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Integrative taxonomy of near-threatened species *Pseudambassis lala* (Hamilton 1822), an ornamental fish of the Gomti river, Uttar Pradesh, India

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ABSTRACT

In the present study, *Pseudambassis lala* (Hamilton, 1822) was identified from Gomti River, Lucknow, Uttar Pradesh through morphological data (Classical Taxonomy) and molecular data (DNA Barcoding). This taxon is listed under the Near Threatened (NT) category of the IUCN Red List, 2024, and belongs to the Ambassidae family. All examined morphometric data were compared with the original description to validate the species' identity. Two mitochondrial gene cytochrome c oxidase subunit I (COI) sequences and sequences of another Indian ambassid species from the NCBI database were generated to prepare a maximum likelihood (ML) phylogenetic tree. Additionally, a radiograph of the specimen was generated and provided details of Indian distribution, habitat, and major threats. This ornamental valued fish could be needed to conserve and manage the Gomti riverine ecosystem. These results suggest that Integrative taxonomy is an effective tool for identifying species for use in the conservation and management of fish genetic resources. Future research could employ environmental DNA (eDNA) methodologies to detect threatened genetic resources from this riverine habitat, offering a non-invasive approach to tracking its presence and distribution pattern. This innovative technique promises efficient and precise species monitoring, enhancing conservation efforts.

Keywords: DNA barcoding, Gomti river, *Parambassis lala*, Radiography, Taxonomy

Integrative taxonomy is a multidisciplinary approach for resolving ambiguity and accurate fish identification through traditional taxonomy (morphological examination) and molecular biology (DNA barcoding) (Dayrat 2005, Lalramliana *et al.* 2018). Traditional taxonomy, relying solely on morphological traits, can sometimes lead to misidentification due to phenotypic plasticity or convergent evolution (Panprommin and Panprommin 2017). DNA barcoding is a powerful tool for taxon recognition based on short DNA sequences (655 bp) of the mitochondrial respiratory COI gene with universal primers (Hebert *et al.* 2003, Sahu *et al.* 2023, Singh *et al.* 2023). This approach enhances precision in distinguishing closely related species and unveils cryptic diversity that might go unnoticed through the traditional approach (Lakra *et al.* 2016).

Radiography is paramount in fish identification, offering a unique window into the internal anatomical features of fish taxa and other animals (Wildgoose 2003, Dayal *et al.* 2022). While external features are often critical in traditional taxonomy, radiography provides a complementary and usually indispensable perspective by revealing intricate details of the skeletal system including the vertebral column, skull, and fin rays. This method is particularly invaluable in distinguishing between closely related fish species that may share similar external morphological traits.

The Gomti River plays a crucial role in the aquatic biodiversity of Uttar Pradesh, India. This ecosystem provides a foothold for various food chains and functions as a breeding nursery and foraging ground for many ichthyofaunal genetic resources. Specimen collections from the exploratory survey conducted under the present study were from Gomti River, Lucknow. In the present study, collected species was confirmed based on traditional taxonomy, Radiography technique, and DNA barcoding. This research aims to provide detailed diagnostic characteristics of the studied fish. Additionally, it will undoubtedly be helpful for researchers, planners, and policymakers in the conservation and management of the ornamental fish fauna of this habitat.

MATERIALS AND METHODS

Description of study area, field surveys, and sample collection: The Gomti River, a major tributary of the Ganga River in northern India was selected for study. For the experimental sampling, fisherman was hired to capture the fish in Mehndi Ghat (Peepe wala Pull), Lucknow (26° 53' 11.9364'' N and 80° 53' 57.3828'' E) (Fig. 1). Specimens were collected from amidst aquatic vegetation consisting of Hydrilla, Pistia, Azolla, Vallisneria, and Water hyacinth in

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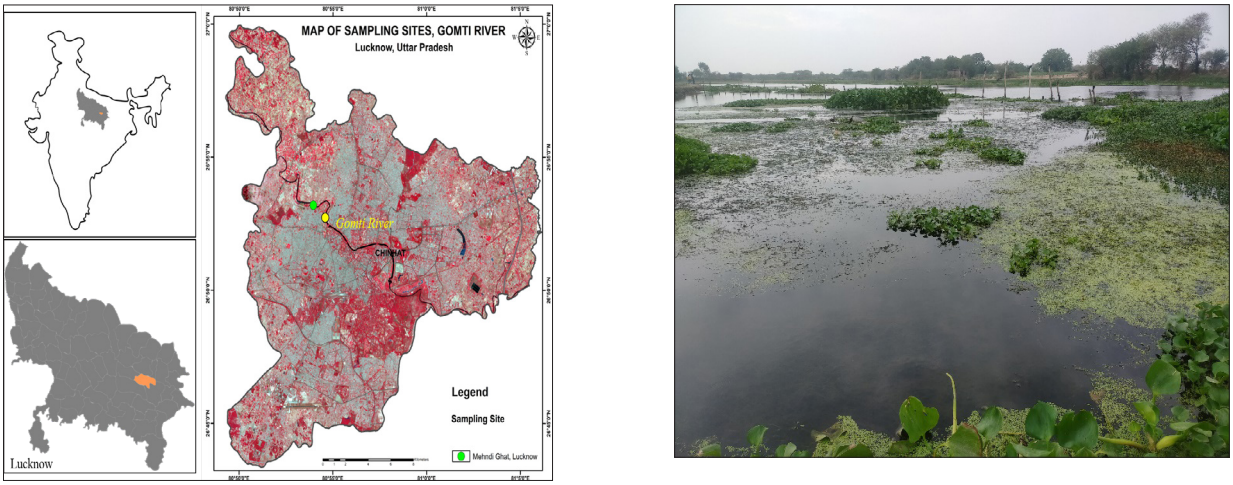

Fig. 1. Map showing the sampling site (Green dot) and collection location of *P. lala* (Mehndi Ghat, Lucknow, 26° 53' 11.9364'' N, and 80° 53' 57.3828'' E).

a slow-flowing bank of the river by using a gill net (locally called *Current jal*). Some specimens of the *Chanda* and *Parambassis* (now *Pseudambassis*) genus were obtained during catch. Still, some fishes looked like another species of the *Pseudambassis* genus. The fisherman identified, them as male and female due to different colour patterns. To validate this observation, all specimens were brought to ICAR-NBFGR (Molecular Phylogenetics Lab) for further investigation. Details of samples and GenBank accession numbers are provided in Table 1.

Table 1. Details of GenBank accession numbers for *COI* sequence of Ambassid group

Species name	Accession number (NCBI)
Pseudambassis lala	NKG-40A PP064994 (Current Study)#
	NKG-40B PP064995 (Current Study)#
	MH918101*, MT812152*, KC774658*
P. ranga	MH918109*, MK572457*, KJ936704*
P. baculis	MH918113*, MH918110*, MK733342*
P. dayi	MG431836*, KC774656*, KC774657*
P, waikhomi	KT896709*, KT896710*, MH918100*
P. bistigmata	MK733345*, MK572441*, MK733348*
P. thomasi	NA
P. serrata	NA
P. tenasserimensis	NA

*, NCBI; NA-COI sequence not available in NCBI; #, Accession numbers

Photography and taxonomic identification: Each specimen was photographed in fresh conditions and labeled. Collected specimens were carefully examined and classical taxonomy was conducted using dichotomous keys and species descriptions based on the standard literature (Jayaram 1981, Talwar and Jhingran 1991, Vishwanath *et al.* 2007) as well as printed literature. The valid scientific name was confirmed by Eschmeyer's Catalog of Fishes and FishBase (Froese and Pauly 2019).

Radiography of collected individuals: After identification, radiographs of the collected individuals were taken, and all countable and measurable characteristics were documented.

Tissue collection and preservation: Right-sided muscle tissues were preserved in 95% v/v ethanol and the vouchers were kept in 5% v/v formaldehyde. A unique code was given to the specimen and voucher specimens were kept in the Molecular Phylogenetics Lab (NBFGR), Lucknow, Uttar Pradesh.

DNA extraction, PCR amplification, and DNA sequencing: Approximately 50 mg of tissue was used for genomic DNA (gDNA) isolation following the standard phenol: chloroform: isoamyl alcohol method with minor modification (Singh *et al.* 2024). The concentration of isolated DNA was estimated using a UV spectrophotometer and diluted to a final concentration of 100 ng/L. The partial mitochondrial *COI* gene was amplified using universal primers, forward primer Fish F1 (5′TCAACCAACCACAAAGACATTGGCAC3′), and reverse primer Fish R1 (5′TAGACTTCTGGGTGGCCAAAGAATCA3′) (Ward *et al.* 2005). The *COI* gene was amplified in a 50 μL volume with 5 μL of 10×Taq polymerase buffer, 2 μL of $MgCl_2$ (50 mM), 0.25 μL of each dNTP (0.05 mM), 0.5 μL of each primer (0.01 mM), 0.6 U of Taq polymerase and 5 μL of genomic DNA. PCR amplifications were performed in the Veriti 96 fast thermal cycler (Applied Biosystems, Inc., Foster City, CA) with an initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 50 s, annealing at 54°C for 30 s, extension at 72°C for 80 s and final extension at 72°C for 10 min. PCR products were visualized on 1% agarose gels stained with ethidium bromide and documented using a gel documentation system (UVP, Upland, CA). Sanger sequencing was performed in a forward direction following the dideoxynucleotide chain termination method using an automated ABI 3500 sequencer (Applied Biosystems, Inc., USA).

Genetic analysis of DNA sequences: To obtain the consensus sequences of each specimen, chromatograms were checked in "FinchTV" software and trimmed at both ends to discard the ambiguous bases and noisy parts. Finally, each sequence was compared in the GenBank database

through nucleotide Basic Local Alignment Search Tool search (BLASTn). Genetic divergence within and between species was calculated using MEGA 11 (Tamura *et al*. 2021). A maximum likelihood (ML) tree was constructed with 1000 bootstrap replicates to assess the reliability of the branching patterns.

RESULTS AND DISCUSSION

In the present study, *Pseudambassis lala* (Hamilton, 1822) was characterized based on molecular signature and morphological examination in the Gomti River, Lucknow, UP.

Taxonomic identification and molecular phylogeny: The collected specimens perfectly matched the description given by Jayaram (1981), Talwar and Jhingran (1991), and Vishwanath *et al.* (2007). The current study indicated that the specimens in the current collection were conspecific with taxa. However, all the morphometric and meristic counts of the current collection were in the prescribed range as provided by Jayaram (1981). Therefore, further confirmation of the identity of the current collections were done using the *COI* gene.

Systemic taxonomy: Pseudambassis lala (Hamilton, 1822), commonly known as the Highfin Glassy Perchlet, Asiatic glassfish, belongs to the order Perciformes and the family Ambassidae. This species, locally referred to as *Chanari* or *Tanbijla* is currently classified as Near Threatened (NT) on the IUCN Red List. The Highfin Glassy Perchlet is notable for its transparent body and distinct high dorsal fin, which makes it a unique and attractive fish within its native habitats. Conservation efforts are crucial for this species due to its declining population, primarily attributed to habitat loss and environmental changes.

The material examined for *P. lala* included specimens NKG-40A and NKG-40B, with a total length ranging from 20.6 to 32.0 mm. Diagnostically, *P. lala* has a small, rounded body with a moderately sized head that is narrower than the body and an oblique mouth. The species is characterized by an elongated second dorsal fin spine, minute scales, and a lateral line consisting of about 90 scales. Additionally, the cheeks possess seven transverse scales, and the fish features two united dorsal fins, with the second dorsal fin having 12-14 soft rays and the anal fin comprising 15 soft rays. There are 16 gill rakers on the lower arm of the first arch.

In terms of colouration, fresh specimens of *P. lala* exhibit a translucent body with an orange-yellowish hue, adorned with three longitudinal dusky bands composed of tiny black dots extending dorsoventrally. The operculum is marked by dark stripes, while the dorsal, anal, and caudal fins are deep orange or reddish-orange with blackish outer margins. This species is distributed across Bangladesh, Myanmar, India (in the states of Odisha, Assam, Tripura, West Bengal, Bihar, and Maharashtra), Nepal, and Pakistan within the Indus River basin. *P. lala* inhabits both freshwater and brackish water habitat. Economically, it holds value both as a food source and as an ornamental fish.

Radiography of collected specimens: The radiography of collected specimens of *Pseudambassis lala* (Fig. 2B) allowed to generate X-ray images and document the measurable and countable characters. The recorded measurements for a single specimen were: a total length of 3.2 cm, body width of 1.4 cm, and an eye orbit area of 0.11 cm². The total length of the vertebrae was 2 cm. The vertebral count of 20 (2 fused vertebrae + 8 trunk vertebrae +10 caudal vertebrae).

Species validation and phylogenetic analysis: The obtained *COI* sequences were submitted to GenBank with accession numbers PP064994 (586 bp) and PP064995 (578 bp). BLAST was used to perform a similarity-based search of the NCBI GenBank databases with maximum identity per cent (ID) scores and query covers were selected for further analysis. Additionally, the 2-3 *COI* sequences of all *Parambassis* spp. were downloaded from the NCBI database for comparison to our species. All *COI* barcodes deal with multiple sequence alignment using Clustal W and

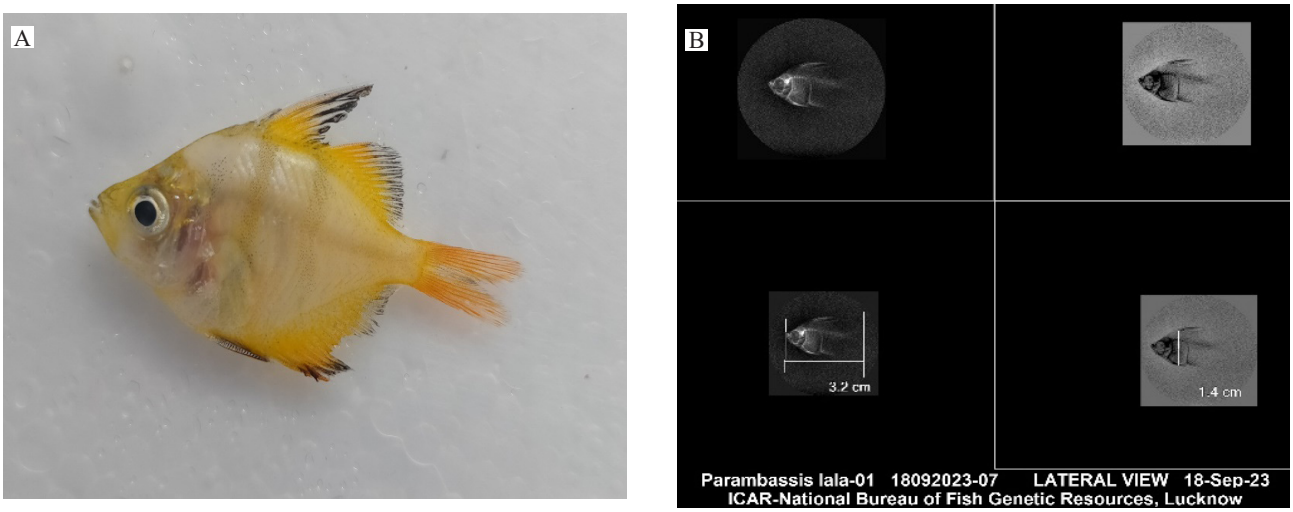

Fig. 2. (A) Fresh specimens of *Pseudambassis lala* (Hamilton, 1822) collected from Gomti River, Uttar Pradesh; (B) Radiograph of *P. lala* (X-Ray photo ID-18092023-07).

find best-fitting models of sequence evolution in MEGA 11. The Hasegawa-Kishino-Yano+ Invariant sites (HKY+G+I) substitution model based on the lowest BIC scores (Bayesian Information Criterion) standard was selected as the best nucleotide model for Bayesian inference (BI) analysis. A phylogeny tree was used to validate all species that clearly distinguished species with the same genus under one cluster with a 1000 bootstrap value. Moreover, the overall genetic distance calculated within the species was also found to be 0.01. A total of 20 nucleotide sequences were involved in the phylogenetic analysis. Moreover, 543 positions were present in the final dataset (Fig. 3). Evolutionary analyses were conducted in MEGA 11 (Tamura *et al.* 2021). Kimura 2-parameter method was used to compute genetic distances. There were no insertions, deletions or stop codons in any sequences indicating that all of the sequences represented the functional mitochondrial *COI* gene.

The nucleotide composition of all fish species was analyzed, focussing on the counts of adenine (A), thymine (T), guanine (G), and cytosine (C), along with the total count of occurrences. Additionally, the percentage of GC content and AT content was calculated for each species. All species of Ambassid nucleotide discrimination revealed varied AT (Adenine + Thiamine) and GC (Guanine + Cytosine) content. It was observed that nucleotide base composition of all analyzed sequences was 47.08% (AT) and 52.92% (GC). The results demonstrated that for these fish species, the total nucleotide composition consisted of more GC than AT bases.

Integrative taxonomy (IT) is one of the valuable tools for species confirmation (Singh *et al.* 2023). In the present investigation, this approach was used to identify *P. lala* from the Gomti River, Uttar Pradesh. During a field survey of the Gomti River, four specimens of an ambassid species were collected, subsequently identified as *Pseudambassis lala*. Three ambassids (*Chanda nama, C. ranga,* and *P. lala*) were reported from this river. Family Ambassidae (freshwater glass perches) are small to medium-sized semitransparent fish, extensively eaten by large predators (Geetakumari and Basudha 2012). Currently, the family consists of five genera including *Chanda, Gymnochanda, Paradoxodacna, Parambassis,* and *Pseudambassis.* Generally, freshwater ambassids are widely distributed in the Indian subcontinent mainland,

Fig. 3. Phylogenetic position of *P. lala* based on Maximum-likelihood (ML) phylogeny tree of a *COI* fragment gene.

peninsular Southeast Asia, and the Australian region. While, as per the literature survey, a total of nine valid species of freshwater ambassids are currently found in India such as *Pseudambassis lala, Parambassis dayi, P. thomassi, P. tenasserimensis, P. waikhomi, P. bistigmata. P. serrata, Chanda nama, C. ranga*. *P. Lala* (Hamilton, 1822) was originally described from "the Gangetic provinces" of India (Musikasinthorn 1998, Geetakumari and Basudha 2012). This taxon had been synonymized with *P. ranga* (Hamilton, 1822) by Day (1875) and Fraser-Brunner (1955) for several decades, but in 1995 resurrected by Roberts (1994). Presently, this tiny fish inhabits different ecosystems of the Indian region (Table 2).

Table 2. Occurrence of *P. lala* (Hamilton, 1822) in the different ecosystems of the India

Types of ecosystem	Location	Reference
Brahmaputra river	Northeast Region	Vishwanath (2017)
Tapi river basin	Maharashtra	Patole and Jadhav (2017)
Ganol river, Damring river (Upper reaches of Brahmaputra and Surma-Meghna river basins)	Meghalaya	Dey *et al.* (2015)
Godavari river basin	Maharashtra	Chowdhury *et al.* (2023)
Ganga river	Uttar Pradesh	Swain *et al*. (2021)
Panchet reservoir	Jharkhand	Sandhya *et al*. (2019)
Gandak river	Uttar Pradesh	Srivastava (2013)
Narmada river basin	Madhya Pradesh	Mondal and Bhat (2020)
Several rivers and tributaries	Tamil Nadu	Mogalekar and Jawahar (2015)
Different aquatic water bodies	Telangana	Prasad and Srinivasulu (2021)
Kole wetland	Kerala	Sharma (2023)

Integrative taxonomy proved to be an effective supplementary tool for accurate species identification and biodiversity research in several studies (Singh *et al.* 2023). In the current study, a detailed account of recorded ornamental fish taxa using classical, radiography, and molecular information has been provided. The translucent *P. lala*, a tiny fish, is highly prized as an aquarium fish due to its fascinating resemblance to a moving crystal in the aquarium (Talwar and Jhingran 1991). The International Union for Conservation of Nature (IUCN) has assessed this taxon as Near Threatened (NT) category. The group is also fished by lower-income fishermen as a subsistence food and ornamental business. This ambassid species emphasizes the ecological significance of the Gomti River.

The findings of the present study contribute valuable information to the regional ichthyofaunal diversity, for effective sustainable management, developing some conservation measures, and further research on the Gomti River ecosystem. In summary, combining morphology, molecular, radiographic and characterization can correctly identify the fish species. Additionally, the eDNA approach holds significant potential for the detection and monitoring of near-threatened species (Sahu *et al.* 2023). It provides an eco-friendly and sensitive method for tracking these faunas through trace DNA present in their natural habitats, which is crucial for informing timely and effective conservation strategies. In the future, this tool could be applied to large-scale conservation efforts, enabling proactive management and protection of fish genetic resources (FGR) by providing real-time data on their distribution and population trends.

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