World Journal of Fish and Marine Sciences 4 (4): 430-435, 2012
ISSN 2078-4589

DNA Bar-Coding of Indian Coldwater Fishes of Genus *Schizothorax* (Family: *Cyprinidae*) from Western Himalaya

[1]*Suresh Chandra*, [1]*Ashoktaru Barat*, [2]*Mahender Singh*, [3]*Birender Kumar Singh and* [1]*Rakesh Matura*

[1]Molecular Genetics Laboratory, Directorate of Coldwater Fisheries Research, (ICAR), Bhimtal-263136, Nainital, Uttarakhand, India
[2]International Centre for DNA Barcoding, National Bureau of Fish Genetic Resources, (ICAR), Dilkusha, Lucknow-226002, (U.P.), India
[3]Department of Zoology, Kumaun University, Nainital, Uttarakhand, India

Abstract: The rapid and accurate characterization of species using morphological data is a critical constraint. To overcome this, species identification using molecular tools has been supplemented in many studies in present era. The present study was designed to test the utility of Cytochrome Oxidase I (COI) DNA barcodes for the identification of two commercially important coldwater species of Genus *Schizothorax* (Snow trout), Family *Cyprinidae*, from Uttarakhand Himalayas. COI gene (655 bp) was amplified using PCR and sequenced. The mean intra-specific nucleotide sequence divergence was 1.75% (range 0.00-3.50%) and inter-specific divergence of *S. richardsonii* is 0.00% (range 0.00040-0.00080%) and *S. progastus* is 0.00% (range 0.00036-0.000206), respectively. Although, DNA bar-coding aims to develop species identification systems, some phylogenetic signal was apparent in the data. It was concluded that COI sequencing or 'bar-coding', was found to be suitable for the identification of coldwater fish species.

Key words: Cytochrome Oxidase Subunit I % Bar-coding % Fish % MtDNA % *Schizothorax*

INTRODUCTION

Accurate and unambiguous identification of fish and fish products, from eggs to adult, is important in many areas. It would enable retail substitutions of species to be detected, assist in managing fisheries for long-term sustainability and improve ecosystem research and conservation. Hitherto, a wide variety of protein and DNA based methods have been used for the genetic identification of fish species [1-4]. DNA sequence analysis has been used for 30 years to assist species identifications, but different sequences have been used for different taxonomic groups and in different laboratories [5]. Proposed that a single gene sequence would be sufficient to differentiate all, or at least the vast majority of, animal species and proposed the use of the mitochondrial DNA gene cytochrome oxidase subunit I (COI) as a global bio-identification system for animals. The sequence was likened to a barcode, with species being delineated by a particular sequence or by a tight cluster of very similar sequences.

Species identification by DNA bar-coding is based on sequencing a short standardized genomic region of the target specimen and comparing this information to a sequence library from known species. The proposed standard barcode sequence for animal species is a 650-bp fragment of the mitochondrial gene cytochrome *c* oxidase I (COI). Many benefits of DNA bar-coding for species identification and discovery have been discussed [6], although the concept continues to be hotly debated [7]. In addition to species identification, the construction of barcode database could expose novel DNA barcodes that may indicate provisional new species [8]. Genetic interrelationships of Cyprinid subfamilies have been extensively investigated from morphological, anatomical and molecular perspectives [9-12]. Two studies mainly based on morphological and anatomical characters have investigated phylogenetic relationships among genera and species and explored the taxonomic status of these

Corresponding Author: Suresh Chandra, Molecular Genetics Laboratory, Directorate of Coldwater Fisheries Research (ICAR), Bhimtal-263136, Nainital, Uttarakhand, India. Tel: +91-9696692623.

fishes [13, 14] but, the molecular identification based on mtDNA COI gene are somewhat understudied for the highly specialized *Schizothorax* species in the Garhwal Himalaya [15].

The fishes of genus *Schizothorax*, members of the family Cyprinidae, commonly known as snow trout, consist of 15 genera and over 100 species all over the world [16]. In India, these species are distributed in the cold waters from Jammu and Kashmir [17], to Assam and Eastern Himalayas through Bhutan and Sikkim at an altitude of 1180-3000m [18]. 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 resources and population increase [19]. This genus consists of a group of species that are remarkably similar in general morphology. The species of *Schizothorax* are often the most difficult to distinguish based on external morphological characters. A DNA bar-coding approach may be useful for the identification of taxa. For these reasons, the utility of the COI barcode sequence for the identification of snow trout was tested. In the present study, an attempt was made to examine COI diversity within and among two fish species, with the goal of determining whether DNA bar-coding can achieve unambiguous species recognition in fishes.

MATERIALS AND METHODS

Samples Collection: The fish Samples were collected by cast net from two different river system (Khanda and Dugadda gad) of pauri Garhwal from Uttarakhand Himalaya (Fig. 1). The Muscle samples were collected through dorsal part and preserved in 95% ethanol. All specimens were fixed in 10% formalin in the field as a voucher. The Rivers (Khanda and Dugadda gad) drain North-Western Garhwal Himalaya. It is a tributary of the River Alakananda, originates from the high altitude zone of the Distt Pauri Garhwal close the distt Chamoli. It travels distance of about 10 to 20 kilometer through the wide valley of the Distt Pauri and Tehri (Table 1).

Laboratory Procedures

DNA Extraction, Amplification and Sequencing:

Total genomic DNA was isolated from muscle tissue by using the standard phenol-chloroform extraction protocol. Approx. 655 bp nucleotide was amplified from the 5' region of the COI gene from mtDNA using different combinations of two pairs of primers: F1 (5'-TCAACCAACCACAAAGACATTGGCAC-3'), R1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'), F2 (5'-TCGACTAATCATAA AGATATCGGCAC-3'), R2 (5'-ACTTCAGGGTGACCGAAGAATCAGAA-3'). Polymerase chain reaction (PCR) amplifications were

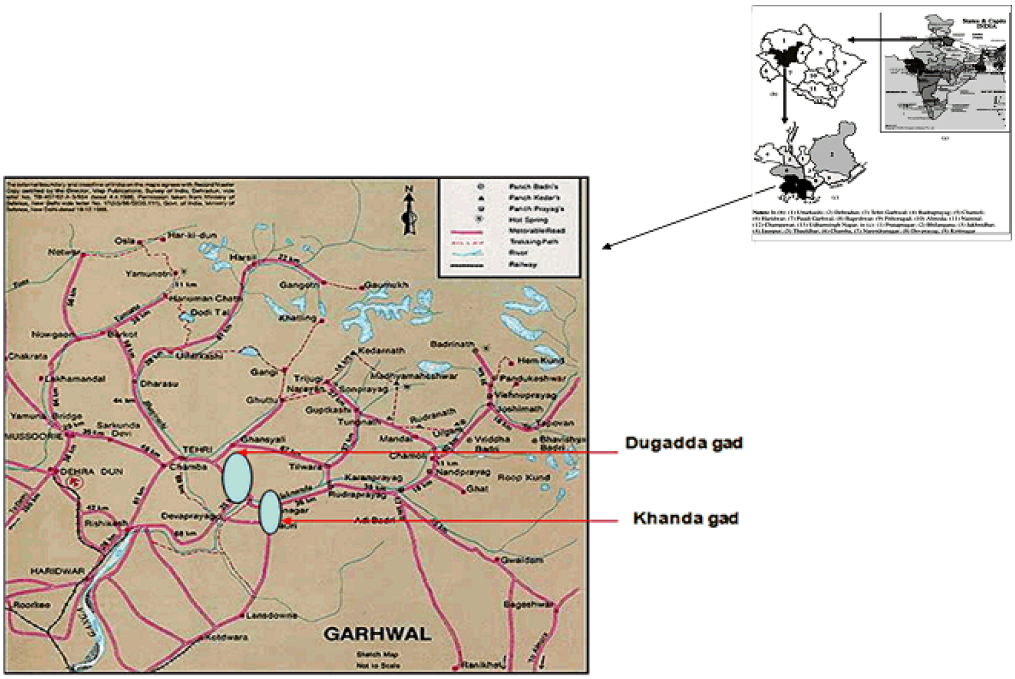

Fig. 1: Map of Uttarakhand (Garhwal region) showing *Schizothorax* sample locations

Table 1: Specimen information, collection dates and collection localities for the Himalayan Snow trout *Schizothorax* species studies

Species	Voucher ID	GenBank accession no.	Collection locality
Schizothorax progastus (Mcclellad)	SK8011-a	FJ170772	Khanda and Dugadda Gad Lat-29° 45'
	SK8011-b	FJ170773	27.0"N and Log-78° 32'22.4"E.
	SK8011-c	FJ170774	
	SK8011-d	FJ170775	
	SK8011-e	FJ170776	
Schizothorax richardsonii (Gray)	SD8012-a	FJ170777	Pauri and Tehri Garhwal,Uttarakhand
	SD8012-b	FJ170778	
	SD8012-c	FJ170779	
	SD8012-d	FJ170780	
	SD8012-e	FJ170781	

performed on a MJ research PTC-200 thermocycler in a 50µl reaction consisting of: 5µl of 10X buffer (100mM Tris, pH 9.0, 500mM KCl, 15mM $MgCl_2$, 0.1% Gelatin) (Genei, India), 200 µM each nucleotide (dNTP) (Genei, India), 5pmole of each primer (Sigma Genosys, USA), 1.5U taq polymerase (Genei, India) and 1-2µl of total genomic DNA. The thermal regime consisted of an initial step of 2 min at 95°C followed by 35 cycles of 0.5 min at 94°C, 0.5 min at 54°C and 1 min at 72°C, followed in turn by 10 min at 72 °C and then held at 4°C. All PCR products were visualized on 1.2% agarose gels and purified using the Mini Elute ™PCR Purification Kit (B. Genei, India) according to the supplier's instructions. The most intense products were selected for sequencing. Purified DNA fragments were directly sequenced using an automated sequencer (Applied Biosystems 377) following the manufacturer's instructions DYEnamic™ET Dye Terminator Cycle Sequencing Kit.

DNA Sequencing Analysis: The DNA sequences were aligned using the Editseq 5.0 and Megalign 5.0 software of the DNA Star package (DNAStar Inc.). To ensure accuracy, strands were sequenced in both directions for each individual. Both DNA strands were checked for ambiguous bases and edited manually. Sequence divergences were calculated using the Kimura two parameter (K2P) distance model [20]. Finally, several matrices were computed from the pair wise distance matrices using the package (K2P), namely the mean, minimum and maximum of the distance within species (D within species) and the distance between species (D between species). DNA sequences were confirmed and edited manually using Edit sequence (DNA STAR software). Neighbour-joining (NJ) and UPGMA trees of K2P distances were created to provide a graphic representation of the patterning of divergence between species [21]. In the two chosen subgroups of fish, bootstrapping was performed in MEGA4 [22] with 500 replications.

RESULT AND DISCUSSION

Amplification and Sequencing of the COI Barcode Region: Our analyses, based on the commonly used mitochondrial genes cytochrome *c* oxidase I (the standard DNA barcode for animal species) are capable of discriminating coldwater species with high accuracy. A total of 10 COI barcodes of 655 bp were thus obtained for 2 species of *Schizothorax* (Fig. 2). Well defined peaks and the absence of stop codons indicated that co-amplification of nuclear pseudo-genes did not occur (Zhang and Hewitt, 1996). In accordance with previous work [5], the sequences aligned with ease due to the absence of insertions and deletions. Nucleotide composition showed a CT bias within *Schizothorax* (mean C = 28.1%, T =28.0%, A = 25.6%, G = 18.2%). All sequences have been deposited in GenBank (Accession no # FJ 170777 to #FJ 170781 and #FJ 170772 to #FJ 170776). Accession numbers for the barcodes, specimen and collection data, sequences, trace files and primers

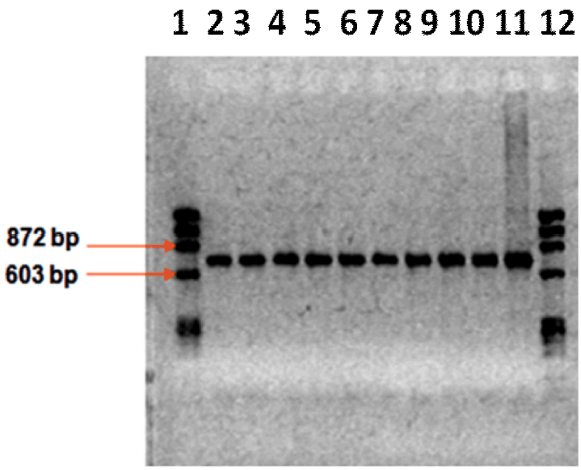

Fig. 2: CO-I mitochondrial gene PCR amplified products Lane-1and12: Ö×174 DNA marker (*Hae III* digested) Lane-2-6: *S. progastus*, Lane- 7- 11: *S. richardsonii*

Table 2: Percentage sequence divergences (K2P) between and within the *Schizothorax* species for the cytochrome oxidase I (COI) barcode region.

Species	Minimum	Mean	Maximum
Schizothorax progastus (With species)	0.00036	0.00121	0.00206
Schizothorax richardsonii (With species)	0.00000	0.00040	0.00080
Schizothorax progastus VS Schizothorax richardsonii (Between species)	0.00000	1.75000	3.50000

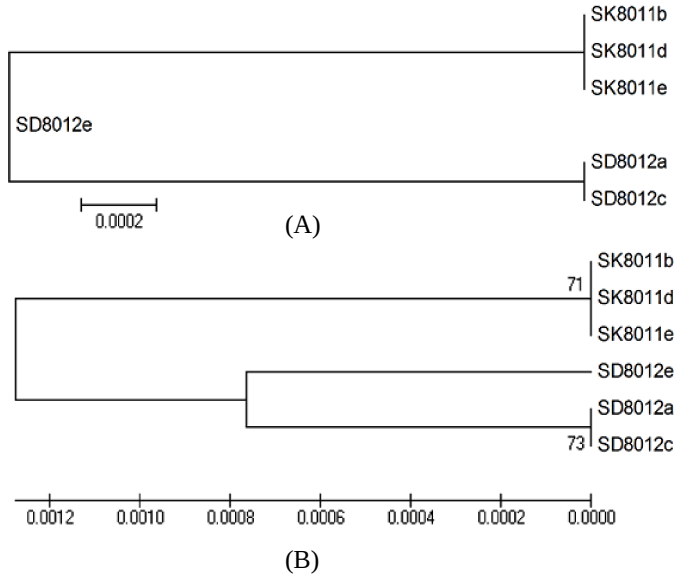

Fig. 3: Neighbour-joining and UPGMA tree of Kimura two-parameter (K2P) distances. Numbers above branches refer to bootstrap proportions among 500 bootstrap replicates. Specimen voucher codes referred to in (Table 1) are shown in parentheses following species names

details were available in NCBI genbank. The average percentage divergence (K2P) distance of individual's species of *S. progastus* is 0.00121% and for *S. richardsonii* is 0.00040%. Furthermore, the sequence divergence between these two species is 1.75000% (Table 2). There is high inter-specific sequence divergence for *Schizothorax* species as compared to intra-specific sequence divergence.

Neighbour-Joining Analysis of COI Barcode Sequences: The purpose of this study was to investigate whether the COI barcode provided sufficient resolution to identify snowtrout of the genus *Schizothorax*. The NJ analysis showed that the COI barcode is an effective tool for identification purposes [5]. All *Schizothorax* species were resolved as reciprocally monophyletic groups, despite low COI divergences between some individuals. Although the COI barcode region alone is not intended to be used to resolve taxonomic relationships, it appears to contain enough phylogenetic signals to delineate close relationships within *Schizothorax* from the Garhwal region of Uttarakhand. Both tree-building methods (NJ and UPGMA) recovered each *Schizothorax* species as a monophyletic group (Fig. 3). The NJ analysis of the COI barcode region placed *S. richardsonii* as a sister to *S. progastus*.

The NJ method has been promoted as the analysis tool of choice for the construction of bar-coding databases, due to its advantage of speed and its performance when sequence divergences are low [5, 23]. However, a comparison of tree-building methods is vital during the development of the bar-coding method, particularly as the suitability of this method for species delineation has been questioned in the past [24]. In some cases, an oversimplified or inadequate phylogenetic analysis may fail to distinguish reciprocally monophyletic groups, whereas an analysis that more realistically models the history of molecular evolution for the COI gene may perform better [25, 26]. To assess this, we compared the NJ tree with UPGMA trees generated from the COI data. Two complications were encountered in this study. In the, two specimens which had been preliminarily identified in the field as *S. richardsonii* (SD8012 b and d from khand,

pauri gharwal) were recovered with *S. progastus* in the COI NJ tree. The second complication concerned two specimen (SK 8011 a and c) identified morphologically as *S. progastus*, but recovered with its closest relative, *S. richarsonii*, in the NJ tree. All the four samples of two species were excluded from the both the trees to maintain the uniformity. These problems may be due to (a) inadequate phylogenetic analysis; (b) an inability of COI to resolve these species and (c) incorrect specimen identification, due to the significant morphological similarities shared by each set of sister species. Because of these close relationships, the possibility of high levels of intra-specific variation, perhaps due to retained ancestral polymorphisms and hybridization, were considered [27].

CONCLUSION

This study has strongly validated the efficacy of COI barcodes for identifying *Schizothorax* species from western Himalaya. The COI barcode region proved straight forward to amplify and sequence, which would facilitate the rapid generation of a barcode database and subsequent identification of specimens. Further, adequate resolution was provided by the COI barcodes to separate coldwater *Schizothorax* species. High COI sequence divergences existed between the species. Mean intra-specific and inter-specific COI sequence divergences differed by more than an order of magnitude. Extremely low sequence divergences between sister species and among species complexes are believed to be indicative of their recent origin. Bar-coding is a technique that could aid the prompt and accurate identification of species that would be enormously beneficial in the application of molecular taxonomy evidence. Based on the results for *Schizothorax*, it is foreseeable that DNA bar-coding could be effective in the identification of other snow trout species. Further investigations should confirm this feasibility and establish the reliability of the technique for routine application in identification cases and other circumstances featuring fishes of applied importance.

ACKNOWLEDGEMEMT

Sincere thanks to Indian Council of Agriculture Research (ICAR) and Department of Biotechnology (DBT), New Delhi, for financial assistance to carry out the present work.

REFERENCES

1. Ward, R.D. and P.M. Grewe, 1994. Appraisal of molecular genetic techniques in fisheries. Rev. Fish Biol. Fisheries, 4: 300-325. (doi:10.1007/BF00042907.)
2. Pe´rez-Martin, R.I. and C.G. Sotelo, 2003. Authenticity of species in meat and seafood products. Eduardo Cabello, Spain: Association International Congress on Authenticity of Species in Meat and Seafood Products.
3. Tautz, D., P. Arctander, A. Minelli, R.H. Thomas and A.P. Vogler, 2002. DNA points the way ahead in taxonomy. Nature, pp: 418-479. (doi:10.1038/418479a.)
4. Tautz, D., P. Arctander, A. Minelli, R.H.Thomas and A.P. Vogler, 2003. A plea for DNA taxonomy. Trends Ecol. Evol., 18: 70-74. (doi:10.1016/S0169-5347(02)00041-1.).
5. Hebert, P.D.N., A. Cywinska, S.L. Ball and De J.R. Waard, 2003. Biological identifications through DNA barcodes. Proc. R. Soc. B., 270: 313-322. (doi:10.1098/rspb.2002. 2218.)
6. Hebert, P.D.N. and T.R. Gregory, 2005. The promise of DNA bar-coding for taxonomy. 254 Systematic Biology, 54: 852-859.
7. Wheeler, Q.D., 2005. Losing the plot: DNA 'barcodes' and taxonomy. Cladistics, 21: 405-407.
8. Hebert, P.D.N., M.Y. Stoeckle, T.S. Zemlak and C.M. Francis, 2004. Identification of birds through DNA barcodes. PLoS Biol, 2: 1657-1663. (doi:10.1371/journal. pbio.0020312.)
9. Cavender, T.M. and M.M. Coburn, 1992. Phylogenetic relationships of North American Cyprinidae. In: R.L. Mayden, (ed.), Systematics, Historical Ecology and North American Freshwater Fishes, Stanford Univ. Press: Stanford, pp: 293-327.
10. Chen, X.L., P.Q. Yue and R.D. Lin, 1984. Major groups within the family Cyprinidae and their phylogenetic relationships. Acta Zootaxon Sinica, 9: 424-440.
11. Gilles, A., G. Lecointre, E. Faure, R. Chappaz and G. Brun, 1998. Mitochondrial phylogeny of the European cyprinids: Implications for their systematics, reticulate evolution and colonization time. Mol. Phyl. Evol., 10: 132-143.
12. Zardoya, R. and I. Doadrio, 1999. Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. J. Mol. Evol., 49: 227-23.

13. Wu, X.W., 1964. The Fauna of Chinese Cyprinidea in China, Shanghai: Shanghai Science and Technology Press.
14. Wu, Y.F. and C.Z. Wu, 1992. The Fishes of the Qinghai-Xizang Plateau, Chengdu: Sichuan Publishing House of Science and Technology.
15. Barat, A., C. Chandra, B.K. Singh, R. Matura and P.C. Mahanta, 2011. Successful Cross-Amplification of Few Microsatellite Loci Isolated from Tor tambroides for Indian Snow Trout, *Schizothorax richardsonii* (Gray, 1832) (Family: Cyprinidae). Global J. Molecular Sciences, 6(1): 22-25.
16. Mirza, M.R., 1991. A contribution to the systematics of the Schizothoracine fishes (Pisces: Cyprinidae) with the description of three new tribes. Pakistan J. Zool, 23: 339-341.
17. Sunder, S. and M.J. Bhagat, 1979. A note on the food of *Schizothorax plagiostomus* (McClelland) in the Chenab drainage of Jammu Province during 1973-74. J. Inland Fish. Soc. India, 11(1): 117-118.
18. Jhingran, V.G., 1982. Fish and Fisheries of India. Hindustan Publishing Corporation, India.
19. Goel, C., A. Barat, V. Pande, S. Ali and R. Kumar, 2011. Length-weight relationship of snow trout (*Schizothorax richardsonii*) based on linear and nonlinear models from hill stream of Uttarakhand, India. World J. Fish and Marine Sci., 3(6): 485-488.
20. Kimura, M., 1980. A simple method of estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol., 16: 111-120. (doi:10.1007/BF01731581.)
21. Saitou, N. and M. Nei, 1987. The neighbour-joining method: a new method for reconstructing evolutionary trees. Mol. Biol. Evol, 4: 406-425.
22. Kumar, S., K. Tamura and M. Nei, 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform, 5: 150–163. (doi:10.1186/1471-2105-5-150.)
23. Ball, S.L., P.D.N. Hebert, S.K. Burian and J.M. Webb, 2005. Biological identifications of mayflies (Ephemeroptera) using DNA barcodes. Journal of the North American Benthological Society, 24: 508-524.
24. DeSalle, R., M.G. Egan and M. Siddall, 2005. The unholy trinity: taxonomy, species delimitation and DNA barcoding. Philosophical Transactions of the Royal Society of London B Biology Sciences, 360: 1905-16.
25. Whelan, S., P. Liò and N. Goldman, 2001. Molecular phylogenetics: state-of-the-art methods for looking into the past. Trends in Genetics, 17: 262-272.
26. Sullivan, J. and P. Joyce, 2005. Model selection in phylogenetics. Annual Review of Ecology, Evolution and Systematics, 36: 455-466.
27. Hajibabaei, M., D.H. Janzen, J.M. Burns, W. Hallwachs and P.D.N. Hebert, 2006. DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences of the United States of America, 103: 968-971.