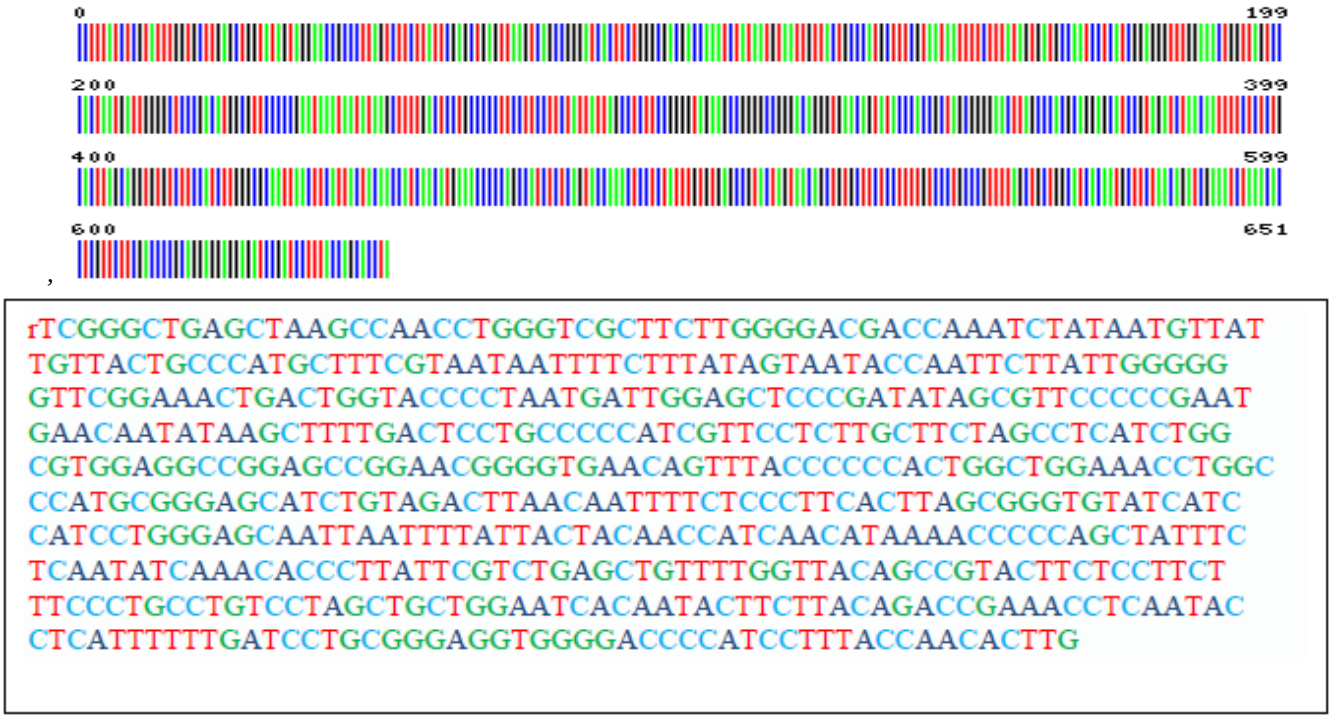

Fig 1.The concept of DNA Barcode. A sequence is converted to a barcode.

Table 1 –Molecular markers and their description

S.No.	Marker	Details
1.	**RFLP** (Restriction Fragment Length Polymorphism)	In this method, one or more restriction enzyme(s) are used to cut a DNA isolated from the desired samples. The DNA digested by these restriction enzymes is then run on a gel which give a unique banding pattern. These patterns are used to a analysis called as RFLP (Restriction Fragment Length Polymorphism). RFLP analysis is used in population studies (Ferguson *et al*., 1995).
2.	**RAPD** (Random Amplification of Polymorphic DNA)	RAPD technique is a PCR based technique. Several primers are used on the same DNA sample and then the amplified regions profile is developed. (Hadry*set al*., 1992).
3.	Dloop region	There is a region in mitochondrial region which is a non-coding region. This region is called as The D-loop region. Variations of this region is mapped in case of studies using D-loop as a marker.
4.	**VNTRs** (Variable number tandem repeats)	When the eukaryote genome was analyzed, it was surprising to note that there were several unique segments of sequences that were repeated several times (from 10 to 100 or more, O'Reilly and Wright, 1995). These repeated units can be of two types – first one called as mini-satellite DNA (9-65 bp long), and second called as microsatellite DNA (4-8 bp long). (Magoulas, 1998).
5.	COI	The mitochondrial genome COI gene is an approximate 656 bp region. The gene encodes part of the terminal enzyme of the respiratory chain of mitochondria.
6.	Cytocrome b	Cytochrome b is a component of electron transport gene and is used in some studies. This gene is too long (about 1,140 bp) and sometimes the DNA sequencing data of longer genes is cumbersome to handle.
7.	16s rRNA	16S rRNA gene has been used extensively for bacterial identification.
8.	ATPase 6/8	A region of mitochondrial ATPase gene

Table 2.Some of research papers in the Uttarakhand region on molecular marker.

Species	References	Marker used	Year	Place/Area
DawkinsiaTambraparniei	karuppiahkannan	Cytocrome b	2014	Uttarakhand
Labeogonius	GrishmaTewari.	RAPD	2013	Uttarakhand
S. richardsonii, T. putitora, B. Bendelisis and G. Gotyla.Danio	Thapliyal M	COI	2013	Uttarakhand
S. richardsonii, T. putitora, B. Bendelisis and G. Gotyla	HimaniPandey.	16SRNA	2013	Uttarakhand
Bariliusbendelisis	A. K. Mishra.	RAPD	2012	Uttarakhand
S. richardsonii and S. progastus.	SureshChandra.	COI	2012	Uttarakhand
Golden Mahseer Tor putitora, Snow trout, Schizothorax richardsonii, Indian trout, Raiamus bola Garra, Garragotyla	G.K.Sivaraman.	RAPD, 12S rRNA	2012	Uttarakhand
Bariliusbendelisis	SeemaSah.	cytocrome b	2011	Uttarakhand
Schizothorax richardsonii	Ashoktaru Barat.	cytocrome b	2011	Uttarakhand
Eutropiichthysvacha	Gyan Chandra.	RAPD	2010	Uttarakhand
Tor putitora	Mamta Singh.	45S and 5S	2009	Uttarakhand

VI. MATERIAL AND METHODS

Study site: The present study was a 200 kilometre radius of Garhwal Himalaya (30N; 78E approx.) Uttarakhand. Sampling sites included Bakot to Ponta Sahib in River Yamuna and from Bhatwari to Rishikesh in river Ganges.

DNA extraction, PCR amplification, and sequencing.

The DNA was isolated from fish fin (Wizard Genomic DNA Purification Kit, Cat# A1120, Qiagen Integrity). The isolated DNAs was then checked on 1% Agarose gel and quantified (Nanodrop 1000 spectrophotometer, Applied bio system). Method used was as per Thapliyal et. al., 2013. In short, Samples were subjected to PCR using universal primers (FF2d (forward): TTCTCCACCAACCACAARGAYATYGGFR, FF1d (reverse): CACCTCAGGGTGTCCGAARAAYCARAA) The thermal cycler program was initial Denaturation at 95^0C for 5min followed by 35 cycles of 95^0C for 30sec of Denaturation, 55^0C of annealing for 30sec and 72^0C of extension for 1min and final extension of 72^0C for 7min and then the samples were stored at 4^0C. The samples were then run at 1.5% of Agarose gel for their quality check. The sample showing one clear band after PCR samples were sorted and purified with EXO1-SAP (Exonuclease1 and Shrimp Alkaline Phosphatase: USB Corp) with the temperature conditions suggested by manufacturer. The purified PCR amplicons were then ladled 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)1µL, Primer (0.8pMol/µL). The cycle sequenced amp icons were then purified with Big Dye (R) X Terminator (TM)(Big Dye Terminator v3.1 clean up Applied Bio systems, USA) each side labelled separately and were sequences on ABI 3130 DNA genetic analyser.2µL, MQ Water4.75µL. The cycle sequencing conditions was Initial Denaturation of 960C for 1min followed by 35 cycles of Denaturation 960C for 10sec, annealing 500C for 5sec, extension 600C for 4min and then the samples were stored at storage temperature of 40C. The sequences were then obtained and analysed in the Sequence Scape software v2.7 for possibilities of indels(Applied Biosystems 3130 Genetic Analyzers).

VII. DATA ANALYSIS

DNA sequence were also submitted to Gene bank (accession numbers included in appendix online tools). MEGA program (XXX) was used for sequence alignment and further interpretation.

VIII. RESULTS

About 350 GenBank submissions have been made during the entire period of study starting from 2013. There were some interesting observations that are becoming evident from our study that the distribution pattern of species, especially the *Schizothorax* species, needs to be redefined based on molecular data. More data is also being added so that statistical validation can be carried out.Some of these submissions of various molecular markers are:

S.No.	Name of the Species	Voucher No	NCBI Accession No
1	*Bariliusbarna*	RS05	JN965191
2	*Bariliusbarna*	GM01	JN965190
3	*Bariliusbendelisis*	KR01	JN965192
4	*Bariliusbendelisis*	KR07	JN965196
5	*Bariliusbendelisis*	KR06	JN965195
6	*Bariliusbendelisis*	KR02	JN965194
7	*Bariliusbendelisis*	GM02	JN965204
8	*Bariliusbendelisis*	KR04	JN965212
9	*Bariliusbendelisis*	KR03	JN965193
10	*Bariliustileo*	GM07	JN965198
11	*Bariliustileo*	GM08	JQ692874
12	*Chaguniuschagunio*	GM10	JN965199
13	*Garragotyla*	BS55	JN965210
14	*Garragotyla*	HD09	JN965211
15	*Garragotyla*	HD10	KC473939
16	*Garragotylagotyla*	RS07	JN965200
17	*Macrognathuspancalus*	BS123	KC473940
18	*Puntiusconchonius*	KR10	JN965201
19	*Puntiusticto*	GM12	JN965202
20	*Puntiusticto*	GM11	JN965203
21	*Schizothorax progastus*	UM01	JN965205
22	*Schizothorax progastus*	UM02	JQ692872
23	*Schizothorax progastus*	RS01	JQ692870
24	*Schizothorax progastus*	HD08	JQ692873
25	*Schizothorax sp.*	HD07	JQ692871
26	*Tor chelynoides*	UM04	JN965207
27	*Tor chelynoides*	RS04	JN965206
28	*Tor putitora*	GM05	JN965209
29	*Tor putitora*	UM05	JN965197
30	*Tor sp.*	BSS01	KC473941
31	*Tor tor*	BS153	KC473942
32	*Tor tor*	GM06	JN965208
33	*Acanthocobitisbotia*	GPCR 281AB	KR809714
34	*Acanthocobitisbotia*	GPCR1AB	KU043312
35	*Acanthocobitisbotia*	GPCR2AB	KU043313
36	*Acanthocobitisbotia*	GPCR3AB	KU043314
37	*Acanthocobitisbotia*	GPCR4AB	KU043315
38	*Badisbadis*	GPCR 141BB	KR809715
39	*Badisbadis*	GPCR 282BB	KR809716
40	*Badisbadis*	GPCR 284BB	KR809717
41	*Badisbadis*	GPCR 287BB	KR809718
42	*Badisbadis*	GPCR5BB	KU043316
43	*Badisbadis*	GPCR6BB	KU043317
44	*Badisbadis*	GPCR7BB	KU043318
45	*Badisbadis*	GPCR8BB	KU043319
46	*Bariliusbarna*	GPCR 438BB	KR809719
47	*Bariliusbarna*	GPCR9BB	KU043320
48	*Bariliusbarna*	GPCR10BB	KU043321
49	*Bariliusbarna*	GPCR11BB	KU043322
50	*Bariliusbarna*	GPCR12BB	KU043323
51	*Bariliusbendelisis*	GPCR 113BB	KR809720
52	*Bariliusbendelisis*	GPCR 114BB	KR809721
53	*Bariliusbendelisis*	GPCR13BB	KU043324
54	*Bariliusbendelisis*	GPCR14BB	KU043325

55	*Bariliusbendelisis*	GPCR15BB	KU043326
56	*Bariliusvagra*	GPCR 112BV	KR809722
57	*Bariliusvagra*	GPCR 115BV	KR809723
58	*Bariliusvagra*	GPCR 270BV	KR809724
59	*Bariliusvagra*	GPCR 272BV	KR809725
60	*Bariliusvagra*	GPCR 273BV	KR809726
61	*Bariliusvagra*	GPCR 274BV	KR809727
62	*Bariliusvagra*	GPCR 385BV	KR809728
63	*Channagachua*	GPCR 142CG	KR809729
64	*Channagachua*	GPCR 16CG	KU043327
65	*Channagachua*	GPCR 17CG	KU043328
66	*Channagachua*	GPCR 18CG	KU043329
67	*Channagachua*	GPCR 19CG	KU043330
68	*Channapunctata*	GPCR 146CP	KR809730
69	*Channapunctata*	GPCR 20CP	KU043331
70	*Channapunctata*	GPCR 21CP	KU043332
71	*Channapunctata*	GPCR 22CP	KU043333
72	*Channapunctata*	GPCR 23CP	KU043334
73	*Cyprinuscarpio*	GPCR 223CC	KR809731
74	*Cyprinuscarpio*	GPCR 225CC	KR809732
75	*Cyprinuscarpio*	GPCR 293CC	KR809733
76	*Cyprinuscarpio*	GPCR 294CC	KR809734
77	*Cyprinuscarpio*	GPCR 295CC	KR809735
78	*Cyprinuscarpio*	GPCR50CC	KR809736
79	*Daniodevario*	GPCR 236DD	KR809737
80	*Daniodevario*	GPCR 237DD	KR809738
81	*Daniodevario*	GPCR24DD	KU043335
82	*Daniodevario*	GPCR25DD	KU043336
83	*Daniodevario*	GPCR26DD	KU043337
84	*Garragotyla*	GPCR144GG	KR809739
85	*Garragotyla*	GPCR 27GG	KU043338
86	*Garragotyla*	GPCR28GG	KU043339
87	*Garragotyla*	GPCR29GG	KU043340
88	*Garragotyla*	GPCR30GG	KU043341
89	*Garralamta*	GPCR 145GL	KR809740
90	*Garralamta*	GPCR31GL	KU043342
91	*Garralamta*	GPCR32GL	KU043343
92	*Garralamta*	GPCR33GL	KU043344
93	*Garralamta*	GPCR34GL	KU043345
94	*Lepidocephalichthysguntea*	GPCR 280LG	KR809741
95	*Lepidocephalichthysguntea*	GPCR 147LG	KR809742
96	*Lepidocephalichthysguntea*	GPCR35LG	KU043346
97	*Lepidocephalichthysguntea*	GPCR36LG	KU043347
98	*Lepidocephalichthysguntea*	GPCR37LG	KU043348
99	*Lepidocephalichthys sp.*	GPCR 289Lsp.	KR809743
100	*Lepidocephalichthys sp.*	GPCR38Lsp.	KU043349
101	*Lepidocephalichthys sp.*	GPCR39Lsp.	KU043350
102	*Lepidocephalichthys sp.*	GPCR40Lsp.	KU043351
103	*Lepidocephalichthys sp.*	GPCR41Lsp.	KU043352
104	*Mystusvittatus*	GPCR 288MV	KR809744
105	*Mystusvittatus*	GPCR42MV	KU043353
106	*Mystusvittatus*	GPCR43MV	KU043354
107	*Mystusvittatus*	GPCR44MV	KU043355
108	*Mystusvittatus*	GPCR45MV	KU043356
109	*Nemacheilusmontana*	GPCR 58NM	KR809745
110	*Nemacheilusmontana*	GPCR46NM	KU043357
111	*Nemacheilusmontana*	GPCR47MV	KU043358
112	*Nemacheilusmontana*	GPCR48MV	KU043359

113	*Nemacheilusmontana*	GPCR49MV	KU043360
114	*Pseudecheneissulcata*	GPCR 197PS	KR809746
115	*Pseudecheneissulcata*	GPCR 292PS	KR809747
116	*Pseudecheneissulcata*	GPCR 63PS	KR809748
117	*Pseudecheneissulcata*	GPCR50PS	KU043361
118	*Pseudecheneissulcata*	GPCR51PS	KU043362
119	*Pseudecheneissulcata*	GPCR52PS	KU043363
120	*Puntiuschelynoides*	GPCR 196PC	KR809749
101	*Puntiuschelynoides*	GPCR 170PC	KR809750
122	*Puntiuschelynoides*	GPCR 171PC	KR809751
123	*Puntiuschelynoides*	GPCR 172PC	KR809752
124	*Puntiuschelynoides*	GPCR 195PC	KR809753
125	*Puntiuschelynoides*	GPCR 221PC	KR809754
126	*Puntiuschelynoides*	GPCR 262PC	KR809755
127	*Puntiuschelynoides*	GPCR 263PC	KR809756
128	*Puntiuschelynoides*	GPCR 267PC	KR809757
129	*Puntiuschelynoides*	GPCR 331PC	KR809758
130	*Puntiuschelynoides*	GPCR 387PC	KR809759
131	*Puntiuschelynoides*	GPCR 390PC	KR809760
132	*Puntiuschelynoides*	GPCR 430PC	KR809761
133	*Puntiuschelynoides*	GPCR 431PC	KR809762
134	*Puntiuschelynoides*	GPCR 432PC	KR809763
135	*Salmotrutta*	GPCR 121BT	KR809764
136	*Salmotrutta*	GPCR 124BT	KR809765
137	*Salmotrutta*	GPCR 126BT	KR809766
138	*Salmotrutta*	GPCR 128BT	KR809767
139	*Salmotrutta*	GPCR 1BT	KR809768
140	*Schizothorax plagiostomus*	GPCR 101SP	KR809769
141	*Schizothorax plagiostomus*	GPCR53SP	KU043364
142	*Schizothorax plagiostomus*	GPCR54SP	KU043365
143	*Schizothorax plagiostomus*	GPCR55SP	KU043366
1444	*Schizothorax plagiostomus*	GPCR56SP	KU043367
145	*Schizothorax progastus*	GPCR 105SP	KR809770
146	*Schizothorax progastus*	GPCR 162SP	KR809771
147	*Schizothorax progastus*	GPCR 356SP	KR809772
148	*Schizothorax progastus*	GPCR 4SP	KR809773
149	*Schizothorax progastus*	GPCR 9SP	KR809774
150	*Schizothorax progastus*	GPCR 100SP	KR809775
151	*Schizothorax progastus*	GPCR 131SP	KR809776
152	*Schizothorax progastus*	GPCR 160SP	KR809777
153	*Schizothorax progastus*	GPCR 227SP	KR809778
154	*Schizothorax progastus*	GPCR 374SP	KR809779
155	*Schizothorax progastus*	GPCR 97SP	KR809780
156	*Schizothorax sinuatus*	GPCR 110SS	KR809781
157	*Schizothorax sinuatus*	GPCR 111SS	KR809782
158	*Schizothorax sinuatus*	GPCR 57SS	KU043368
159	*Schizothorax sinuatus*	GPCR 58SS	KU043369
160	*Schizothorax sinuatus*	GPCR 59SS	KU043370
161	*Schizothorax richardsonii*	GPCR 1SR	KU695217
162	*Schizothorax richardsonii*	GPCR 2SR	KU695218
163	*Schizothorax richardsonii*	GPCR 3SR	KU695219
164	*Schizothorax richardsonii*	GPCR 4SR	KU695220
165	*Schizothorax richardsonii*	GPCR 5SR	KU695221
166	*Tor putitora*	GPCR 151TP	KR809783
167	*Tor putitora*	GPCR 382TP	KR809784
168	*Tor putitora*	GPCR 383TP	KR809785
169	*Tor putitora*	GPCR 384TP	KR809786
170	*Tor putitora*	GPCR 51TP	KR809787

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REFERENCES

1. Adams, C.I.; Knapp, M.; Gemmell, N.J.; Jeunen, G.-J.; Bunce, M.; Lamare, M.D.; Taylor, H.R. (2019) Beyond Biodiversity: Can Environmental DNA (eDNA) Cut It as a Population Genetics Tool? Genes, 10, 192
2. Barat A, et al., (2011). Phylogenetic analysis of fishes of the subfamily Schizothoracinae (Teleostei: Cyprinidae) from Indian Himalayas using cytochrome b gene, Indian J. Fish., 59(1): 43-47.
3. Chandra S et al., (2012). DNA Bar-Coding of Indian Coldwater Fishes of Genus Schizothorax (Family: Cyprinidae) from Western Himalaya, World Journal of Fish and Marine Sciences 4 (4): 430-435, 2012 ISSN 2078-4589 © IDOSI Publications,
4. Ferguson, A., Taggart, J.B., Prodohl, P.A., McMeel, O., Thompson, C., Stone, C., McGinnity, P. and Hynes, R.A. (1995).The application of molecular markers to the study and conservation of fish populations with special reference to *Salmo*.*Journal of Fish Biology*, 47(A), 103-126.
5. Hadrys, H., Balick, M. and Schierwater, B. (1992). Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol., 1: 55–63.
6. Hansen, M.M. (2003). Application of molecular markers in population and conservation genetics, with special emphasis on fishes. DSc Thesis, Faculty of Natural Sciences, University of Aarhus, 68 pp.
7. Hebert, P. D., Cywinska, A., Ball, S. L., &Dewaard, J. R. (2003).Biological identifications through DNA barcodes.Proceedings of the Royal Society of London. Series B: Biological Sciences, 270(1512), 313-321.
8. Hebert, P. D., Stoeckle, M. Y., Zemlak, T. S., & Francis, C. M. (2004).Identification of birds through DNA barcodes.PLoS biology, 2(10).
9. Krehenwinkel, H.; Pomerantz, A.; Prost, S. (2019) Genetic Biomonitoring and Biodiversity Assessment Using Portable Sequencing Technologies: Current Uses and Future Directions.Genes, 10, 858.
10. Magoulas, A. (1998). Application of molecular markers to aquaculture and broodstock management with special emphasis on microsatellite DNA. Cahiers Options Mediterrannes, 34: 153- 168
11. McConnell, S.K., O'Reilly, P., Hamilton, L., Wright, J.N. and Bentzen, P. (1995).Polymorphic microsatellite loci from Atlantic salmon (*Salmosalar*) – genetic differentiation of North American and European populations.Can. J. Fish.Aquat.Sci., 52: 1863– 1872.
12. Parker, Patricia G. et al., (1998). "What Molecules Can Tell Us about Populations: Choosing and Using a Molecular Marker". *Ecology* **79** (2): 361–382.
13. Petzold, A., &Hassanin, A. (2020). A comparative approach for species delimitation based on multiple methods of multi-locus DNA sequence analysis: A case study of the genus Giraffa (Mammalia, Cetartiodactyla). PloS one, 15(2), e0217956.
14. Siozios, S., Massa, A., Parr, C. L., Verspoor, R. L., & Hurst, G. D. (2020). DNA barcoding reveals incorrect labelling of insects sold as food in the UK. PeerJ, 8, e8496.
15. Thapliyal M., Sati B.K., Kumar R., Chandra T., Thapliyal A., (2013).Molecular taxonomy of fresh water fishes from song river Uttarakhand using mitochondrial cytochrome-c oxidase-1 gene, Environment Conservation Journal, 14(3).
16. Xing, B., Zhang, Z., Sun, R., Wang, Y., Lin, M., & Wang, C. (2020). Mini-DNA barcoding for the identification of commercial fish sold in the markets along the Taiwan Strait. Food Control, 107143.