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MITOCHONDRIAL COI GENE ANALYSIS FOR TAXONOMIC IDENTIFICATION OF PAKISTANI SPECIES

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Abstract

Accurate species identification is essential for biodiversity assessment, conservation management, and taxonomic verification. This study utilizes DNA barcoding based on the mitochondrial cytochrome c oxidase subunit I (COI) gene to enable molecular identification of selected species from Pakistan. Specimens were collected from diverse habitats, after which genomic DNA was extracted, and the COI region was amplified via PCR and sequenced.

The obtained sequences were analyzed using BLAST and compared with reference sequences available in public databases, including GenBank and the Barcode of Life Data System (BOLD). Phylogenetic analyses were subsequently performed to evaluate genetic relationships and confirm species-level identifications.

The results demonstrated high sequence similarity with authenticated reference records, allowing clear discrimination among closely related taxa. Observed patterns of genetic divergence supported distinct species boundaries, reaffirming the reliability of the COI gene as a standard DNA barcode marker.

Overall, this study provides validated molecular data that enhance regional biodiversity records and underscores the value of COI-based DNA barcoding as a rapid, accurate, and cost-effective tool for species identification and biodiversity monitoring in Pakistan..

INTRODUCTION

DNA barcode base molecular identification used as efficient technique for identification of species.1 Fish identification is essential to fishery management and conservation. Fish molecular identification is performed consistently using mitochondrial DNA.2 Since conventional techniques of species identification are less accurate and lead to misunderstandings when identifying species that are physically similar, DNA barcode base molecular identification is employed as an alternative way to compare with traditional methods.3 Because mitochondrial COI DNA barcoding is maternally transmitted, has a high mutation rate, high nucleotide variability, and can quickly discriminate between closely related fish species with comparable morphologies, it is very useful for species identification.4 The fast and reliable technique for identifying species of fish is DNA barcoding, which is carried out by the help of DNA sequences from complete genome. The strategy of DNA barcoding can likewise be utilized for the distinguishing proof of an original example as indicated by the new arrangement. DNA barcoding specially involves amplification and sequencing of short universal mitochondrial COI for identification. Cytochrome C sequences are very useful to identify the genetic

structures, features and peculiarity in fish. There were nucleotides with amino acids obtained with gene sequencing.5 The COI, followed by Cyt c, is widely used for broad level identification than the Cyt b. The Cyt c has been very useful for freshwater fishes.6 The technique that is mostly applied in the fish species detection will be DNA based. The DNA based methods used for efficiently good quality of DNA. Then DNA quantification, purity, PCR amplification will do and then barcoding of DNA is to identify the accuracy of labelling.7 The main objective of present work was to carry out molecular based identification of C. reba by CO1 gene from Pakistan.

2. MATERIALS AND METHODS

Samples of C. reba were collected from Chenab River of Pakistan and were transported to Fisheries Lab, Institute of Zoology, BZU Multan. The standard taxonomic key, the scale count, fin count and lateral line count 7 and morphological characters were used to identify all samples. DNA was extracted using a modified phenol-chloroform technique and kept at -20°C until further examination.9 DNA quantity and quality measurements were made using a nanophotometer set to absorbance of A260/280 nm. The isolated DNA identifying region was amplified using polymerase chain reaction (PCR). The reaction was carried out under general conditions, which included one cycle of denaturation at 95 °C, 40 cycles of the denaturation phase at 94 °C, annealing at 55 °C, and extension at 72 °C with a single cycle of the final extension lasting seven minutes. Table 1 contains the primer sequence used for cytochrome c oxidase-I by PCR and then gene sequencing.9 The product of PCR which is amplified DNA were powered by 2% gel and then visualized on the Gel Doc which gave the quality of the DNA. Molecular product was extracted from all the samples, but only clear and DNA of good quality and quantity was used for further analysis, for sequence purpose. The barcode of life data (BOLD) system was used to analyse obtained sequence and blast on National center for Biotechnology Information (NCBI) to find out the unknown sequence to the known product. Neighbour joining (NJ) tree was conducted with MEGA X software.10 Sequence accession number as following is submitted on NCBI Genbank.

Table 1. Primer sequences used for PCR amplification

Sr. no	Primer	Sequence	Temp. ^{0}C	GC%	Primer size
1	Cyt c forward	5'TCAACCAACCACAAAGACATTGGAAC3'	64.7	46.15	26
2	Cyt c reverse	5'TAGACTTCTGGGTGGCCAAAGAATCA3'	66.3	46.15	26

3. RESULTS AND DISCUSSION

3.1 Morphometric Identification

The fish was identified physically by its slender, elongated body, which is laterally compressed, with a convex dorsal side. It was observed that dorsal profile was more compressed as compared to convex than that of abdomen. It has a terminal mouth. The mouth inferior and a pair of rostral barbels were seen. It was observed that it has a projecting snout, which is the common feature of immature fishes. The lips of the species are quite fleshy unlike others. The whole body is covered with hexagonal and cycloid scales.11 The color of the body is silver. The dark scales in color lower and upper and edges. The length was observed 60cm. Head is SL (23.9%) and TL (19.1%), as Height was 26.6% for SL and 21.3%. for TL. Eyes were 24.4% HL. It was observed that the lateral lines were present and complete with about 36-38 scales.

D. 24-26/30-49; A. 3/31-46; P.19; C. 12 12
D. 10-11 (2-3/8): P1. 16-17: P2. 9: A. 8(3/5) 11
D. 32-39/74-90: A. 3/75-88: P. 23 13

3.2 Molecular Identification

In the present study, a total of 15 fish specimens were used for DNA extraction and PCR analysis as shown in Figure 1. Result of the present study revealed that identification of C. reba, and the read length of sequence was found 496 base pair (bp) as 100% similarity index was found from the barcode of life

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data system which confirm as C. reba and Neighbour joining tree in Figure 2.

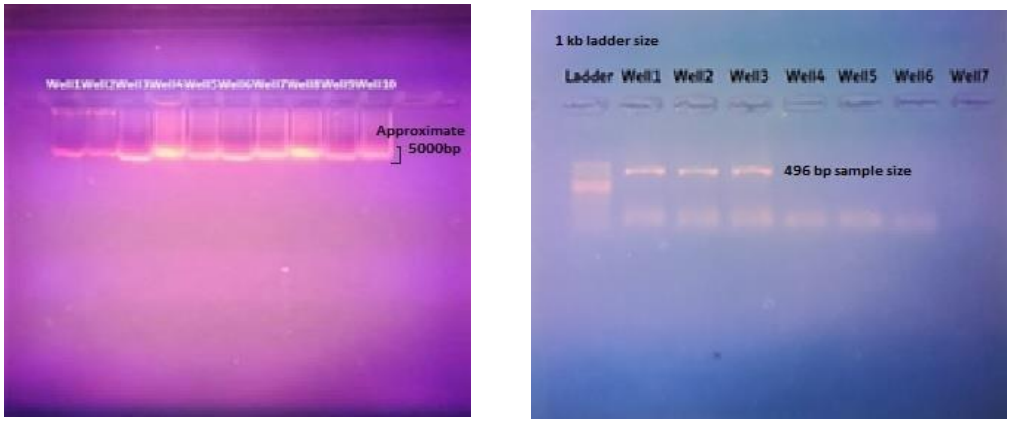

Figure 1. DNA extraction (Left) and PCR product (Right) on Gel documentation

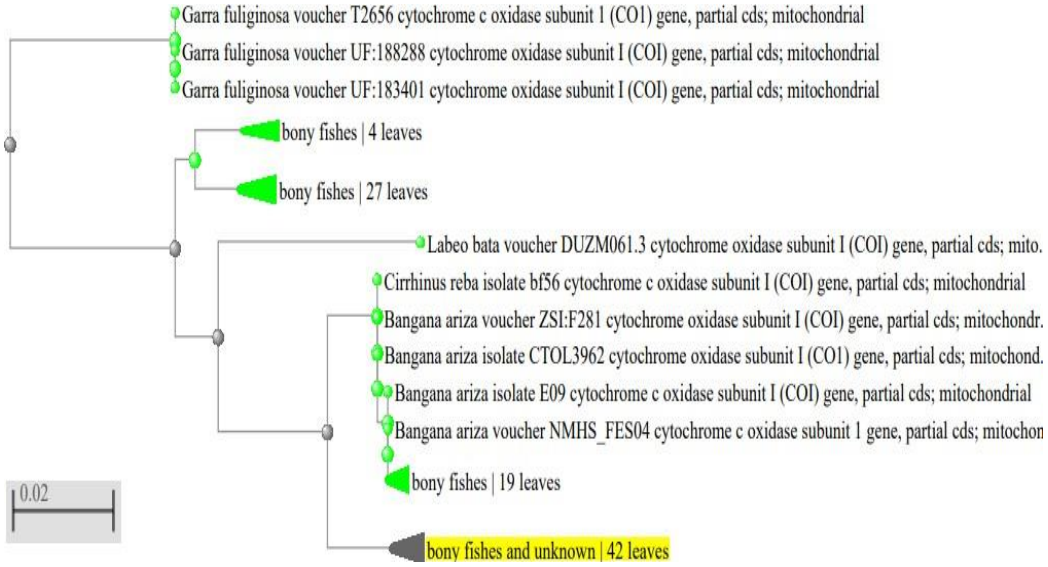

Figure 2. Neighbour joining tree of *Cirrhinus reba*

Fifteen C. reba specimens from the current investigation were barcoded using unknown and sequence was obtained of 496 base pairs, confirming the fish species identification as C. reba, as Lohman et al. (2009) utilised 650 base pairs for species identification.14 The GenBank database contains the C. reba barcode sequences generated in this work, which might be used as a reference in other research. DNA barcoding's primary goal is to preserve records.15 Once organised, the GenBank database is beneficial from a scientific and functional aspect.16

According to the experience of Taxonomists' DNA encoding can be used as an important tool for identifying animal species and this is very helpful for the study of interspecies differences as compared to use of traditionally used morphological classification system. The COI gene of mitochondria can be utilized as species barcode and identification of species because it revealed high degree of conservation and genetic variations among different species. The use of mitochondrial COI gene sequencing or barcoding can be used as a global aspect for identification of unknown

organism. In the previous studies several freshwater fish species have been clearly identified from the different regions of the world such as Canada, Australia, Thailand, and India etc.17-18 The findings of the present study concluded that DNA barcoding is a successful tool for proper identification of freshwater fish C. reba. The molecular identification is important when traditionally used morpho-taxonomic methods failed, especially with specimens unable to identify due to morphologically ambiguities and damages. Nevertheless, several limitations are also founded in molecular identification. The qualification and quantification were displayed in the form of ratio A260/280 and A260/230. The results of quantification showed that good quality DNA is present jn almost all the samples. The significant values of results cleared that the workers handled the procedure so well that they got such results. For determination of DNA quality absorbance ratios were calculated the results showed that most of the samples were in the specified range of 1.7–2.0. The values other than this range cleared that the samples could be anyhow contaminated with proteins or other impurities. The mean standard deviation and range at quality 260/230 was found to be 1.5754±0.107458 and 1.447-1.712 respectively. Similarly at quality 260/280, the standard deviation was 1.6615±0.075657 with range 1.556-1.772 was observed.

The accession number was issued by submitting gene sequences to Gene bank. Checking the data by putting the accession number in NCBI website. The sequence which was found in our data is Sanger sequence. The length of the sequence is found to be 496 Base pair. After the extraction of DNA the gel electrophoresis clearly shows the presence of DNA in specimens. The gel electrophoresis revealed that a fine quantity of DNA present in samples. The samples loaded in well 4 and 7 revealed the presence of DNA in good quantity while in well 1and 2 was not contain good quantity of DNA after extraction. In a study of 250 bp COI fragment were used to screen billfish stomach contents but this cannot utilize BOLD reference library due to overlapping with 655 bp barcode region.18 Given the large number of successive variations that differentiate many species, the internal barcode regions as short as 100 or 200 bp retain a large discriminatory ability. 19

The phenol-chloroform technique produces a variety of DNA quantities and qualities. Despite this, the appropriateness and type of DNA were evaluated using PCR. But after DNA extraction, 3 millilitres of high-quality DNA was added to the reaction mixture to optimise the PCR. The concentration and quality of DNA could highly affect the efficiency of Polymerase Chain Reaction as it may be inhibited by low or very high concentration of DNA. 20 The quality and quantity of extracted DNA indicate standardized method, and present study suggest that phenol-chloroform method can give good yield of DNA than other extraction methods.

4. CONCLUSION

To be the best of our knowledge, present study was used to access Molecular Based Approach for the Identification of C. reba by CO1 gene from Pakistan. The freshwater fish C.reba belongs to Cyprinidae family. The reba carp is commonly found in Pakistan, India, Bangladesh, Nepal, and Myanmar. DNA Barcoding facilitates rapid and typically accurate species identification, eliminating the requirement for morphological identification—that is, taxonomic competence. Identifying species when they are deteriorated or just partially present is also made feasible by barcoding. The CO1 gene of mitochondrial DNA serves as the foundation for DNA barcoding, and it has enough variation to distinguish across species. Fish species identification can be aided using DNA barcoding.

Conflict of interest

Authors declare no conflict of interest.

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