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Research Article

Genetic confirmation of morphological identified endangered ornamental loach *Botia lohachata* by mitochondrial CO-I gene, found from Kirthar mountain range Sindh, Pakistan

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ABSTRACT

The *Botia lohachata* were collected from the Ranikot Stream for morphological and molecular characterization. *B. lohachata* belong to family Botiidae, commonly known as freshwater Loaches and generally as ‘tiger loach’. Currently, it is enlisted in is in IUCN Red List It is a tropical bottom dwelling loach of small to medium-sized riverine loach. The cytochrome coxidase I (CO-I) gene was used to evaluate the species. CO-I gene sequence resulted 707bp. For all the samples, the percentage of Thymine was significantly higher compared to the other nucleotides i.e., 31.54% followed by Adenine and Cytosine i.e 25.74%. While the GC contents were recorded 42.7%. In further, the molarity (1µg/µl) was recorded 2.29µM. The present findings will be helpful in the identification of the Endangered and Vulnerable Botia species. Further, it will be beneficial for future genetic population studies and developing a conservation-scheme approach for managing the fish diversity in their natural habitats.

Keywords: Cytochrome coxidase I (CO-I) gene; conservation; mitochondrial DNA; *Botia lohachata*.

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INTRODUCTION

Pakistan encompasses a rich diversity of landscapes, from the soaring Pamirs to a maze of mountain ranges and a complex of valleys to inhospitable plateaus to the fertile plains. These unique topographical and climatic conditions have resulted in high biodiversity in Pakistan. The Kirthar Mountain Range is located in southern Pakistan; that extends southward for about 190 miles from the east-central Baluchistan to west of Karachi on the Arabian Sea (Ali and Qaiser, 1986). Differences of latitude, climate, elevation and soil type have favored a variety of animals and plants, either aquatic or terrestrial. There are about 183 freshwater fish species belonging to 83 genera has been reported all around the Pakistan (Mirza, 2003). Most of fishes found in Rivers, Reservoirs and Lakes. While, few species found in Mountainous streams or brooks. However, many remote stream habitats remain unexplored and their biodiversity under-documented because of difficult access and other logistical constraints. However, recently a loach species had morphologically identified and reported (Wakeela, 2024). Loaches belong to the superfamily Cobitoidei, and their taxonomy is often ambiguous due to morphological similarity; they are considered one of the most taxonomically complex fish groups. These fishes are isolated and inhabitant of limited to stream water. Severe loss of genetic variation could be observed due to environmental degradation and habitat loss. In general, due to elevated inbreeding causes isolation of population and size fall below the critical levels. In further, the increasing random genetic drift and reduced gene flow is result of habitat degradation. Which leads to increasing inter population genetic divergence and erosion of genetic variation (Young et al., 1996). Hence, fishes go through the adaptive modifications of characters including feeding habit and swimming. Furthermore, these fishes tend to produce similar characters of community species living to similar environmental ecology (Menon, 1987). Likewise, similarity bands, lack of good reviews and poor quality of specimen of species would leads to ambiguities and uncertain identification (Pandey et al., 2012). Fish identification is generally dependent on morphological characteristics. But, in several conditions such as diverse developmental stages of fishes, to recognize by morphological features only is very difficult (Victor et al., 2009). Modern developed molecular approach of DNA barcoding provides accurate species identification based on standard gene region. Scientists use mitochondrial cytochrome oxidase subunit 1 (CO-I) for zoological specimens' taxonomic authentication (Hebert et al., 2003). However, through the blood groups supported to analyze population structure such in tunas, salmonids and cod. While, for the fisheries research the application of molecular genetics approaches was stated in 1950 (Ligny, 1969). Further, the analytically powerful techniques of DNA-based identification was developed (Zhang et al., 2004 and Teletchea, 2009). Hence, the technique DNA barcoding is applied to uncover biological diversity. Hence, it is to be considered as a standardized application that is adopted worldwide in these years (Hebert et al., 2003 & 2004). The loach *Botia lohachata* is enlisted by IUCN as one of the endangered fish species (Pal & Daniels, 2022). The taxonomic confirmation of the endangered loach species has gained significant attention due to its unique genetic characteristics and ecological importance. The endangered loach species, *Botia lohachata* plays a vital role in its ecosystem, particularly within freshwater habitats. As part of the benthic community, these loaches contribute to the health and balance of their aquatic environments by participating in sediment disturbance and nutrient cycling. As research continues to evolve, it becomes increasingly apparent that comprehensive genetic accordingly. The present study was designed to authenticate the morphologically identified *B. lohachata* (Wakeela et al., 2023) using COI-based DNA barcoding. Since these loaches inhabit restricted ecological systems, molecular identification will complement traditional taxonomy and help resolve taxonomic confusion.

MATERIALS AND METHODS

Sample Collection and Preservation

Samples of *Botia lohachata* were collected from the Ranikot stream (Kirthar Mountain Range) for morphological and molecular analyses (Wakeela et al., 2022). All morphometric measurements were taken following standard methods (Wakeela et al., 2022). The morphological identification was performed using the diagnostic key of Wakeela et al., (2023) (Figure 1). For molecular analysis, muscle tissue was excised and stored at −80 °C until DNA extraction.

Genomic DNA Isolation

Genomic DNA was isolated using the phenol–chloroform method (Sambrook & Russell, 2001). DNA quantity and purity were assessed by spectrophotometry and agarose gel electrophoresis. Extracted DNA was stored at −20 °C until PCR amplification.

PCR Amplification and Sequencing

The mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified by polymerase chain reaction (PCR). Amplifications were performed in 50 µL reactions containing 25 µL of 2× GoTaq® G2 Master Mix (M7822, Promega), 1 µL (10 pmol μL^{-1}) of each primer, 2 µL of genomic DNA (~50 ng μL^{-1}), and nuclease-free water to final volume. PCRs were run on an Applied Biosystems 2720 thermal cycler. Thermal cycling conditions followed Goswami et al. (2022).

PCR products were separated on 1.5% agarose gels and visualized under UV illumination using a gel documentation system. Representative PCR products were purified and sequenced by Bionics Pvt. Ltd. (Korea) using Sanger dideoxy sequencing.

Universal primer used in thermal reaction:

Primers:

FishF1-5′-TCAACCAACCACAAAGACATTGGCAC-3′

FishR1-3′-TAGACTTCTGGGTGGCCAAAGAATCA-5′ (Ward et al., 2005)

Molecular Data Analyses

Sequence Analysis

Raw sequence chromatograms were inspected and edited using sequence-editing software (e.g., BioEdit). Consensus COI sequences were queried against the NCBI database using BLASTn to assess homology and identity. The confirmed COI sequence was deposited in GenBank (Accession No. OR567500). The top 14 BLAST hits were selected for phylogenetic analysis. Sequences were aligned using MUSCLE implemented in MEGA12. A neighbor-joining (NJ) phylogenetic tree was built with 1,000 bootstrap replicates, and genetic distances were calculated using the Kimura 2-parameter model (Tamura et al., 2013).

RESULTS AND DISCUSSION

In this study, we obtained mitochondrial DNA sequences for authentication of morphological base identified loach (*Botia lohachata*) through molecular data. The DNA sequence, CO-I gene, resulted a total of 707 base pairs in length (NCBI gene bank accession No. OR567500). This fragment of gene sequence consisted of 182, 223, 120, 182 number of Adenine (A), Thymine (T), Guanine (G) and Cytosine (C) nucleotides respectively.

The AT contents of sequence were higher (57.28%) then GC contents (42.7%). While, the percentage of Thymine was significantly higher (31.54%) followed by Adenine and Cytosine i.e 25.74% each (Table 1 and Figure 2). In further the molarity was recorded 2.29µM in 1µg/µl (Table 1).

Table 1. Showing the GC contents of CO-I gene for the *Botia lohachata*, compared to related 14 species during blast at NCBI.

Sr.No.	Accession number (NCBI)	Total base pairs (bp)	Adenine (A) (%)	Cytosine (C) (%)	Guanine (G) (%)	Thymine (T) (%)	%G~C (%)	Molarity
1	KX455912	696	26	25	17	31	42.80	2.33
2	PQ248504	651	25	25	18	32	42.90	2.47
3	MW002485	657	25	25	18	32	43.20	2.46
4	MW002486	657	25	25	18	31	43.20	2.46
5	MH087044	655	25	25	18	32	42.90	2.47
6	MK572072	655	25	25	18	32	42.90	2.47
7	KU568771	652	25	25	18	32	42.9	2.48
8	KU568773	657	25	25	18	32	43	2.48
9	KU568770	652	25	25	18	32	43	42.9
10	PQ409587	666	26	25	18	32	42.60	2.43
11	*OR567500	707	25	25	18	32	43	42.7
12	MK572071	652	25	25	18	32	43.10	2.48
13	MK962672	670	32	18	25	25	42.80	2.42
14	MK993523	7500	26	26	17	32	43	2.39
15	MN178273	679	32	18	24	25	43.30	2.38

*NCBI No. of current experimental fish.

Nucleotide Composition and Frequency

The CO-I length resulted 707bp with the average nucleotide frequencies of A: 25.74, C: 25.74, G: 16.96 and T: 31.56 with GC percentage of 42.7% (Table 1).

BLAST Analyses

The DNA sequences of CO-I genes was checked for their quality, trimmed and scanned in the NCBI BLAST database to ensure the gene and to confirm the species (NCBI No. OR567500). The present result of DNA sequence of loach was scanned to the blast results at online data base of NCBI. In further, for the validity an increase accuracy, one representative DNA sequence from each of these ten Loaches species was repeatedly scanned to before finalizing the BLAST results. Blast resulted varying percentage identity of loach species for CO-I gene sequence with same species sequences. Hence, species was confirmed and top 14 higher similarity sequences were selected for phylogenic studies in further (Table1).

Genetic Distances/Phylogenetic Analysis

All 14 sequences in the tree share a common ancestor at the root of the tree. The tree is rooted, meaning evolutionary direction from the ancestor to the most recent lineages is implied. Bootstrap values or support values of the percentages at each node (e.g., 95%, 92%, etc.) represent bootstrap support values measure of how strongly supported each

Figure 1. The specimen of *Botia lohachata*.

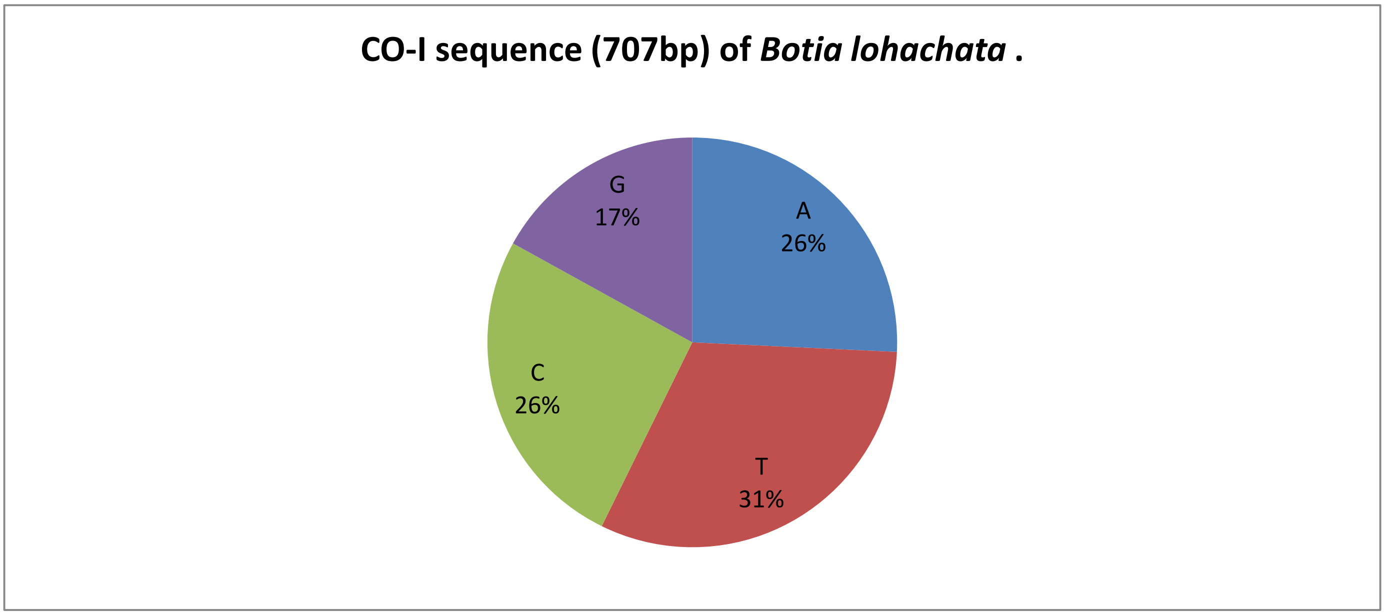

Figure 2. Shows the nucleotide percentage of CO-I gene in *Botia lohachata*.

branching pattern is values above 70% are generally considered statistically reliable. This tree shows mostly high support values, indicating a well-supported tree topology (Figure.3).

Clades and relationships of certain sequences form clades (groups of sequences with a common ancestor). For example, PQ248504.1, MN083135.1, LC823320.1 form a clade with 88% support. OQ472408.1 and OR567500.1 cluster together with 94% support. PQ409587.1, KF742423.1, and MK572070.1 form another clade with 92% support (Figure.3). Genetic similarity of closely clustered sequences (on short branches) suggests high sequence similarity and closer evolutionary relationships. For example, MK572072.1 and MW002486.1 are closely related. OQ472407.1 and PQ409587.1 are also closely related as shown in Figure (2). Possible Duplicate/closely related sequences of some sequences with very similar accession numbers such as MK572071.1 and MK572072.1 may be variants of the same species or closely related isolates. Findings of the tree show multiple strongly supported clades, indicating good confidence in the inferred relationships. Sequences cluster into meaningful evolutionary groups, suggesting shared ancestry and possibly similar biological characteristics. The presence of high bootstrap values (mostly above 90%)

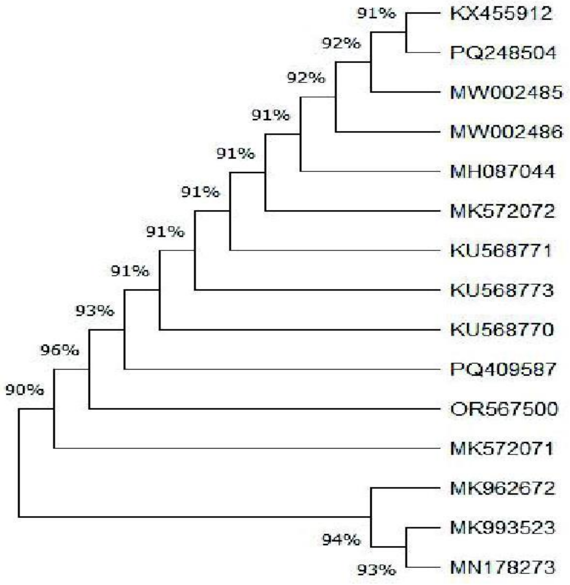

Figure.3. Phylogenetic tree of *Botia lohachata.*

implies the tree is reliable and robust. The present study successfully identified and confirmed the loach species (*Botia lohachata*) found from the Kirthar Mountain Range through molecular data first time from the Pakistan. The morphological identification authenticated through CO-I gene by blasting on NCBI website nucleotide blast, then affirmed by the MEGA software 100 bootstraps by selecting initial 14 gene sequence of *Botia lohachata* from that blast result. Generally, phylogeny made two different clads writing population *Botia lohachata* while a clear separate clade can be observed MN178273 that was of *Botia lohachata*.

Hence, morphological identified fish *Botia lohachata* is authenticated by molecular data. Further, the study supports the view that the use of CO-I is a powerful tool for species identification (Kerr et al., 2007). Using this method would clearly allow the identification of individually isolated fishes. This is best tools for the practice of conservation and forensics genetic in the fisheries research. In fact, the CO-I sequence provide a fast approach for screening the taxonomy of species (Palandačić et al., 2017). The phylogenetic analysis demonstrates that the sequences analyzed are evolutionarily related and can be grouped into several distinct clades with strong bootstrap support values, indicating reliable branching patterns. Most clades exhibit high genetic similarity, suggesting close evolutionary relationships among the grouped sequences. Notably, several accession numbers such as MK572071.1, MK572072.1, and KU568773.1 form tight clusters, reflecting potential strain or variant-level similarities. The overall high bootstrap values (mostly above 90%) support the robustness and reliability of the inferred evolutionary relationships.

CONCLUSION

The present findings will contribute in resolving taxonomic ambiguities arising due to morphological closeness in Loch species. Further, generated molecular data from present study will be useful in biodiversity assessment in the region and their conservation.

AUTHOR'S CONTRIBUTION

The manuscript was written by Wakeela Gul Mughal and Summaiya Rajput with help of inputs from Punhal Khan Lashari, Ikram Hussain and revised by Muhammad Younis Laghari. Field work and sample collection was done by Wakeela Gul Mughal, Zameer Palh and Dilawer.

FUNDING

No funding was received for the study.

AVAILABILITY OF DATA AND MATERIAL

The datasets supporting this study are included in the article. Extended methodological details and raw data can be accessed by contacting the corresponding author, subject to ethical and institutional guidelines.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study did not involve human subjects or animal models. In accordance with institutional guidelines, ethical approval and informed consent requirements were formally waived for this research.

CONSENT FOR PUBLICATION

I, the undersigned, consent to the publication of my identifiable information.

CONFLICT OF INTERESTS

The authors declare no competing or financial interests.

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