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RESEARCH PAPER

Efficacy of Dna Barcoding for the Identification of Fish in the Mahanadi River, India

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Abstract

Freshwater fish diversity in the Mahanadi River basin remains poorly resolved at the molecular level, and the absence of a curated DNA barcode reference library has limited accurate species identification, biodiversity monitoring, and conservation planning in this ecologically important system. To address this gap, we generated a comprehensive COI-based DNA barcode dataset comprising 421 specimens representing 121 morphologically identified species collected across eight locations in the basin. Species delimitation analyses revealed high concordance among taxa, with most species forming distinct genetic clusters and a clear barcode gap that is mean intraspecific divergence was 0.20% and interspecific divergence around 11.84% confirming the effectiveness of COI for species discrimination. Notably, multiple taxa exhibited deep intraspecific divergence, indicating the presence of potential cryptic diversity within the basin. In addition, several non-native species were detected, highlighting ongoing biotic homogenization and emerging ecological risks to native fish communities. This study establishes the first basin-scale DNA barcode reference library for the Mahanadi River and reveals previously underrecognized lineage-level diversity. The dataset provides a critical molecular baseline for future taxonomic revision, environmental DNA (eDNA)-based monitoring, and evidence-based conservation and fisheries management in tropical freshwater ecosystems.

Introduction

The Mahanadi River, one of the major river systems in peninsular India, supports a diverse assemblage of freshwater fishes and plays a critical role in regional biodiversity and fisheries-based livelihoods. (Tyagi et al., 2021; Sajina et al., 2024). Despite several faunal surveys, the ichthyofauna of this basin remains insufficiently resolved at the molecular level. In particular, the absence of a curated DNA barcode reference library limit accurate). Despite identification, obscures cryptic diversity, and constrains the application of modern biodiversity monitoring tools such as environmental DNA (e-DNA) (Yu et al., 2026; Aprilia et al., 2025).

Accurate species identification is fundamental to biodiversity assessment and conservation planning, yet it remains challenging in freshwater fishes due to phenotypic plasticity, morphological convergence, and the presence of cryptic species (Bickford et al., 2007; Adams & Rohlf, 2000). These limitations are further exacerbated by incomplete taxonomic keys, difficulties

in identifying early life stages, and declining taxonomic expertise (Wheeler et al., 2004; Ko et al., 2013). As a result, morphology-based approaches alone are often insufficient to reliably resolve species boundaries in diverse tropical river systems (Hebert et al., 2003; Ward et al., 2005).

DNA barcoding, based on standardized sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene (Hebert et al., 2003; Ward et al., 2005) has emerged as a powerful tool for species identification and biodiversity assessment. This approach enables rapid and accurate identification across all life stages and has proven effective in detecting cryptic diversity, validating taxonomy, and identifying invasive species (Hebert et al., 2003; Ward et al., 2005). Its applicability to degraded samples and its integration with global reference databases such as BOLD further enhance its utility in ecological monitoring and conservation (Murugaiah et al*.,* 2009; Wilson-Wilde et al., 2010; Hebert et al., 2004; Melo et al*.,* 2016).

Despite its widespread application globally and within India, no comprehensive COI-based barcode reference library currently exists for the Mahanadi River basin, representing a critical gap in molecular resources (Ratnasingham & Hebert, 2007; Lakra et al*.,* 2016). This limits the accuracy of biodiversity assessments and the application of emerging tools such as eDNA-based monitoring, which depend on curated reference databases (Taberlet et al*.,* 2012). In this context, COI-based DNA barcoding provides a standardized framework for species identification (Hebert et al*.,* 2003), while integrative species delimitation approaches such as BIN, ABGD, and PTP offer robust tools to evaluate genetic divergence and species boundaries (Ratnasingham & Hebert, 2013; Puillandre et al*.,* 2012; Zhang et al*.,* 2013).

Materials and Methods

Sample Collection and Morphological Identification

Fish specimens were collected from eight geographically distinct sites across the Mahanadi River basin between February 2015 and August 2018 using cast nets (Figure 1) (Sutherland, 2006; Fischer & Bianchi, 1984). A total of 430 specimens were initially sampled, and caudal fin tissues were excised and preserved for molecular analysis.

Nine specimens were excluded due to inconsistencies in morphological identification, suggesting potential misidentification or sample contamination. The remaining samples were re-examined and curated, resulting in a final dataset of 421 specimens for downstream analyses. Detailed information on sampling sites, including geographic coordinates and collection periods, is provided in Table 1.

All specimens were initially identified to species level using standard taxonomic keys and authoritative ichthyological literature (Table 2). Taxonomic

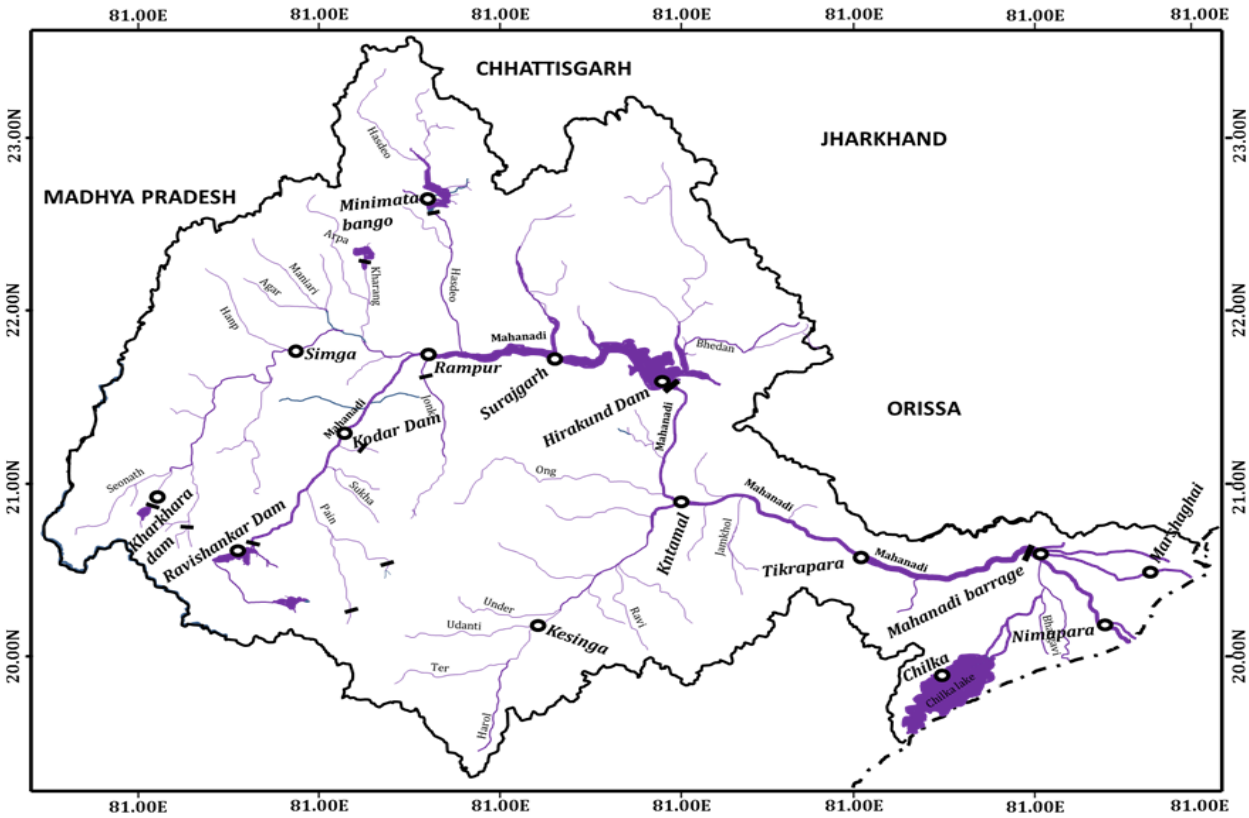

Figure 1. Map of sampling sites in the Mahanadi River basin. Blank circles indicate sampling locations.

assignments were further validated using updated references and the *Catalog of Fishes* database (Jayaram, 2010; Talwar & Jhingran, 1991., Vishwanath et al., 2014., Eschmeyer, 2012; Fricke et al., 2012).

DNA Extraction, COI Amplification, and Sequencing

Genomic DNA was extracted from caudal fin tissues using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's protocol. DNA concentration and purity were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and extracted DNA was stored at -20 °C until further processing.

A 620 bp fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using fish-specific primers Ward et al. (2005) as listed in Table 3.

Polymerase chain reaction (PCR) was performed in 25 µL reaction volumes containing 10× buffer (1.5 mM), 25 mM $MgCl_2$, 10 mM dNTPs, 3 U Taq DNA polymerase,

Table 1. Sampling sites in the Mahanadi River basin

S.N.	Sampling Site	Latitude	Longitude	Elevation(m)	Specimen collected
1	Arang	21° 11' 43.8'' N	81° 57' 43.92'' E	277	31
2	Charama	20° 29' 55.96''N	81° 22' 31.03'' E	356	19
3	Cuttack	20° 28' 10.60'' N	85° 54' 39.33'' E	9	127
4	Dhamtari	20° 40' 39.26'' N	81° 34' 37.82'' E	326	24
5	Gidhori	21° 15' 55.14'' N	82° 3' 9.15'' E	219	62
6	Jharsuguda	21° 51' 0'' N	83° 57' 0'' E	197	55
7	Musadia	20° 19' 48'' N	86° 37' 12'' E	4.26	67
8	Narsinghpur	20° 24' 11.36'' N	86° 34' 50.43'' E	56.08	27

Table 2. Taxonomical distribution of orders, families, genera and species recorded in the present study based on DNA barcoding identification. Taxonomic assignment follows FishBase (Froese & Pauly, 2024) and current ichthyological classification systems

Orders	Families	Genera	*Species*
Acanthuriformes	1	2	3
Anabantiformes	4	4	7
Anguilliformes	1	1	1
Atheriniformes	1	1	1
Aulopiformes	1	1	1
Beloniformes	2	2	2
Carangiformes	1	5	5
Characiformes	1	1	1
Cichliformes	1	1	2
Clupeiformes	3	5	6
Cypriniformes	3	22	37
Gobiiformes	2	2	2
Istiophoriformes	1	1	1
Kurtiformes	1	1	1
Moroniformes	2	2	2
Mugiliformes	1	1	1
Osteoglossiformes	1	1	1
Ovalentaria	1	3	4
Perciformes	7	8	11
Scombriformes	1	1	1
Siluriformes	9	15	21
Spariformes	3	4	5
Synbranchiformes	1	1	3
Tetraodontiformes	1	2	2
24	50	91	121

Table 3. List of primers used in the present study

Primer	Sequence (5'-3')	Reference
Fish F1	TCAACCAACCACAAAGACATTGGCAC	Ward *et al*., 2005
Fish R1	TAGACTTCTGGGTGGCCAAAGAATCA	Ward *et al*., 2005
Fish F2	TCGACTAATCATAAAGATATCGGCAC	Ward *et al*., 2005
Fish R2	ACTTCAGGGTGACCGAAGAATCAGAA	Ward *et al*., 2005

100 nM of each primer, and approximately 60 ng of template DNA. Thermal cycling conditions consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 45 s, and extension at 72°C for 10 s, with a final extension at 72°C for 10 min. Amplified products were verified on a 1.2% agarose gel.

PCR products were purified using ExoSAP-IT reagent and sequenced bidirectionally using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Singapore) on a Veriti™ 96-Well Thermal Cycler. Sequencing reactions included an initial denaturation at 96°C for 2 min, followed by 35 cycles of 96°C for 30 s, 55°C for 15 s, and 60°C for 4 min.

Molecular Data Analysis

High-quality COI sequences were assembled and edited using CodonCode Aligner. Species identities were validated through BLAST searches against the NCBI nucleotide database (Altschul et al., 1990) and the Barcode of Life Data Systems (BOLD) identification engine (Ratnasingham & Hebert, 2007). These platforms assign taxonomic identities at family, genus, and species levels and are widely recognized for supporting DNA barcoding applications (Alcantara & Yambot, 2016; Bhattacharjee et al., 2012; Cerutti Pereyra et al., 2012; Filonzi et al*.,* 2010; Joly et al., 2014; Kress & Erickson, 2012; Ward, 2009; Zhang & Hanner, 2012).

Taxonomic classification was further verified using global fish taxonomy references (Fricke et al*.,* 2023), and conservation status was assessed based on the IUCN Red List (IUCN, 2012; Sadovy de Mitcheson et al., 2013) as presented in Table 4.

All sequences were deposited in the BOLD system under the project DNA Barcoding of Fish from the Mahanadi River and in GenBank, with accession numbers provided in Table 5. Barcode Index Number (BIN) discordance reports from BOLD were examined to identify potential cases of low genetic divergence or ambiguous species assignments.

Table 4. Comparative performance of three species delimitation methods used for species delineation

Method	OUT	Match/Mismatch *species*	Mismatch *species*
BIN	121	121/0	-
ABGD	121	121/0	-
PTP	122	121/1	*Siganus sutor(n=2)*

Table 5. List of studied fish species with their taxonomic classification, Barcode Index Number (BIN), GenBank accession numbers, and IUCN Red List status

Species No.	Order	Family	Species	N	BIN	Genbank Accession No.	IUCN Status
1	Acanthuriformes	Sciaenidae	*Johnius amblycephalus*	1	BOLD:AAF2803	OR532197	LC
2			*Nibea maculata*	3	BOLD:AAZ8464	OQ880818 to 20	LC
3			*Otolithes ruber*	3	BOLD:ABV8824	OQ730275	LC
4	Anabantiformes	Anabantidae	*Anabas testudineus*	3	BOLD:AAA8511	OQ880655,57,58	LC
5		Channidae	*Channa marulius*	4	BOLD:AAB6483	OQ632659,60,64	LC
6			*Channa orientalis*	3	BOLD:AAB7311	OQ632666, 70, 72	VU
7			*Channa punctata*	7	BOLD:AAB7684	OQ632673, 74,76,81, 83,85	LC
8			*Channa striata*	3	BOLD:AAB8391	OQ632686,87	LC
9		Nandidae	*Nandus nandus*	12	BOLD:AAZ3137	OQ539529,31,33 to 38, 40 42, 44, 46	LC
10		Osphronemidae	*Trichogaster fasciata*	1	BOLD:AEB8888	OR532239	LC
11	Anguilliformes	Muraenesocidae	*Muraenesox bagio*	2	BOLD:AAY5233	OQ880805, OR533553	LC
12	Atheriniformes	Atherinidae	*Hypoatherina valenciennei*	1	BOLD:AAE6698	OQ880763	NE
13	Aulopiformes	Synodontidae	*Saurida undosquamis*	6	BOLD:ADF9352	OQ730377 to 82	LC
14	Beloniformes	Belonidae	*Xenentodon cancila*	2	BOLD:AEH8569	OQ555360,61	LC
15		Hemiramphidae	*Rhynchorhamphus malabaricus*	1	BOLD:ACW5482	OR532232	NE
16	Carangiformes	Carangidae	*Alepes kleinii*	2	BOLD:AEB7987	OQ569908, 909	LC
17			*Ferdauia ferdau*	1	BOLD:AAD7996	OQ880734	LC
18			*Megalaspis cordyla*	2	BOLD:AAX7390	OQ880801, 02	LC
19			*Parastromateus niger*	2	BOLD:ACC0078	OQ730307, 309	LC
20			*Scomberoides commersonnianus*	2	BOLD:ADG2635	OQ730383,84	LC
21	Characiformes	Serrasalmidae	*Piaractus brachypomus*	1	BOLD:ACC3337	OQ730316	DD
22	Cichliformes	Cichlidae	*Oreochromis mossambicus*	1	BOLD:ABA9363	OQ726307	VU
23			*Oreochromis niloticus*	4	BOLD:ABA9683	OQ726311, 12, 15, 16	LC
24	Clupeiformes	Chirocentridae	*Chirocentrus dorab*	1	BOLD:AAC0383	OR533538	LC
25		Clupeidae	*Gonialosa manmina*	3	BOLD:AAE3237	OR532193 to 95	LC
26			*Hilsa kelee*	1	BOLD:AAE5518	OR532196	LC
27			*Nematalosa nasus*	2	BOLD:AAZ3554	OQ880806, 809	LC
28		Engraulidae	*Stolephorus waitei*		BOLD:ADL8809	OQ730401	DD
29			*Thryssa malabarica*	1	BOLD:AEB5275	OR532237	DD
30	Cypriniformes	Cobitidae	*Lepidocephalichthys guntea*	1	BOLD:AAU2862	OR532198	LC
31		Cyprinidae	*Amblypharyngodon mola*	5	BOLD:AAA7175	OQ880646,47,51,52,54	LC
32			*Bangana ariza*	1	BOLD:AAB2256	OQ880674	LC

Table 5. Continued

Species No.	Order	Family	Species	N	BIN	Genbank Accession No.	IUCN Status
33			*Barbonymus gonionotus*	1	BOLD:AAB3884	OQ880676	LC
34			*Barilius barila*	1	BOLD:AAB5271	OQ880679	LC
35			*Cirrhinus mrigala*	13	BOLD:AAC0856	OR532175 to 87	LC
36			*Cirrhinus reba*	29	BOLD:AAC1279	OQ536243,45,48,50 to 54, 57 to 61, 63,66, 71,73,74,81,82,84,88 to 91	LC
37			*Ctenopharyngodon idella*	2	BOLD:AAC5812	OQ880695,OQ880701	LC
38			*Cyprinus carpio*	5	BOLD:AAC6086	OQ880707 to 11,	VU
39			*Devario devario*	3	BOLD:AAC7492	OQ880715 to 17	LC
40			*Esomus danrica*	1	BOLD:AAD2813	OR533537	LC
41			*Gibelion catla*	8	BOLD:AAD9382	OQ880742 to 46,49,51	LC
42			*Hypophthalmichthys molitrix*	1	BOLD:AAE7640	OQ880768	NT
43			*Hypophthalmichthys nobilis*	1	BOLD:AAE7811	OQ880769	DD
44			*Hypselobarbus curmuca*	2	BOLD:AAE8814	OQ880773,75	EN
45			*Hypselobarbus lithopidos*	1	BOLD:AAF2047	OQ880776	DD
46			*Labeo bata*	1	BOLD:AAF3096	OQ622018	LC
47			*Labeo boggut*	2	BOLD:AAF3393	OQ622020,23	LC
48			*Labeo calbasu*	14	BOLD:AAF3499	OR533533, OQ622030, 31,33,36,39,41,45, 47, 48 49, 51, 52	LC
49	Cypriniforme	Cyprinidae	*Labeo gonius*	6	BOLD:AAF5455	OQ622057, 59, 60, 61,64,66	LC
50			*Labeo rohita*	23	BOLD:AAF6633	OQ536318 to 23, 26, 27,28, 30, 31, 34 to 38, 44,46 to 49, 54, 57	LC
51			*Opsarius barna*	2	BOLD:ABA8489	OR533550, 51	LC
52			*Opsarius bendelisis*	1	BOLD:ABA8625	OR532222	LC
53			*Osteobrama cotio*	1	BOLD:ABA9729	OQ730269	LC
54			*Osteobrama vigorsii*	9	BOLD:ABU9035	OR532223 to 30, OR533541	LC
55			*Puntius chola*	6	BOLD:ACK1995	OQ730330, 31, 33, 34 to 36	LC
56			*Puntius mahecola*	1	BOLD:ACK7558	OQ730338	DD
57			*Puntius sophore*	3	BOLD:ACK8065	OQ730340 to 42	LC
58			*Puntius terio*	1	BOLD:ACL1923	OQ730345	LC
59			*Rasbora daniconius*	1	BOLD:ACR6962	OQ730348	LC
60			*Rasbora rasbora*	2	BOLD:ACS5708	OQ730349, 50	LC
61			*Salmostoma phulo*	1	BOLD:ADB8074	OQ730371	LC
62			*Salmostoma sladoni*	4	BOLD:ADB9997	OR532234, OR533543, OQ730375, 76	LC
63			*Securicula gora*	2	BOLD:ADG5739	OQ730386, 87	LC
64			*Systomus sarana*	6	BOLD:ADM3581	OQ555179,80,84,88 to 90	LC
65			*Tor tor*	1	BOLD:AEB5811	OR532238	DD
66		Nemacheilidae	*Paracanthocobitis botia*	2	BOLD:ABY2938	OQ730296, 97	LC
67	Gobiiformes	Gobiidae	*Glossogobius guiris*	2	BOLD:AAE1290	OQ880758, 60	LC
68		Oxudercidae	*Awaous ocellaris*	14	BOLD:AAB0409	OR532163,64,66 to 72,OQ880669 to 672	LC
69	Istiophoriformes	Sphyraenidae	*Sphyraena chrysotaenia*	1	BOLD:ADL7684	OQ730398	NE
70	Kurtiformes	Apogonidae	*Ostorhinchus fasciatus*	1	BOLD:ABU9910	OQ730274	LC
71	Moroniformes	Drepaneidae	*Drepane longimana*	1	BOLD:AAC9904	OQ880718	LC
72		Ephippidae	*Platax teira*	1	BOLD:ACG7154	OQ730322	LC
73	Mugiliformes	Mugilidae	*Planiliza tade*	2	BOLD:ACE0522	OQ730318, 19	NE
74	Osteoglossiformes	Notopteridae	*Notopterus notopterus*	22	BOLD:AAZ9696	OQ536403 to 06, 08, 09, 13, 14,17 to 23, 25, 26, 28, 29,31, 32, OR532213	LC
75			*Ambassis ambassis*	6	BOLD:AEV4672	OQ880639 to 42,44,45	LC
76	Ovalentaria	Ambassidae	*Chanda nama*	11	BOLD:AAB6417	OQ539629 to 33, 35 to 39, OR532173	LC
77			*Parambassis lala*	5	BOLD:ABY3071	OQ730298 to 302	NT
78			*Parambassis ranga*	3	BOLD:ACB7973	OQ730303, 304, OR533535	LC
79		Gerreidae	*Gerres filamentosus*	1	BOLD:AAD8911	OR532192	DD
80			*Gerres infasciatus*	1	BOLD:AAD9286	OQ880735	LC
81		Latidae	*Lates calcarifer*	11	BOLD:AAK2267	OQ880779	LC
82		Leiognathidae	*Leiognathus equulus*	2	BOLD:AAM2632	OQ880781, 82	LC
83			*Photopectoralis bindus*	1	BOLD:ACC0807	OQ730312	DD
84	Perciformes	Lutjanidae	*Lutjanus johnii*	2	BOLD:AAU4330	OQ880787,88	LC
85			*Lutjanus rivulatus*	1	BOLD:AAV2898	OQ880790	LC
86		Polynemidae	*Polydactylus sextarius*	1	BOLD:ACI0806	OQ730328	LC
87		Serranidae	*Epinephelus coioides*	1	BOLD:AAD0400	OQ880723	LC
88			*Epinephelus latifasciatus*	1	BOLD:AAD1940	OQ880728	LC
89		Siganidae	*Siganus sutor*	2	BOLD:ADJ5771	OQ730390, 91	LC
90	Scombriformes	Stromateidae	*Pampus chinensis*	7	BOLD:ABX1828	OQ730287 to 93	NE
91	Siluriformes	Ariidae	*Arius arius*	5	BOLD:AAA9421	OQ880659,60,64,66	LC
92			*Plicofollis layardi*	1	BOLD:ACH1421	OQ730324	NE
93		Bagridae	*Mystus bleekeri*	4	BOLD:AAY8322	OR532201, 202, OR533539, 542	LC
94			*Mystus cavasius*	5	BOLD:AAZ1771	OQ554957,61, 63,65,70	LC
95			*Mystus gulio*	1	BOLD:AAZ2536	OR532203	LC
96			*Mystus malabaricus*	2	BOLD:AAZ3076	OR532204, 05	NT
97			*Mystus vittatus*	2	BOLD:AAZ3112	OR532206 to 212	LC
98			*Sperata seenghala*	7	BOLD:ADL4240	OQ730397	LC
99		Clariidae	*Clarias gariepinus*	5	BOLD:AAC4119	OQ880684,85, 87 to 89	LC
100		Horabagridae	*Pachypterus atherinoides*	4	BOLD:ABX1827	OQ730281, OQ730283 to 285	LC
101		Loricariidae	*Pterygoplichthys anisitsi*	1	BOLD:ACJ3337	OQ730329	NE
102		Pangasiidae	*Pangasianodon hypophthalmus*	1	BOLD:ABX6594	OQ730295	EN
103		Ritidae	*Rita chrysea*	4	BOLD:ACX7514	OQ730366 to 69	LC
104			*Rita kuturnee*	1	BOLD:ADA7852	OR532233	LC

Table 5. Continued

Species No.	Order	Family	Species	N	BIN	Genbank Accession No.	IUCN Status
105	Siluriformes	Schilbeidae	*Ailia coila*	4	BOLD:AEB1793	OQ526062, 63, 65,66,OQ569908,09	NT
106			*Clupisoma garua*	4	BOLD:AAC5682	OQ880690 to 92	LC
107			*Eutropiichthys burmannicus*	1	BOLD:AAD6423	OQ880733	LC
108			*Silonia silondia*	2	BOLD:ADL2110	OQ730394,95	LC
109		Siluridae	*Ompok bimaculatus*	5	BOLD:ABA2575	OR532214 to 18	NT
110			*Ompok pabda*	4	BOLD:ABA7419	OR532219 to 21, OR533534	NT
111			*Wallago attu*	10	BOLD:AEC6518	OQ555342,43,45,47,49,50, 52 to 55	VU
112	Spariformes	Nemipteridae	*Nemipterus japonicus*	3	BOLD:AAZ3699	OQ880812, 15,17	LC
113		Sillaginidae	*Sillago indica*	1	BOLD:ADJ9734	OQ730392	DD
114			*Sillago vincenti*	1	BOLD:ADK6840	OQ730393	NE
115		Sparidae	*Acanthopagrus berda*	1	BOLD:AEA1389	OQ569906	LC
116			*Rhabdosargus sarba*	4	BOLD:ACW3537	OQ730352, 56 58 60	LC
117	Synbranchiformes	Mastacembelidae	*Macrognathus aral*	1	BOLD:AAV6679	OQ880792	LC
118			*Macrognathus guentheri*	5	BOLD:AAX3611	OQ880793,95 to 98	LC
119			*Mastacembelus oatesii*	2	BOLD:AAX5453	OR532199, 200	EN
120	Tetraodontiformes	Tetraodontidae	*Lagocephalus guentheri*	2	BOLD:AAJ3231	OQ880777, 78	LC
121			*Takifugu oblongus*	2	BOLD:ADX0539	OR532235,36	LC

Species Delimitation Analysis (ABGD)

Species-level operational taxonomic units (OTUs) were inferred using Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) under Jukes-Cantor (JC), Kimura 2-parameter (K2P), and simple distance models with varying prior intraspecific divergence values (Table 6).

Genetic divergence and phylogenetic relationships were assessed using the Kimura Two-Parameter (K2P) model implemented in BOLD v4. Pairwise genetic distances were calculated at multiple taxonomic levels, including within species, among genera, and among families. The final dataset comprised 421 COI sequences representing 121 species. Associated specimen metadata and sampling site information are summarized in Table 1**.**

Results

COI Sequence Characteristics

A total of 421 mitochondrial COI sequences were successfully generated using Sanger sequencing, representing 121 species, 91 genera, 50 families, and 24 orders (Table 1). The average sequence length was approximately 620 bp. No insertions, deletions, or stop codons were detected, confirming that all sequences corresponded to functional mitochondrial DNA (Ward, 2009; Ivanova et al., 2007).

The overall GC content was 45.1%, with codon position-specific GC values of 55.6%, 42.8%, and 36.9% at the first, second, and third codon positions, respectively (Table 7). These values are consistent with typical mitochondrial genome characteristics of teleost fishes (Meyer, 1993; Saccone et al*.,* 2002; Lavoue & Sullivan, 2004).

DNA Barcoding Distance Analysis

Intraspecific and nearest-neighbour genetic distances were calculated using standard barcode gap analysis methods (Hebert et al., 2003). The nearest neighbour approach follows procedures implemented in BOLD systems (Ratnasingham & Hebert, 2007) (Table 8).

Species Identification and Phylogenetic Analysis

All sequences were validated using NCBI BLAST and the Barcode of Life Data Systems (BOLD) (Altschul et al., 1990; Ratnasingham & Hebert, 2007) showing 97-100% similarity with reference sequences. Neighbour-Joining (NJ) analysis (Saitou & Nei, 1987) revealed that most species formed well-defined monophyletic clusters (Figure 2), demonstrating strong concordance between morphological and molecular identifications (Meyer & Paulay, 2005; Ward, 2009).

Barcode gap analysis indicated clear separation between intra- and interspecific variation for most taxa However, two species lacked a distinct barcode gap, suggesting possible taxonomic complexity, recent divergence, or incomplete lineage sorting (Funk & Omland, 2003).

Genetic Divergence

Kimura Two-Parameter (K2P) analysis (Kimura, 1980) revealed mean genetic distances of 0.20% within species, 11.84% among genera, and 17.04% among families (Meier et al., 2008., Suryawanshi et al., 2025). (Table 9; Figure 3). These results demonstrate a clear hierarchical increase in genetic divergence with increasing taxonomic rank.

The lowest interspecific divergence was observed in the genus *Hypophthalmichthys* (5.37%), whereas the highest divergence occurred in *Channa* (24.61%) (Khedkar et al*.,* 2014). On average, interspecific divergence among congeners was approximately 59-fold higher than intraspecific divergence, indicating strong discriminatory power of COI barcodes. This progressive increase in divergence further supports the presence of a well-defined barcode signal within the dataset.

Taxonomic Assignment and Species Delimitation

All 421 sequences were assigned to operational taxonomic units (OTUs) using Barcode Index Number (BIN), Automatic Barcode Gap Discovery (ABGD), and Poisson Tree Processes (PTP) methods (Ratnasingham & Hebert, 2013; Puillandre et al*.,* 2012; Zhang et al., 2013). (Table 4).

BIN analysis identified 121 BINs, including 70 concordant BINs and 51 singleton BINs. While this indicates generally high species resolution, the relatively high proportion of singleton BINs suggests either under-sampling of regional diversity or incomplete global reference coverage for certain taxa (Dellicour & Flot, 2018., Clare et al., 2011; Dahruddin et al*.,* 2017) ABGD recovered 121 OTUs under initial partitioning and 121-171 OTUs under recursive partitioning, reflecting sensitivity to prior intraspecific divergence values (P=0.001-0.0599). This range suggests the presence of potential hidden genetic structure within some taxa (Figure 4 and Table 10).

PTP analysis delineated 122 OTUs, with *Siganus sutor* split into two distinct lineages, indicating possible cryptic diversity or deep intraspecific divergence.

Table 6. OTU delimitation of 421 COI sequences using Automatic Barcode Gap Discovery (ABGD) under different substitution models (Jukes-Cantor, Kimura 2-parameter, and simple distance). Analyses were performed following Puillandre et al. (2012)

Substitution Model	Relative gap width(X)	Partitions	Prior Intraspecific divergence(P)								
			0.0599	0.0359	0.0215	0.01291	0.00774	0.004642	0.00278	0.001668	0.001
Jukes Cantor (JC)	1.0	Initial	121	121	121	121	121	121	121	121	121
		Recursive	171	170	131	123	123	123	121	121	121
Kimura 2parameter (K2P)	1.0	Initial	121	121	121	121	121	121	121	121	121
		Recursive	171	170	131	123	123	123	121	121	121
Simple Distance	1.0	Initial	121	121	121	121	121	121	121	121	0
		Recursive	123	123	123	121	121	121	121	121	0

Table 7. Mean nucleotide composition and GC content of COI sequences from fishes of the Mahanadi River. Values are consistent with mitochondrial COI barcoding data reported in previous studies (Ward et al., 2005; Hebert et al., 2003)

	Minimum	Mean	Maximum	Standard error
G %	14.45	17.34	22.35	0.0593
C %	22.86	27.79	33.22	0.0788
A %	19.02	25.48	28.96	0.0955
T %	25.62	29.39	34.83	0.0663
GC %	39.72	45.13	52.35	0.1014
GC % Codon Pos 1	48.31	55.60	59.68	0.0848
GC % Codon Pos 2	40.33	42.84	48.20	0.0469
GC % Codon Pos 3	22.07	36.93	57.07	0.2882

Table 8. Mean and maximum intraspecific genetic distances for each species compared with nearest-neighbour genetic distances. For singleton species, intraspecific values are not applicable (N/A)

Species	Mean Intra-Sp	Max Intra-Sp	Nearest Neighbour	Nearest *Species*	Distance to NN
Nibea maculata	0	0	MAHAN1567-18	*Johnius amblycephalus*	21.61
Otolithes ruber	N/A	N/A	MAHAN1723-18	*Ambassis ambassis*	20.26
Anabas testudineus	0.42	0.81	MAHAN1633-18	*Scomberoides commersonnianus*	20.64
Channa marulius	0	0	MAHAN2087-18	*Channa punctata*	17
Channa orientalis	0.16	0.23	MAHAN1051-16	*Channa punctata*	20.12
Channa punctata	0.29	0.76	MAHAN1503-18	*Channa marulius*	17
Channa striata	0.53	0.8	MAHAN870-15	*Channa punctata*	17.75
Nandus nandus	0.11	0.46	MAHAN1140-16	*Acanthopagrus berda*	20.59
Trichogaster fasciata	N/A	N/A	MAHAN2136-18	*Anabas testudineus*	21.7
Muraenesox bagio	0.97	0.97	MAHAN794-15	*Cyprinus carpio*	19.84
Hypoatherina valenciennei	N/A	N/A	MAHAN1048-16	*Rita kuturnee*	18.95
Saurida undosquamis	0.06	0.19	MAHAN1149-16	*Drepane longimana*	21.32
Xenentodon cancila	0.22	0.22	MAHAN1616-18	*Pampus chinensis*	19.63
Rhynchorhamphus malabaricus	N/A	N/A	MAHAN1780-18	*Rhabdosargus sarba*	17.44
Alepes kleinii	0	0	MAHAN1601-18	*Parastromateus niger*	14.73
Ferdauia ferdau	N/A	N/A	MAHAN1601-18	*Parastromateus niger*	12.11
Megalaspis cordyla	0	0	MAHAN1691-18	*Parastromateus niger*	15.12
Parastromateus niger	0.32	0.32	MAHAN1612-18	*Ferdauia ferdau*	12.11
Scomberoides commersonnianus	0	0	MAHAN1800-18	*Sillago vincenti*	18.71
Piaractus brachypomus	N/A	N/A	MAHAN1048-16	*Rita kuturnee*	18.22
Oreochromis mossambicus	N/A	N/A	MAHAN878-15	*Oreochromis niloticus*	5.77
Oreochromis niloticus	0.07	0.2	MAHAN1320-16	*Oreochromis mossambicus*	5.77

Table 8. Continued

Species	Mean Intra-Sp	Max Intra-Sp	Nearest Neighbour	Nearest *Species*	Distance to NN
Chirocentrus dorab	N/A	N/A	MAHAN1387-16	*Hilsa kelee*	24.51
Gonialosa manmina	0.08	0.23	MAHAN2065-18	*Nematalosa nasus*	17.7
Hilsa kelee	N/A	N/A	MAHAN1837-18	*Gonialosa manmina*	20.54
Nematalosa nasus	0	0	MAHAN1888-18	*Gonialosa manmina*	17.7
Stolephorus waitei	N/A	N/A	MAHAN2054-18	*Rasbora daniconius*	20.82
Thryssa malabarica	N/A	N/A	MAHAN1613-18	*Clarias gariepinus*	20.84
Lepidocephalichthys guntea	N/A	N/A	MAHAN683-15	*Salmostoma sladoni*	19.41
Amblypharyngodon mola	0.45	0.76	MAHAN1451-18	*Rasbora rasbora*	14.77
Bangana ariza	N/A	N/A	MAHAN1785-18	*Cirrhinus reba*	11.19
Barbonymus gonionotus	N/A	N/A	MAHAN1070-16	*Labeo rohita*	12.3
Barilius barila	N/A	N/A	MAHAN289-15	*Salmostoma sladoni*	16.86
Cirrhinus mrigala	0.43	1.47	MAHAN1655-18	*Labeo bata*	8.23
Cirrhinus reba	0.39	2.44	MAHAN1069-16	*Labeo calbasu*	10.21
Ctenopharyngodon idella	0.22	0.22	MAHAN1406-16	*Hypophthalmichthys molitrix*	10.71
Cyprinus carpio	0.04	0.18	MAHAN1655-18	*Labeo bata*	8.89
Devario devario	0	0	MAHAN685-15	*Rasbora rasbora*	24.81
Esomus danrica	N/A	N/A	MAHAN1764-18	*Cirrhinus reba*	17.86
Gibelion catla	0.23	0.61	MAHAN1509-18	*Labeo rohita*	5.94
Hypophthalmichthys molitrix	N/A	N/A	MAHAN1739-18	*Hypophthalmichthys nobilis*	4.61
Hypophthalmichthys nobilis	N/A	N/A	MAHAN1406-16	*Hypophthalmichthys molitrix*	4.61
Hypselobarbus curmuca	0	0	MAHAN1881-18	*Hypselobarbus lithopidos*	10.9
Hypselobarbus lithopidos	N/A	N/A	MAHAN1897-18	*Tor tor*	9.18
Labeo bata	N/A	N/A	MAHAN394-15	*Labeo boggut*	5.73
Labeo boggut	0	0	MAHAN1655-18	*Labeo bata*	5.73
Labeo calbasu	0.07	0.61	MAHAN1685-18	*Labeo gonius*	5.25
Labeo gonius	0	0	MAHAN1611-18	*Labeo calbasu*	5.25
Labeo rohita	0.06	0.44	MAHAN1685-18	*Labeo gonius*	5.73
Opsarius barna	0.33	0.33	MAHAN1744-18	*Barilius barila*	17.83
Opsarius bendelisis	N/A	N/A	MAHAN1764-18	*Cirrhinus reba*	17.52
Osteobrama cotio	N/A	N/A	MAHAN1620-18	*Osteobrama vigorsii*	14.28
Osteobrama vigorsii	0.18	0.54	MAHAN1542-18	*Osteobrama cotio*	14.28
Puntius chola	0.47	1.45	MAHAN355-15	*Puntius terio*	9.26
Puntius mahecola	N/A	N/A	MAHAN794-15	*Cyprinus carpio*	14.09
Puntius sophore	0	0	MAHAN355-15	*Puntius terio*	11.36
Puntius terio	N/A	N/A	MAHAN2141-18	*Puntius chola*	9.26
Rasbora daniconius	N/A	N/A	MAHAN1451-18	*Rasbora rasbora*	11.25
Rasbora rasbora	0.96	0.96	MAHAN2054-18	*Rasbora daniconius*	11.25
Salmostoma phulo	N/A	N/A	MAHAN683-15	*Salmostoma sladoni*	14.17
Salmostoma sladoni	0.26	0.48	MAHAN1544-18	*Salmostoma phulo*	14.17
Securicula gora	0.2	0.2	MAHAN683-15	*Salmostoma sladoni*	16.37
Systomus sarana	0	0	MAHAN1386-16	*Labeo rohita*	13
Tor tor	N/A	N/A	MAHAN1881-18	*Hypselobarbus lithopidos*	9.18
Paracanthocobitis botia	1.52	1.52	MAHAN794-15	*Cyprinus carpio*	20.26
Glossogobius guiris	0.56	0.56	MAHAN1622-18	*Siganus sutor*	19.25
Awaous ocellaris	0.05	0.36	MAHAN1661-18	*Oreochromis niloticus*	17.35
Sphyraena chrysotaenia	N/A	N/A	MAHAN2055-18	*Rhynchorhamphus malabaricus*	22.23
Ostorhinchus fasciatus	N/A	N/A	MAHAN1149-16	*Drepane longimana*	22.48
Drepane longimana	N/A	N/A	MAHAN1460-18	*Lutjanus johnii*	18.31
Platax teira	N/A	N/A	MAHAN1183-16	*Lutjanus rivulatus*	20.26
Planiliza tade	0.37	0.37	MAHAN1124-16	*Awaous ocellaris*	20.08
Notopterus notopterus	0.41	1.19	MAHAN1183-16	*Lutjanus rivulatus*	19.31
Ambassis ambassis	0.11	0.18	MAHAN2093-18	*Plicofollis layardi*	18.64
Chanda nama	0.19	0.73	MAHAN1797-18	*Parambassis lala*	16.87
Parambassis lala	0.03	0.16	MAHAN1392-16	*Chanda nama*	16.87
Parambassis ranga	0.07	0.2	MAHAN1715-18	*Parambassis lala*	17.65
Gerres filamentosus	N/A	N/A	MAHAN1463-18	*Gerres infasciatus*	6.2
Gerres infasciatus	N/A	N/A	MAHAN1462-18	*Gerres filamentosus*	6.2
Lates calcarifer	N/A	N/A	MAHAN1585-18	*Arius arius*	24.35
Leiognathus equulus	0	0	MAHAN1167-16	*Photopectoralis bindus*	19.05
Photopectoralis bindus	N/A	N/A	MAHAN1152-16	*Leiognathus equulus*	19.05
Lutjanus johnii	0	0	MAHAN1183-16	*Lutjanus rivulatus*	14.15
Lutjanus rivulatus	N/A	N/A	MAHAN1460-18	*Lutjanus johnii*	14.15
Polydactylus sextarius	N/A	N/A	MAHAN1596-18	*Lagocephalus guentheri*	20.39
Epinephelus coioides	N/A	N/A	MAHAN1169-16	*Epinephelus latifasciatus*	11.52
Epinephelus latifasciatus	N/A	N/A	MAHAN1180-16	*Epinephelus coioides*	11.52
Siganus sutor	1.52	1.52	MAHAN1136-16	*Alepes kleinii*	18.51
Pampus chinensis	0.15	0.35	MAHAN1460-18	*Lutjanus johnii*	18.83
Arius arius	0.21	0.43	MAHAN2093-18	*Plicofollis layardi*	10.27
Plicofollis layardi	N/A	N/A	MAHAN1679-18	*Arius arius*	10.27
Mystus bleekeri	0.19	0.38	MAHAN1645-18	*Mystus malabaricus*	12.95
Mystus cavasius	0.35	0.73	MAHAN1491-18	*Mystus bleekeri*	13.88

Table 8. Continued

Species	Mean Intra-Sp	Max Intra-Sp	Nearest Neighbour	Nearest *Species*	Distance to NN
Mystus gulio	N/A	N/A	MAHAN995-15	*Mystus bleekeri*	13.94
Mystus malabaricus	0	0	MAHAN662-15	*Mystus vittatus*	10.88
Mystus vittatus	0.22	0.45	MAHAN1645-18	*Mystus malabaricus*	10.88
Sperata seenghala	N/A	N/A	MAHAN1075-16	*Pachypterus atherinoides*	17.81
Clarias gariepinus	0.5	0.94	MAHAN1048-16	*Rita kuturnee*	17.81
Pachypterus atherinoides	0	0	MAHAN995-15	*Mystus bleekeri*	14.67
Pterygoplichthys anisitsi	N/A	N/A	MAHAN1048-16	*Rita kuturnee*	16.97
Pangasianodon hypophthalmus	N/A	N/A	MAHAN296-15	*Silonia silondia*	16.77
Rita chrysea	1.25	2.24	MAHAN1048-16	*Rita kuturnee*	7.35
Rita kuturnee	N/A	N/A	MAHAN346-15	*Rita chrysea*	7.35
Ailia coila	0.08	0.16	MAHAN296-15	*Silonia silondia*	14.91
Clupisoma garua	0.82	1.65	MAHAN296-15	*Silonia silondia*	8.31
Eutropiichthys burmannicus	N/A	N/A	MAHAN1719-18	*Clupisoma garua*	10.01
Silonia silondia	0	0	MAHAN1719-18	*Clupisoma garua*	8.31
Ompok bimaculatus	0.07	0.18	MAHAN1093-16	*Ompok pabda*	13.73
Ompok pabda	0.23	0.4	MAHAN987-15	*Ompok bimaculatus*	13.73
Wallago attu	0.2	0.39	MAHAN2091-18	*Ompok bimaculatus*	13.98
Nemipterus japonicus	0	0	MAHAN1179-16	*Lutjanus johnii*	18.15
Sillago indica	N/A	N/A	MAHAN1056-16	*Awaous ocellaris*	20
Sillago vincenti	N/A	N/A	MAHAN1633-18	*Scomberoides commersonnianus*	18.71
Acanthopagrus berda	N/A	N/A	MAHAN2092-18	*Rhabdosargus sarba*	14.25
Rhabdosargus sarba	0.47	0.95	MAHAN1140-16	*Acanthopagrus berda*	14.25
Macrognathus aral	N/A	N/A	MAHAN109-15	*Mastacembelus oatesii*	15.12
Macrognathus guentheri	0	0	MAHAN109-15	*Mastacembelus oatesii*	14.84
Mastacembelus oatesii	0	0	MAHAN1556-18	*Macrognathus guentheri*	14.84
Lagocephalus guentheri	0.56	0.56	MAHAN1159-16	*Polydactylus sextarius*	20.39
Takifugu oblongus	0	0	MAHAN1460-18	*Lutjanus johnii*	19.09

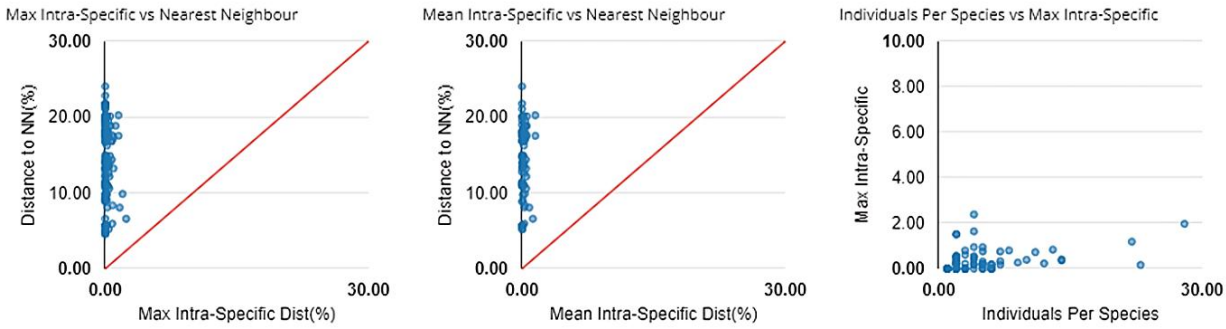

Figure 2. DNA barcoding gap showing maximum intraspecific Kimura 2-parameter (K2P) distances compared with minimum interspecific K2P distances in fishes from the Mahanadi River. The plot illustrates the relationship between mean and maximum intraspecific distances and nearest-neighbour (NN) interspecific distances.

Table 9. Summary of genetic divergences (K2P%) within and between taxonomic levels of fishes from the Mahanadi River. Distance calculations were performed using the Kimura 2-parameter model following standard DNA barcoding methods (Hebert et al., 2003; Kimura, 1980)

Comparison within	K2P genetic distance %			Standard Error
	Minimum	Mean	Maximum	
Species	0	0.20	2.38	0.00
Genera	4.58	11.84	24.47	0.00
Families	5.94	17.04	32.70	0.00

Overall, the recovery of 121-171 OTUs compared to 121 morphologically identified species suggests that an additional 10-30% of hidden diversity may exist within the dataset. The high level of congruence among K2P, BIN, ABGD, and PTP approaches demonstrates methodological robustness and supports the reliability of single-locus COI barcoding for species delimitation in tropical freshwater systems.

Biodiversity Insights and Species Composition

Five introduced species namely *Cyprinus carpio*, *Ctenopharyngodon idella*, *Hypophthalmichthys molitrix*, *Oreochromis mossambicus*, and *Oreochromis niloticus* were detected, confirming the presence of non-native taxa with potential ecological impacts on indigenous fish communities (Singh, 2014; Gozlan et al*.,* 2010). Endemic

taxa, including *Rita chrysea*, were successfully resolved, demonstrating the effectiveness of COI barcoding in recovering native biodiversity. Additionally, species endemic to the Mahanadi River basin, such as *Mahanadi rita* and *Rita chrysea*, were documented (Dahanukar et al*.,* 2011). Previous taxonomic assessments of the Mahanadi River (Tyagi et al*.,* 2021) reported 121 fish species, including 66 food fishes, 22 ornamental species, and 33 species of dual importance. The present study corroborates these findings while providing a molecular framework for their validation (Lakra et al*.,* 2016).

Phylogenetic Structure

Phylogenetic reconstruction using the NJ tree, combined with OTU-based clustering, revealed cohesive grouping of individuals within species, with most taxa forming well-supported monophyletic clusters across analytical methods. A few taxa exhibited deep intraspecific divergence or clustering inconsistencies, potentially reflecting population structuring, restricted gene flow, or early stages of lineage diversification (Meyer & Paulay, 2005; Dellicour & Flot, 2018). Overall, the combined evidence from sequence similarity (BLAST), genetic distance analyses, and species delimitation approaches demonstrates a strong and consistent taxonomic signal within the COI dataset. These results validate DNA barcoding as a reliable tool for species identification and biodiversity assessment in the Mahanadi River basin, while also highlighting the presence of potential incipient evolutionary lineages requiring further investigation.

Discussion

The present study demonstrates the strong efficacy of mitochondrial COI barcoding for species identification and delimitation across a taxonomically diverse assemblage of freshwater fishes from the Mahanadi River basin. The recovery of 121 species spanning 24 orders represents substantial coverage of the regional ichthyofauna, indicating that the generated barcode library captures a significant proportion of known diversity within this system. The presence of a pronounced barcode gap, with low intraspecific divergence (0-2.38%) relative to high interspecific divergence (4.58-24.47%), provides robust support for the effectiveness of COI in resolving species boundaries in tropical freshwater fishes (Hebert et al*.,* 2003; Ward et al*.,* 2005).

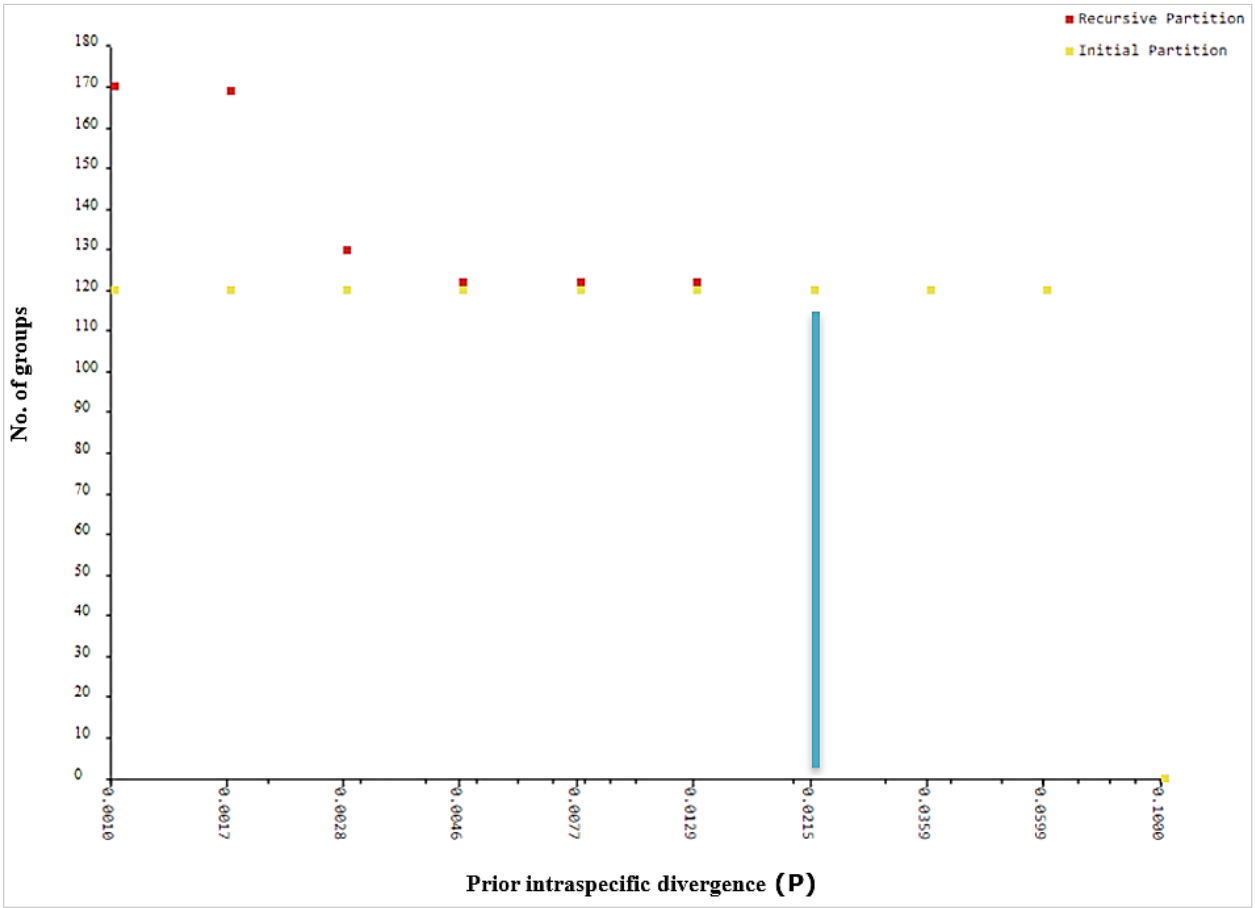

Figure 3. Automatic Barcode Gap Discovery (ABGD) partitioning of the dataset. The number of groups in initial and recursive partitions is shown as a function of the prior intraspecific divergence (P). Initial partitions are indicated by yellow squares, and recursive partitions by red squares. The dotted line represents the threshold value (P = 0.0235) used to define species boundaries in fishes from the Mahanadi River based on DNA barcodes.

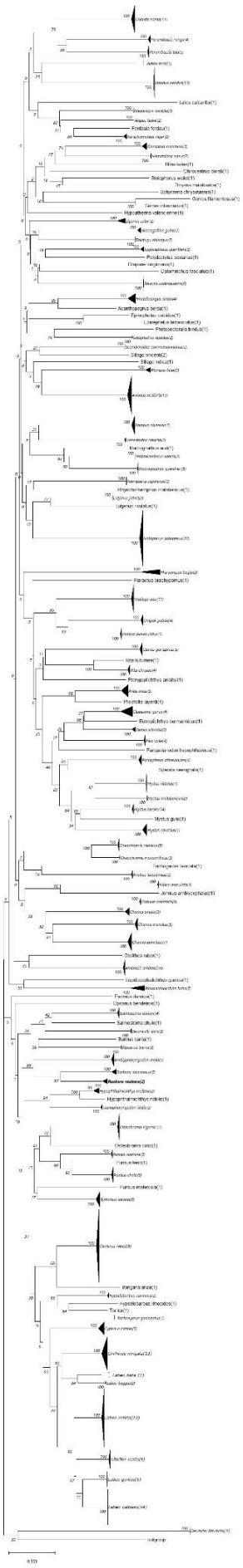

Figure 4. Neighbor-joining tree based on Kimura 2-parameter (K2P) distances of 421 COI sequences representing 121 fish species from the Mahanadi River basin.

Table 10. Number of groups delimited by Automatic Barcode Gap Discovery (ABGD) analysis under different prior intraspecific divergence (P) values

Partition	Group Number	Prior maximal distance (P)
1	171	0.001000
2	170	0.001668
3	131	0.002783
4	123	0.004642
5	123	0.007743
6	123	0.012915
7	121	0.021544
8	121	0.035938
9	121	0.059948
10	1	0.100000

Neighbour-joining phylogenetic reconstruction revealed well-supported monophyletic clustering for most taxa, demonstrating strong concordance between morphological and molecular identifications. However, the occurrence of a few polyphyletic or deeply structured lineages indicates that taxonomic resolution remains incomplete in certain groups, particularly within complex genera. Integrative species delimitation approaches (BIN, ABGD, and PTP) further supported species boundaries while revealing hidden genetic structure in several taxa, including *Labeo gonius, Puntius sophore, Nematolosa spp., and Mystus spp*. The concordance of these signals across multiple analytical frameworks strongly suggests the presence of cryptic species complexes or previously overlooked evolutionary lineages, indicating that current taxonomy underestimates true species diversity in the basin (Hebert et al., 2004; Melo et al., 2016).

Patterns of genetic divergence observed in this study are consistent with those reported for other freshwater and marine fishes, where interspecific divergence significantly exceeds intraspecific variation (Chakraborty et al., 2014; Hubert et al*.,* 2008., Suryawanshi et al*.,* 2024). The approximately 60-fold difference between mean intra- and interspecific distances observed here exceeds values reported in several previous studies, potentially reflecting greater taxonomic breadth or unresolved diversity within the Mahanadi system. This pronounced divergence pattern further reinforces the reliability of COI as a primary marker for species discrimination in diverse ichthyofaunal assemblages.

The detection of five non-native species, including Oreochromis niloticus, highlights ongoing processes of biotic homogenization driven by aquaculture expansion and anthropogenic connectivity. The establishment of such invasive taxa poses significant ecological risks, including competition, habitat alteration, and potential hybridization with native species (Ardura et al*.,* 2013; Liang et al*.,* 2018). The repeated detection of aquaculture-associated species suggests multiple introduction pathways and underscores the need for systematic monitoring and management interventions.

In contrast, the successful genetic validation of endemic taxa such as *Rita chrysea* confirms the persistence of native evolutionary lineages within the basin. However, the presence of deep intraspecific divergence in taxa such as *Cirrhinus reba* and *Rita chrysea* suggests the existence of species complexes undergoing incipient divergence, potentially driven by hydrological fragmentation, ecological specialization, and restricted gene flow. These findings indicate that the Mahanadi River basin represents not only a reservoir of known biodiversity but also a previously underrecognized hotspot of cryptic diversity.

From a conservation perspective, the results underscore the importance of incorporating molecular tools into biodiversity assessment and management frameworks. Taxa exhibiting significant genetic structuring should be prioritized for integrative taxonomic revision, while the presence of invasive species necessitates immediate monitoring to mitigate ecological impacts. Furthermore, the establishment of a validated DNA barcode reference library provides a critical foundation for emerging applications such as environmental DNA (eDNA)-based monitoring, which can enable rapid and non-invasive assessment of biodiversity in freshwater ecosystems (Bohmann et al*.,* 2014; Deiner et al*.,* 2017).

Overall, this study demonstrates that the ichthyofauna of the Mahanadi River basin is both taxonomically complex and dynamically structured, shaped by ongoing evolutionary processes and increasing anthropogenic pressures. The integration of molecular and morphological approaches not only improves species resolution but also reveals hidden diversity with important implications for taxonomy, conservation prioritization, and sustainable fisheries management.

Conclusion

This study establishes the first comprehensive COI barcode reference library for freshwater fishes of the Mahanadi River basin, providing a robust framework for accurate species identification using integrative DNA barcoding and multiple delimitation approaches (BIN, ABGD, PTP). The findings confirm the reliability of COI-based identification while revealing cryptic diversity and previously unrecognized lineages. The 421 validated

sequences substantially enrich reference databases and provide a foundation for eDNA monitoring, population studies, and biodiversity assessments under increasing anthropogenic pressure. The detection of non-native species further emphasizes the need for continued molecular surveillance. Overall, this work highlights the value of integrative taxonomy in resolving species boundaries and supports informed conservation and management of freshwater biodiversity in the Mahanadi River basin and comparable tropical ecosystems.

Ethical Statement

We declare that the fish used in this study are not protected under wildlife conservation act and are regularly caught by professional fisherman and sold as a food fish in markets. No specific permission is required for obtaining these fish in India, and no in vitro experiment was conducted in the laboratory.

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Author Contribution

RMS: Conceptualization, Data curation, Funding acquisition, Methodology, Validation, Writing - original draft, Writing - review & editing.

GDK: Writing - review & editing.

AK: Software.

SJ: Methodology, Writing - original draft.

DK: Methodology.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional or personal conflict that could have appeared to influence the work report in this paper.

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