



DNA barcoding and phylogenetic relationship of two fish species of genus *Garra* (Family: Cyprinidae) from Aravalli region of Southern Rajasthan based on mtDNA COI sequences

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ABSTRACT

The genus *Garra* (stone sucker), Family *Cyprinidae*, consists of a group of species that are remarkably similar in morphology. These species are often difficult to distinguish based on external morphological approach. To resolve the existing uncertainty about the relationships and groups of these fishes, an attempt has been made to study the phylogenetic relationships of *Garra gotyla* and *Garra mullya* using mtDNA COI gene sequences from Aravalli region of Southern Rajasthan. The sequences were submitted to NCBI GenBank to establish and validate the taxonomical identification of the samples. A total of 5 COI sequences were generated. The overall GC content of *Garra gotyla* and *Garra mullya* were 44.13 and 43.00% respectively. The genetic distance within groups was 0.001 and 0.01 for *G. gotyla* and *G. mullya* respectively. The Neighbour-joining tree of two fish species using COI gene data revealed two distinct groups with 0.157 divergence. DNA barcode discriminated congeneric species without any confusion. The study strongly validated the efficiency of COI as an ideal marker for DNA barcoding of Indian freshwater fishes.

Keywords: Cytochrome C Oxidase I (COI), DNA barcoding, *Garra*, mtDNA

The Aravalli hill range is conspicuous physiographic feature. It divides the Rajasthan into two parts -South West and North East. The smaller Southern part was undertaken for the study which is rocky and served by rivers like Banas, Mahi, Chambal, Berach, and Sisarma. During present investigation, the specimens were collected from Banas River.

As compared with the traditional morphological classification methods, DNA barcoding technology is useful mainly because some species have extremely similar external morphological characteristics; therefore, it is difficult to distinguish them from each other merely by morphological characteristics. DNA barcoding technology can help accurately distinguish such species. The DNA barcoding is manifested as an effective method for species identification and systematics research on freshwater fishes in India and abroad (Lakra *et al.* 2016, Staffen *et al.* 2017, Barman *et al.* 2018, Laskar *et al.* 2018, Qayoom *et al.* 2018, Tiwari *et al.* 2019, Basudha *et al.* 2019, Sobita *et al.* 2019, Ude *et al.* 2020 and Pandey *et al.* 2020).

Garra is commonly known as stone sucker and bears well developed adhesive disc on its ventral surface. It is an

inhabitant of fast flowing streams and a bottom dweller fish. The species of genus *Garra* have extremely similar external morphological characteristics; therefore, it is difficult to distinguish them from each other merely by morphological characteristics. Hence COI barcode sequence for the identification of these fishes were tested with a goal of whether DNA barcoding can achieve unambiguous species recognition in fishes.

In the present study, an attempt was made to examine COI diversity within and among two fish species of genus *Garra*.

MATERIALS AND METHODS

The fishes were collected from Banas River during May 2019. Fin-clip of the samples were stored in 70% ethanol for further studies. Total genomic DNA was isolated from 50 mg of preserved tissue. DNA extraction was carried out through the phenol-chloroform extraction technique (Crandall *et al.* 1999). The amplification reaction was performed in a total volume of 25 µl, including 16.25 µl ultra-pure water, 2.25 µl 10X PCR buffer, 1.25 mM MgCl₂, each dNTP at 0.2 mM, each primer at 2 mM, 1.25 U Taq DNA polymerase and 1 µl DNA template. The thermal cycling conditions consisted of an initial step of 2 min at 95°C followed by 35 cycles of denaturing (94°C, 30 sec), annealing (54°C, 30 sec) and extension (72°C, 1 min), with a final extension at 72°C for 10 min; the samples were then

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held at 4°C. The primers used for the amplification of the COI gene were:

FishF1–5′ TCAACCAACCACAAAGACATTGGGCAC 3′
FishR1–5′ TAGACTTCTGGGTGGCCAAAGAATCA 3′

The PCR products were visualized on 1% agarose gels, and the most intense products were selected for sequencing after quantification through spectrophotometry. The products were purified using Exosap IT. Sequencing reactions were performed using single capillary sequencer ABI 310 Genetic Analyzer to generate sequences with accurate DNA base calling following manufacturer's instructions.

Sequences were aligned using CLUSTAL W and submitted to GenBank (Accession No. MK814513, MK814516, MK814517, MK814518 and MK814519). The extent of sequence difference between species was calculated by averaging pairwise comparisons. The average nucleotide frequencies along with standard error were calculated. Pairwise evolutionary distance among groups was determined by the Kimura two-parameter method using the software program MEGA 7 (Kumar *et al.* 2016). The Neighbour-joining (NJ) tree was constructed using MEGA 7 and to verify the robustness of the internal nodes of NJ tree, bootstrap analysis was carried out using 1000 pseudo replications. Sequencing of amplified PCR products were done from Aquaculture Research and Seed Unit, Directorate of Research RCA Campus MPUAT-Udaipur (Rajasthan).

RESULTS AND DISCUSSION

The mitochondrial *Cytochrome C Oxidase I* (COI) region of fish was successfully amplified using PCR and barcoded. The barcodes were submitted to the NCBI Gene Bank. The DNA barcodes were used for species level identification based upon the findings of the closed match of the query sequences with database sequences (BOLD and NCBI BLAST). Both databases revealed definitive identity matches in the range of 99–100% for consensus sequences of all the species. GenBank-based identification yielded an alignment E-value of 0.0. BOLD-IDS results were in agreement with GenBank results in the identification of these species, yielding 100% identity. The NCBI BLAST and BOLD indicated that all sequenced samples have been correctly identified because the e-values were low or zero and high sequence similarity scores > 99%.

All amplified sequences of the mtDNA COI gene were >500 bp. DNA barcoded species along with GenBank accession numbers is given in Table 1. The species wise nucleotide composition is presented in Table 2. The overall GC content of *G. gotyla* and *G. mullya* was 44.13 and 43% respectively. Similar GC content in fishes has been reported earlier based on complete mtDNA genome ranging from 38.4–43.2% and 42.2–47.1% with COI alone (Ward *et al.* 2005, Qayoom *et al.* 2018). The respective genetic distance within groups was 0.001 and 0.01 for *G. gotyla* and *G. mullya*. Further, the genetic distance between groups was 0.157. The evolutionary divergence (K2P) between sequences of *G. gotyla* and *G. mullya* is presented in Table 3.

The Analysis of molecular variance (AMOVA) was performed to test the significance of the partitioning of genetic variances resulting from different groupings of the populations. Analysis of molecular variance among two groups revealed no significant subdivision ($F_{ST}=0.02$). Results of AMOVA indicated that majority of the genetic variations in mtDNA COI gene was contributed by variations among populations (20%). Genetic variation within the population was comparatively high at 120%. Genetic differentiation between two fishes was assessed using F_{ST} pair wise comparisons. Pairwise F_{ST} values for genetic differentiation among the populations are presented in Table 4. The pairwise F_{ST} comparison of population samples using COI gene sequences showed non-significant genetic difference ($P=0.99$) between the populations. Further, the phylogenetic analysis of the mitochondrial sequences using Neighbor- Joining Tree (NJ) method was performed and the tree generated is presented in Fig.1. The

Table 1. DNA barcoded species along with GenBank accession numbers

Fish	GenBank Accession no	No of individuals	Order	Family
<i>Garra gotyla</i>	MK814513 MK814518 MK814519	3	Cypriniformes	Cyprinidae
<i>Garra mullya</i>	MK814516 MK814517	2	Cypriniformes	Cyprinidae

Table 2. Nucleotide composition of *Garra gotyla* and *Garra mullya* (All frequencies are given in percent)

Domain: Data	T(U)	C	A	G	GC	Total
<i>Garra gotyla</i>	28.86	27.06	27	17.06	44.13	531
<i>Garra mullya</i>	30.4	25.85	26.6	17.15	43	545

Table 3. Estimates of evolutionary divergence (K2P) between sequences of *Garra gotyla* and *Garra mullya*

<i>Seq32_Garra_gotyla_</i> {Gp_1}					
<i>Seq35_Garra_mullya_</i> {Gp_2}	0.1718				
<i>Seq36_Garra_mullya_</i> {Gp_2}	0.1697	0.0207			
<i>Seq37_Garra_gotyla_</i> {Gp_1}	0.0022	0.1688	0.1667		
<i>Seq38_Garra_gotyla_</i> {Gp_1}	0.0000	0.1718	0.1697	0.0022	

Table 4. Population pairwise F_{ST} (distance method) and F_{ST} P values of *Garra gotyla* and *Garra mullya*

Fish	<i>G. gotyla</i>	<i>G. mullya</i>
<i>G. gotyla</i>	0.00 (0.00)	0.00 (0.00)
<i>G. mullya</i>	0.02 (0.99)	0.00 (0.00)

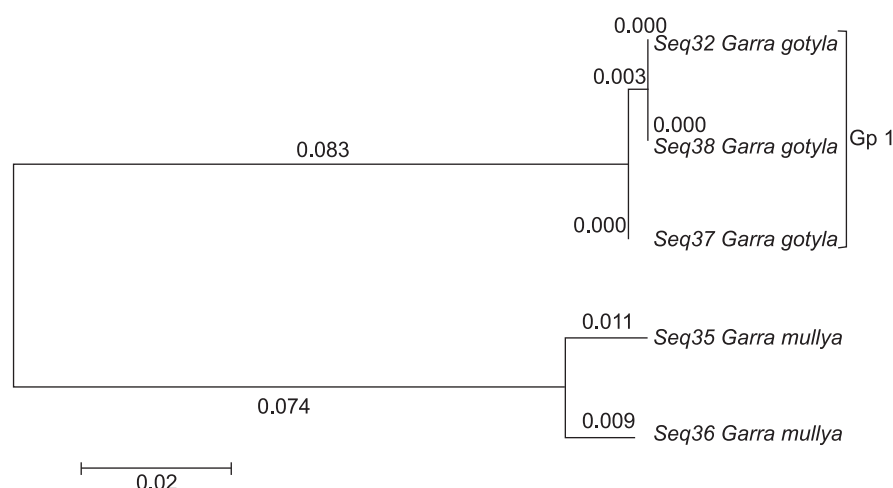


Fig. 1. Neighbor-joining tree of *Garra gotyla* and *Garra mullya* using COI gene.

Neighbor-joining tree of two fish species using COI gene data revealed two distinct groups with 0.157 divergence.

The results in the present study showed conformity with previous studies in other fishes (Lakra *et al.* 2015, Lasker *et al.* 2018, Basudha *et al.* 2019, Sobita *et al.* 2019, Ude *et al.* 2020 and Pandey *et al.* 2020). The morphology and DNA barcode based assessment reported the range expansion of *Garra qiaojiensis* from Indo-Myanmar biodiversity hotspot (Barman *et al.* 2018). Tiwari *et al.* 2019 also found intra-genus genetic distance between *Garra litanensis* (0.068), and *Garra lamta* (0.090) in Alaknanda river of Garhwal region.

The use of barcode technique for species level identification is of utmost importance since the routine species identification based on morphological and meristic features has following four limitations: (i) incorrect identification of species due to phenotypic plasticity and genetic variability in the characters involved; (ii) inability to recognize morphologically cryptic taxa; (iii) keys designed for a particular life-history stage or gender may not be effective for the others, and (iv) misdiagnoses due to lack of expertise and the dwindling pool of taxonomist's signal-ling the need for a new approach to taxon recognition (Jarman and Elliott 2000, Hebert *et al.* 2013, Subhasree and Sumit 2013). In the present study, the target region (mitochondrial COI) of two fishes of genus *Garra* were successfully amplified. The sequences were found to be conserved enough to allow amplification by a single set of forward and reverse primers in different fish families, yet diverse enough to permit unambiguous identification of fish species. No insertion, deletion or stop codons were observed in any of the sequences, which confirmed all amplified sequences being functional mitochondrial COI sequence.

The present study clearly demonstrated that there is considerable genetic differentiation among populations along the river Banas in Southern Rajasthan. The current study revealed more intra and less of inter-population variations. The high genetic differentiation was supported by hierarchical level analysis using AMOVA which indicated substantial variation (120%) among populations.

As revealed by AMOVA the majority of the genetic variations in different populations suggested a geographical structure within populations.

The results of the present study suggest that COI barcoding can be taken up as a pragmatic approach for resolving unambiguity in the identification of the fish fauna with applications in its management and conservation. This could be an invaluable tool for fisheries managers, fisheries ecologists and fish retailers, and for those wanting to develop fish identification microarrays.

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REFERENCES

- Barman A S, Singh M, Singh S K, Saha H, Singh Y J, Laishram M and Pandey P K. 2018. DNA barcoding of freshwater fishes of Indo-Myanmar biodiversity hotspot. *Science Report* **8**: 8579.
- Basudha Ch, Sobita N, Rawat A and Prakash N. 2019. Molecular characterization of an indigenous minor carp *Bangana dero* (Cyprinidae: Labeoninae) of Manipur and its relationship with Labeonin fishes of North East India based on mitochondrial 16SrRNA gene sequences. *Indian Journal of Animal Research* **53**(5): 594–98.
- Crandall K A, Fetzner J W (Jr), Lawler S H, Kinnersley M and Austin C M. 1999. Phylogenetic relationships among the Australian and New Zealand genera of freshwater crayfishes (Decapoda: Parastacidae). *Australian Journal of Zoology* **47**: 199–214.
- Hebert P D N, Cywinska A, Ball S L and DeWard J R. 2013. Biological identification through DNA barcodes. *Proceedings of the Royal Society of London Biological Sciences* **270**: 313–22.
- Jarman S N and Elliott N G. 2000. DNA evidence for morphological and cryptic Cenozoic speciations in the Anaspididae, 'living fossils' from the Triassic. *Journal of Evolutionary Biology* **13**: 624–33.
- Kumar S, Stecher G and Tamura K. 2016. MEGA7: Molecular

- Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**: 1870–74.
- Lakra W S, Singh M, Goswami M, Gopalakrishnan A, Lal K K, Mohindra V, Sarkar U K, Punia P P, Singh K V, Bhatt J P and Ayyappan S. 2015. DNA barcoding Indian freshwater fishes. *Mitochondrial DNA*, Early Online: 1–8.
- Laskar B A, Kumar V, Kundu S, Darshan, Tyagi K and Chandra K. 2018. DNA barcoding of fishes from River Diphlu within Kaziranga National Park in northeast India. *Mitochondrial DNA Part A*: 1–9.
- MEGA. <http://www.megasoftware.net>.
- Pandey P K, Singh Y S, Tripathy P S, Kumar R, Abujam S K and Parthi J. 2020. DNA barcoding and phylogenetics of freshwater fish fauna of Ranganadi River, Arunachal Pradesh. *Gene* **754**: 144860.
- Qayoom U, Pawar R A, Mohite S A, Sawant M S, Nirmale V H, Pawar S P, Goswami M and Lakra W S. 2018. DNA barcoding of some commonly exploited fishes from the northern Western Ghats, India. *Indian Journal of Animal Sciences* **88**(2): 245–50.
- Sobita N, Basudha Ch and Sharma S K. 2019. Molecular characterization of an indigenous barb *Pethia manipurensis* (Cyprinidae: Barbinae) from Manipur, India and its relationship with other cyprinid fishes inferring from mitochondrial cox1 gene sequences. *Indian Journal of Animal Research* **53**(2):181–86.
- Staffen C F, Staffen M D, Becker M L, Lofgren S E, Muniz Y C N and de Freitas R H A. 2017. DNA barcoding reveals the mislabeling of fish in a popular tourist destination in Brazil. *Peerj* <https://doi.org/10.7717/peerj.4006> PubMed PMID: WOS:000416519600002.PMID:29201560.
- Subhasree S and Sumit H. 2013. DNA barcodes of some threatened freshwater indigenous fishes in India. *Current Science* **105**(1): 84–90.
- Tiwari P, Bhatt A and Bahuguna S N. 2019. Molecular phylogeny of a hillstream fish *Garra Gotyla Gotyla* using mtDNA Cytochrome Oxidase I (COI) gene. *International Journal of Research in Engineering, IT and Social Sciences* **9**(3): 270–75.
- Ude G N, Igwe D O, Brown C, Jackson M, Bangura A, Alor O O, Onyinye C, Iheahuru O C, Okoro M, Ene C, Chieze V, Unachukwu M, Onyia C, Acquah G, James Ogbonna J and Das A. 2020. DNA barcoding for identification of fish species from freshwater in Enugu and Anambra states of Nigeria. *Conservation Genetics Resources* **12**: 643–58.
- Ward R D, Zemlak T S, Innes B H, Last P R and Hebert P D N. 2005. DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society, London B* **360**: 1847–57.