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Integrative Taxonomy Clarifies the Historical Flaws in the Systematics and Distributions of Two *Osteobrama* Fishes (Cypriniformes: Cyprinidae) in India

Boni Amin Laskar [1], Dhriti Banerjee [1,2], Sangdeok Chung [3], Hyun-Woo Kim [4,5], Ah Ran Kim [5,*] and Shantanu Kundu [6,*]

1 High Altitude Regional Centre, Zoological Survey of India, Solan 173211, Himachal Pradesh, India; boniamin.laskar@gmail.com (B.A.L.); dhritibanerjee@gmail.com (D.B.)
2 Zoological Survey of India, Prani Vigyan Bhawan, Kolkata 700053, West Bengal, India
3 Distant Water Fisheries Resources Research Division, National Institute of Fisheries Science, Busan 46083, Republic of Korea; sdchung@korea.kr
4 Department of Marine Biology, Pukyong National University, Busan 48513, Republic of Korea; kimhw@pknu.ac.kr
5 Research Center for Marine Integrated Bionics Technology, Pukyong National University, Busan 48513, Republic of Korea
6 Institute of Fisheries Science, Pukyong National University, Busan 48513, Republic of Korea
* Correspondence: ahrankim@pukyong.ac.kr (A.R.K.); shantanu1984@pknu.ac.kr or shantanu1984@gmail.com (S.K.)

Abstract: The taxonomy and geographical distributions of *Osteobrama* species have historically posed challenges to ichthyologists, leading to uncertainties regarding their native ranges. While traditional taxonomy has proven valuable in classification, the utility of an integrated approach is restricted for this particular group due to limitations in combining information from biogeography, morphology, and genetic data. This study addresses the taxonomic puzzle arising from the recent identification of *Osteobrama tikarpadaensis* in the Mahanadi and Godavari Rivers, casting doubt on the actual distribution and systematics of both *O. tikarpadaensis* and *Osteobrama vigorsii*. The research reveals distinctions among specimens resembling *O. vigorsii* from the Krishna and Godavari riverine systems. Notably, specimens identified as *O. vigorsii* from the Indian Museum exhibit two pairs of barbels, while those from the Godavari River in this study are identified as *O. tikarpadaensis*. Inter-species genetic divergence and maximum likelihood phylogeny provide clear delineation between *O. vigorsii* and *O. tikarpadaensis*. The study suggests that *O. vigorsii* may be limited to the Krishna River system in southern India, while *O. tikarpadaensis* could potentially extend from the Mahanadi River in central India to the Godavari River in southern India. Proposed revision to morphological features for both species, accompanied by revised taxonomic keys, aim to facilitate accurate differentiation among *Osteobrama* congeners. The data generated by this research provide a resource for future systematic investigations into cyprinids in India and surrounding regions. Further, the genetic diversity information obtained from various riverine systems for *Osteobrama* species will be instrumental in guiding aquaculture practices and formulating effective conservation action plans.

Keywords: cyprinids; distribution; genetic divergence; key characters; phylogeny; systematics

Key Contribution: The current investigation resolves a longstanding taxonomic quandary concerning two Indian cyprinids through a comprehensive morphological reassessment; fortified by corroborative molecular data. Furthermore, the research contributes updated morphological keys for the identification of species within the genus *Osteobrama*, offering valuable tools for subsequent systematic studies in the future.

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1. Introduction

Freshwater fishes represent a crucial zoogeographical group due to their confinement to drainage systems, conceptualized as dendritic water islands surrounded by land and bordered by saltwater barriers [1]. According to the Animal Discoveries of India 2022 [2], the country hosts 3439 fish species, encompassing both freshwater and marine varieties, with approximately 206 being endemic and ~18 introduced species. A global fish database search (https://www.fishbase.org, accessed on 15 January 2024) has identified approximately 1064 freshwater fish species reported from India and its islands [3]. Accurate identification of organisms at lower taxonomic levels is crucial for ecosystem understanding and conservation applications, but existing identification systems need advancement to address gaps and enhance precision [4,5]. The DNA barcoding technique, standardized for lower-level taxonomic identification, employs a partial mitochondrial cytochrome oxidase c subunit-I gene (mtCOI), consisting of approximately 648 base pairs near the 5′ end of the gene [6,7]. The molecular technique, proven efficient in biodiversity assessment globally, has resolved taxonomic issues in Indian riverine systems [8–15]. It complements traditional taxonomy by swiftly comparing specimens with reference sequences in global databases [16]. Furthermore, the technique has advanced with various species-level delineation methods.

Fishes belonging to the genus *Osteobrama* Heckel 1843 (Cypriniformes: Cyprinidae), with the type species being *Cyprinus cotio* Hamilton 1822, exhibit a laterally compressed body, an elevated dorsum, the absence of a procumbent pre-dorsal spine, a rounded abdomen in front of the pelvic fin, and a keeled abdominal edge from the pelvic-fin origin to the vent. Additionally, they possess a long anal fin with more than 10 branched rays [17]. The genus currently consists of 11 described species [18]. Notably, *O. cotio* (Hamilton, 1822) is widely distributed in eastern India and Bangladesh, extending to northern and central India up to Pakistan. In southern India, five species—*O. peninsularis* Silas, 1952, *O. vigorsii* (Sykes, 1839), *O. dayi* (Hora and Misra, 1940), *O. neilli* (Day, 1873), and *O. bakeri* (Day, 1873)—are found, while three species—*O. cunma* (Day, 1888), *O. belangeri* (Valenciennes, 1844), and *O. feae* Vinciguerra, 1890—are distributed in Southeast Asia, Myanmar, and China [17,19,20]. A recent addition to the genus is *O. tikarpadaensis* (Shangningam, Rath, Tudu and Kosygin, 2020), described from the Mahanadi River in Odisha, central India, and reported in the Erai River, Godavari drainages, Maharashtra [21,22]. Although *O. alfredianus* (Valenciennes, 1844) was originally documented in Mysore, peninsular India [18], subsequent taxonomic assessments have synonymized it with *O. vigorsii* [17]. Later, *O. alfredianus* has been reported in the Salween Basin, Southeast Asia, which is a location distant from its type locality; however, comprehensive taxonomic descriptions are lacking. The absence of compelling literature supporting the validity of *O. alfredianus* and its accurate distribution restricts any definitive statements within the scope of this study.

In the realm of systematics, the presence or absence of barbels stands as a crucial taxonomic trait in *Osteobrama* [21,23]. When present, these barbels may manifest as a single pair of maxillary barbels or include both maxillary and rostral varieties, sometimes being minute or rudimentary in certain species. Rostral barbels may either remain concealed in a groove or be visible only under microscopic examination, while in other species, they can be significantly longer, extending to the base of maxillary barbels [17,19,21]. The type locality of *O. vigorsii* is the Bhima River (a tributary of the Krishna River) at Pairgaon, Maharashtra, but there are also reports in the Krishna and Godavari Rivers [24,25]. However, the recent discovery of *Osteobrama* in the Mahanadi and Godavari Rivers has introduced a taxonomic quandary for ichthyologists regarding the actual distribution and systematics of *O. tikarpadaensis* and *O. vigorsii*. This study aims to resolve this taxonomic challenge through development and presentation of revised taxonomic keys and genetic information. In this study, discrepancies in prevailing diagnostic features for *O. vigorsii* were noted in specimens from the Krishna and Godavari riverine systems, including those examined at the Indian Museum. Genetic divergence analysis on partial mtCOIs among *Osteobrama* species from south India revealed distinctions between specimens resembling *O. vigorsii* from the Krishna and Godavari Rivers. Consequently, based on existing morphological descriptions,

distinguishing between the two distinct species from the Krishna and Godavari Rivers as *O. vigorsii* is perplexing. This study identifies specimens resembling *O. vigorsii* from the Godavari River as *O. tikarpadaensis*. Therefore, we hypothesized that *O. vigorsii* is limited to the Krishna River system in southern India, whereas *O. tikarpadaensis* is distributed from the Mahanadi River in central India to the Godavari River in south India.

2. Material and Methods

2.1. Material Examined

The following specimens were taken for morphological investigations—*O. vigorsii*: FBRC/ZSI/F3550, (*n* = 1), 119.5 mm SL; India: Telangana, Nagarkurnool District, Krishna River: near Somasila, 16°2′46″ N 78°19′34″ E; B. A. Laskar, 28 Jul 2020. FBRC/ZSI/F3551, (*n* = 2), 116.0–132.0 mm SL and FBRC/ZSI/F3552, (*n* = 3), 90.5–109.0 mm SL; collection details same as F3550. FBRC/ZSI/F2783, (*n* = 1), 95.0 mm SL; India: Telangana, Nagarkurnool District, Krishna River: near Somasila, 16°01′12″ N 78°19′37″ E; B. A. Laskar, 18 July 2018 (Figure 1A). The study specimens of *O. vigorsii* were collected from the same river basin as its type locality in the Bhima River, a tributary of the Krishna River. *O. cotio*: FBRC/ZSI/DNA907/F3880, (*n* = 1), 40.0 mm SL; India: Telangana, Jurala project: Krishna River Basin, Kistampally. FBRC/ZSI/F/2707, (*n* = 2), 62.0–64.0 mm SL; India: Maharashtra, Darna River: near Bhagur. *O. cotio* iconotype figure from Hamilton plate 207 [26] (Figure S1A). *O. neilli*: FBRC/ZSI/F/3548, (*n* = 2), 68.0–69.0 mm SL; India: Telangana, Nagarkurnool District, Krishna River: near Somasila (Figure S1B). *O. peninsularis*: FBRC/ZSI/F/3549, (*n* = 1), 68.0 mm SL; India: Telangana, Wyra Lake, Godavari River drainage, Khammam District (Figure S1C). *O. tikarpadaensis*: FBRC_ZSI_F_2616, (*n* = 4), 101.0–102.0 mm SL; India: Telangana, Godavari River, 17.7431° N, 80.8798° E. FBRC/ZSI/F/3416, (*n* = 1), 77.0 mm SL; India: Telangana, East Godavari District, confluence of Sabri River and Godavari River. (Figure 2A,B). Specimens of *O. tikarpadaensis* were collected from the Godavari River and morphologically compared with the type specimen from the Mahanadi River. *O. dayi*: FBRC-ZSI-F 3795, India: Telangana, Godavari River (Figure S1D). In the current study, the urohyal bone structure was examined for two specimens each of *O. vigorsii* and *O. tikarpadaensis*.

2.2. Sampling and Morphological Investigation

Morphometric and meristic data were documented in accordance with the methodology established in prior investigations [19,21,25]. Measurements were obtained using digital calipers, with precision up to 0.1 mm, with the exception of fin rays and scale counts, which were conducted under transmitted light utilizing a stereomicroscope. Enumeration of all pored scales was undertaken to report the number of lateral line scales. The various components of the body are expressed as a percentage of standard length (SL), while subunits of the head are presented as a percentage of head length (HL). Notably, morphometric data and scale counts for two specimens (voucher No. FBRC_ZSI_F2783_DNA301, (*n* = 1), 95.0 mm SL; FBRC_ZSI_F3551, DNA814, (*n* = 1), 116.0 mm SL) were omitted due to injuries sustained during the collection process. Nonetheless, their DNA data have been included in the subsequent analysis. Additionally, DNA data for one specimen were not generated, as it was promptly preserved in formalin. The specimens examined have been deposited at the Freshwater Biology Regional Centre, Zoological Survey of India (ZSI), Hyderabad, India.

2.3. Molecular Experiments

Tissue samples were procured from seven recently collected specimens of *O. vigorsii* and one specimen of *O. neilli* from the Krishna River; and two specimens each of *O. tikarpadaensis* and *O. cotio* and one specimen of *O. peninsularis* from the Godavari River. Genomic DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The previously published primer pair [27] FishF1-5′–TCAACCAACCACAAAGACATTGGCAC–3′ and FishR1-5′–TAGACTTCTGGGTGGCCAAAGAATCA–3′ was employed to amplify a partial segment of mtCOI. The PCR mixture (30 µL) comprised 10 pmol of each primer, 100 ng of DNA

template, 1 × PCR buffer, 1.0–1.5 mM of $MgCl_2$, 0.25 mM of each dNTPs, and 1U of *Taq* polymerase (Takara BIO Inc., Otsu, Japan). The thermal profile involved an initial denaturation at 95 °C for 2 min; followed by 35 cycles of 0.5 min at 94 °C, 0.5 min at 54 °C, and 1 min at 72 °C, and a final extension at 72 °C for 10 min; with a subsequent hold at 4 °C. Purification of PCR products was accomplished using the QIAquickR Gel extraction Kit (Qiagen, Valencia, Santa Clarita, CA, USA). Commercial cycle sequencing and Sanger sequencing were employed, and both forward and reverse chromatograms were scrutinized using SeqScanner V1.0 (Applied Biosystems Inc., Foster City, CA, USA), nucleotide BLAST (https://blast.ncbi.nlm.nih.gov, accessed on 15 January 2024), and ORF finder (https://www.ncbi.nlm.nih.gov/orffinder, accessed on 15 January 2024) to eliminate low-quality reads and gaps. The resulting sequences of *O. cotio*, *O. peninsularis*, *O. vigorsii*, *O. neilli*, and *O. tikarpadaensis* were deposited in GenBank (https://www.ncbi.nlm.nih.gov, accessed on 15 January 2024) and BOLD Systems (https://www.boldsystems.org, accessed on 15 January 2024) to obtain unique accession numbers (Table 1).

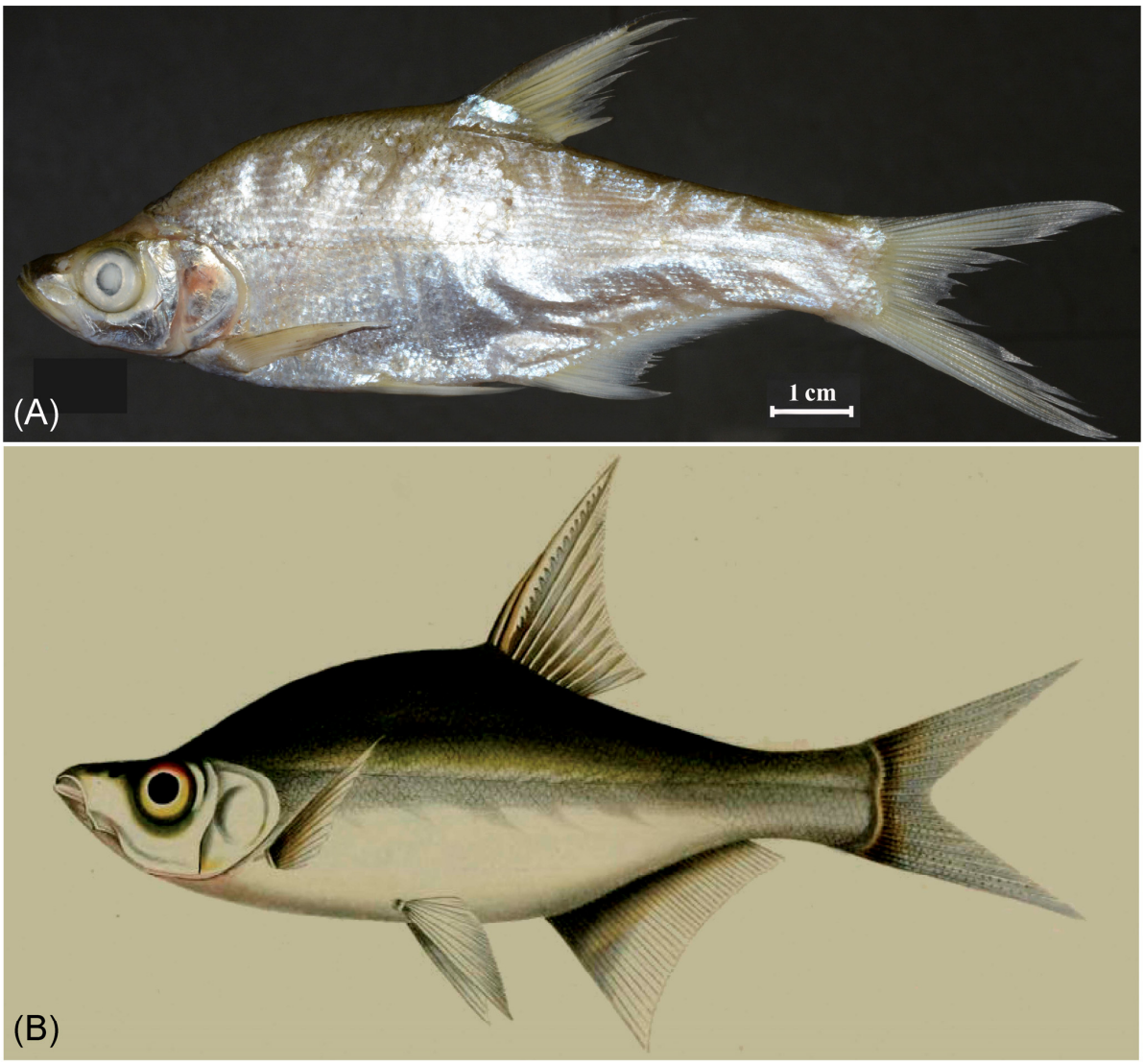

Figure 1. (**A**) *Osteobrama vigorsii*, FBRC/ZSI/ F3550, 119.5 mm SL; India: Telangana, Krishna River: near Somasila. Photo credit @Boni Amin Laskar; (**B**) original drawing of *O. vigorsii*, reproduced laterally reversed from Sykes, 1841.

Figure 2. (**A**) *Osteobrama tikarpadaensis* immediately after collection from the Godavari River; (**B**) *O. tikarpadaensis*, FBRC/ZSI/F/3416, India: Telangana, Bhadradri Kothagudem District, Godavari River: near KTPS Intake Well at Burgampadu. Photo credit @Boni Amin Laskar.

Table 1. The voucher IDs, locality information, GenBank (https://www.ncbi.nlm.nih.gov/nuccore) accession numbers, and BOLD-IDs (https://www.boldsystems.org/) of the generated mtCOI sequences of *Osteobrama* species and out-group taxa, *Rasbora daniconius* (Hamilton 1822).

Species	Museum Registration	Locality	GenBank Accession Number	BOLD-IDs
Osteobrama cotio	FBRC_ZSI_F_2707	Maharashtra, 20.450° N, 74.403° E	MH795978	BOLD:AAE6868
Osteobrama cotio	FBRC_ZSI_DNA907_F3880	Jurala project, Kistampally, Telangana, 16.370° N, 77.694° E	MW506822	-
Osteobrama tikarpadaensis	FBRC_ZSI_F_2616	Godavari River, Telangana, 17.7431° N, 80.8798° E	MH395748	BOLD:ABY3071
Osteobrama tikarpadaensis	FBRC_ZSI_DNA616_F3416	Godavari River, Andhra Pradesh, 17.5721° N, 81.2587° E	MT654653	BOLD:ABY3071

Table 1. *Cont.*

Species	Museum Registration	Locality	GenBank Accession Number	BOLD-IDs
Osteobrama neilli	FBRC_ZSI_DNA833_F3548	Krishna River at somasila near temple, Telangana, 16.046° N, 78.326° E	MT896378	BOLD:ACR7173
Osteobrama peninsularis	FBRC_ZSI_DNA864_F3549	Wyra lake, Telangana, 17.252° N, 80.384° E	MT896379	BOLD:ACJ3278
Osteobrama vigorsii	FBRC_ZSI_DNA861_F3550	Krishna River somasila near temple, Telangana, 16.046° N, 78.326° E	MT896380	BOLD:ACM5411
Osteobrama vigorsii	FBRC_ZSI_F2783_DNA301	Tungabhadra River, Andhra Pradesh, 16.02° N, 78.327° E	MK336909	BOLD:ACM5411
Osteobrama vigorsii	FBRC_ZSI_DNA814_F3551	Krishna River at somasila, Telangana, 16.048° N, 78.334° E	MT896381	BOLD:ACM5411
Osteobrama vigorsii	FBRC_ZSI_DNA862_F3552	Krishna River somasila near temple, Telangana, 16.046° N, 78.326° E	MT896382	BOLD:ACM5411
Osteobrama vigorsii	FBRC_ZSI_DNA863_F3552	Krishna River somasila near temple, Telangana, 16.046° N, 78.326° E	MT896383	BOLD:ACM5411
Osteobrama vigorsii	FBRC_ZSI_DNA836_F3552	Krishna River somasila near temple, Telangana, 16.046° N, 78.326° E	MT896384	BOLD:ACM5411
Osteobrama vigorsii	FBRC_ZSI_DNA897_F3872	Jurala project, Kistampally, Telangana, 16.370° N, 77.694° E	MW506815	-
Rasbora daniconius	FBRC_ZSI_DNA326_F3464	Andhra Pradesh, 18.0733° N, 82.9505° E	MK681752	-

2.4. Dataset Preparation and Genetic Analyses

The representative COI sequences of three genera within the Smiliogastrinae subfamily, namely *Osteobrama*, *Rohtee* (Sykes, 1839), and *Mystacoleucus* (Günther, 1868), were acquired from the GenBank database. Consistent with prior research [20], uncertain sequences of *O. cotio* from the Narmada River Basin, Karnafuli, and Sangu Rivers were excluded from the dataset. Additionally, a maximum of five representative sequences from three congeners (*O. belangeri*, *O. cunma*, and *O. feae*) were incorporated into the dataset [20]. The COI sequences for *O. dayi*, sourced from GenBank, were included in the study. However, no COI data for *O. bakeri* were found in the database, and no specimens could be collected for the study. GenBank accession numbers are indicated alongside the organism's name in the phylogenetic tree, as well as detailed specimen information, including accession numbers for de novo sequences (Table 1). The dataset was aligned using CLUSTAL X, and genetic distances were estimated using MEGA X [28,29]. The model 'GTR + G + I' was chosen based on the lowest Bayesian information criterion (BIC) scores determined using PartitionFinder 2 [30] on the CIPRES Science Gateway v3.3 [31] and JModelTest v2 [32]. The PhyML 3.0 [33] was employed to construct the maximum likelihood (ML) phylogeny, with 1000 bootstrap support. Furthermore, the Bayesian (BA) tree was created using Mr. Bayes 3.1.2 [34], employing one cold and three hot Metropolis-coupled Markov chain Monte Carlo (MCMC) chains. The analysis extended over 10,000,000 generations, with tree sampling occurring every 100th generation, and 25% of the samples were discarded as burn-in. Visualization of both ML and BA trees was carried out using the iTOL v4 web server (https://itol.embl.de/login.cgi, accessed on 15 January 2024) [35].

3. Results and Discussion

3.1. Morphological Amendment of O. vigorsii

Prior to this study, the taxonomic characters of *O. vigorsii* were followed after Hora and Misra [23]. In dealing with the taxonomy of *O. vigorsii*, Hora and Misra [23] examined specimens from diverse locations, including the Darna River, the Mutha-Mula River, and the Kistna (now Krishna) River (ZSI Cat No. 888), and from Deccan and Odisha (with no precise locality specified). The data presented for *O. vigorsii* were derived from specimens in the Indian Museum collection obtained from various locales within the Krishna River Basin, with the exception of one specimen from Orissa, which was acquired from Dr. F. Day. The dorsal profile of *O. vigorsii* was characterized by a distinct concavity extending from the snout to over the nape, consistent with the original descriptions [23]. Additionally, the taxonomic key to species highlighted the presence of only two rudimentary maxillary barbels in *O. vigorsii*. Subsequently, a new species, *O. dayi* [23], characterized by two rudimentary maxillary barbels, was proposed based on specimens collected from the Godavari River. These two species are distinguishable by differences in anal fin length and the number of lateral line scales. Notably, *O. vigorsii* has since been consistently characterized by the presence of two maxillary barbels, among other morphological characters.

In the absence of any designated types, the original illustration proves highly valuable [36] (Figure 1B), exhibiting a notable similarity to the specimens of *O. vigorsii* in the present study (Figure 1A), as well as to Day's illustration of *O. vigorsii* from 1889 [37]. Jayaram (1995) reproduced Day's illustration [24]. Subsequently, Singh and Yazdani (1992) claimed a striking resemblance between their newly identified species, *Osteobrama bhimensis*, and *O. vigorsii*. However, they primarily differentiated the two based on the absence of barbels, the number of transverse scales, and the shape of the urohyal bone [38]. Notably, Singh and Yazdani [38] did not directly examine specimens of *O. vigorsii* displaying the purported resemblance to their new species, but rather utilized measurements and counts from a previous study [23]. Although they differentiated the two species based on urohyal shape, they failed to specify which specimen of *O. vigorsii* was studied for the urohyal [38]. Jadhav et al. [25] criticized the lack of retrievability in Singh and Yazdani's [38] urohyal study. However, the form of urohyal drawn by Singh and Yazdani [38] for *O. bhimensis* was observed in specimens of *O. vigorsii* from near its type locality area, in both Jadhav et al.'s [25] study and the present investigation (Figure 3A). Jadhav et al. [25] did not observe unequal dorsal spreads of the urohyal in their specimens. Surprisingly, Jadhav et al. [25] did not examine the urohyal in freshly collected specimens from the Godavari River drainage. Although the other form of urohyal was not observed by Jadhav et al. [25], it warrants examination in specimens resembling *O. vigorsii* from the Godavari Basin. Jadhav et al. [25] indicated the presence of one pair of barbels in the type specimens of *O. bhimensis* and in comparative materials of *O. vigorsii* in their study. Remarkably, Jadhav et al. [25] identified a significant resemblance among images of the types of *O. bhimensis*, *O. vigorsii* from various sources, and Sykes' illustration [36]. Consequently, *O. bhimensis* is herein regarded as a junior synonym of *O. vigorsii*.

Sykes [36] provided detailed characteristics of *O. vigorsii*, emphasizing the upturned snout, straight upper line of the head, and the lower line curving upwards from below. The mouth structure observed in all specimens of *O. vigorsii* in the current study markedly differs from its congeners, representing a superior mouth type. The lower jaw exhibits strength with a hook-like structure at its distal tip, fitting into a small concavity at the distal tip of the upper jaw (Figure 3C). Day [37] noted the presence of a very rudimentary pair of maxillary barbels for *O. vigorsii*. The revision by Hora and Misra [23] provided additional insights, characterizing the species by the presence of a distinct concavity from the snout to over the nape and two rudimentary maxillary barbels. Simultaneously, Hora and Misra [23] described another new species, *O. dayi*, possessing two maxillary barbels. The presence or absence of barbels is considered a significant taxonomic trait in *Osteobrama* [21,23]. Based on this, Hora and Misra [23] grouped the species into three categories: (i) with four well-defined barbels, (ii) with two rudimentary maxillary barbels, and (iii) without barbels.

Morphological features for *O. vigorsii* have mostly been derived from Hora and Misra [23]. Despite Jadhav et al.'s [25] extensive examination of *O. vigorsii* specimens from the Krishna Basin, they failed to detect the presence of rostral barbels. In contrast, the present study reveals the presence of both maxillary and rostral barbels in *O. vigorsii* (Figure 3C), placing it in Group-(i) alongside *O. bakeri*, *O. feae*, *O. neilli*, and *O. tikarpadaensis*. Consequently, there is a need to revise the key to species within the genus *Osteobrama*. A revised key, adapted from Shangningam et al. [21], is provided below. Furthermore, the body morphometrics of *O. vigorsii* from the Krishna River (this study) are presented in Table 2.

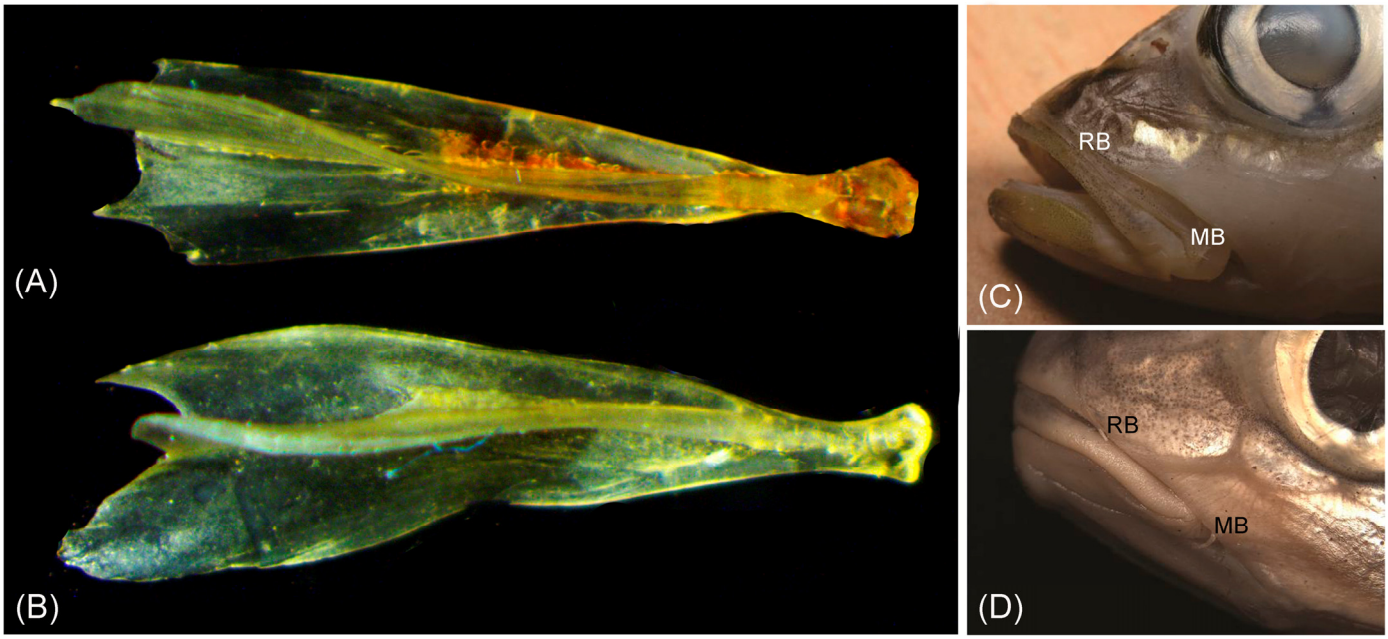

Figure 3. Dorsal view of urohyal bone in (**A**) *O. vigorsii* Krishna River, and (**B**) *O. tikarpadaensis* Godavari River, (**C**) *O. vigorsii*, FBRC/ZSI/ F3550, 119.5 mm SL; India: Telangana, Krishna River, showing the presence of barbels, (**D**) reproduced from Shangningam et al. [21] *O. tikarpadaensis* showing presence of barbels, reproduced with permission from the copyright holder ©Magnolia Press, and authorization for the utilization of the photograph was secured through direct communication with the corresponding author, Shibananda Rath. RB = rostral barbel, MB = maxillary barbel.

Table 2. Morphometric data of *O. vigorsii* from Krishna River (current study). SE, standard error.

Parameters	Range	Mean ± SE
Standard Length	90.5–132.0 mm	
In % SL		
Head length	24.1–28.0	25.8 ± 0.95
Head depth	17.6–22.0	18.9 ± 1.03
Head width	9.9–10.6	7.8 ± 2.59
Mouth width	6.3–7.4	5.2 ± 1.73
Body depth	31.5–35.2	33.2 ± 0.84
Body width	8.6–11.0	9.8 ± 0.49
Pre-dorsal length	51.8–56.9	55.0 ± 1.15
Pre-anal length	59.4–64.3	61.3 ± 1.14
Pre-pelvic length	32.6–42.0	38.6 ± 2.12
Pre-pectoral length	24.8–28.2	26.6 ± 0.75
Pelvic–anal distance	16.5–21.6	19.0 ± 1.21
Dorsal fin base length	11.3–12.4	11.8 ± 0.22
Anal fin base length	22.9–27.5	24.9 ± 0.93

Table 2. *Cont.*

Parameters	Range	Mean ± SE
Caudal peduncle length	12.6–16.5	13.9 ± 0.87
Caudal peduncle depth	11.5–13.8	12.1 ± 0.56
Snout length	6.4–8.3	7.3 ± 0.40
Eye diameter	7.0–7.4	7.3 ± 0.07
Inter-orbital distance	6.0–6.4	6.3 ± 0.11
Inter-narial space	4.3–4.9	4.6 ± 0.13
Dorsal fin height	28.3–34.3	30.5 ± 1.40
Pectoral fin length	19.8–20.2	20.0 ± 0.08
Anal fin height	16.5–19.9	18.1 ± 0.70
Pelvic fin length	20.5–23.2	21.4 ± 0.61
In % HL		
Eye diameter	26.3–30.0	28.3 ± 0.82
Interorbital width	23.0–26.7	24.6 ± 0.77
Head depth	62.7–82.8	73.8 ± 4.14
Head width	37.9–40.9	39.1 ± 0.73
Mouth width	24.8–30.5	26.6 + 1.34

3.2. Note on O. tikarpadaensis, with Urohyal Features

Shangningam et al. [21] delineated *O. tikarpadaensis* as a novel species, highlighting its unique features, particularly the oblique black streak on the anterior body immediately posterior to the opercle, parallel to the upper opercular margin, which distinguished it from all congeners. In the current investigation, it was noted that none of the *Osteobrama* specimens, with the exception of those resembling *O. vigorsii* from the Godavari River Basin, exhibited the distinct oblique black streak precisely described for *O. tikarpadaensis* (Figure 2A). The urohyal morphology in these *O. vigorsii*-like specimens from the Godavari River displayed two unequal ends posteriorly, with the left side being longer and thickened (Figure 3B), akin to one of the urohyal forms illustrated by Singh and Yazdani [38]. Consequently, the *O. vigorsii*-like specimens from the Godavari River Basin differ from their counterparts in the Krishna River Basin due to the presence of the oblique black streak immediately posterior to the opercle, urohyal characteristics with two unequal ends posteriorly, and a combination of other morphological features. These variations, previously overlooked, challenge the previous taxonomic assessments. Singh and Yazdani [38], despite noting some variations, failed to accurately identify true *O. vigorsii*, rendering their proposed new species (*O. bhimensis*) invalid. Following the discovery of *O. tikarpadaensis* by Shangningam et al. [21], it is evident that the taxonomic characteristics of *O. vigorsii* were confounded by the representation of characters from two distinct species. Consequently, the *O. vigorsii*-like specimens from the Godavari River are now identified as *O. tikarpadaensis*, a distinction further supported by genetic divergence analysis highlighting the dissimilarity between *O. tikarpadaensis* and *O. vigorsii*.

Despite the documented pre-dorsal distance for *O. tikarpadaensis* being reported as 37.8–40.4% of standard length (SL) in its descriptions [21], this measurement seems either exceptionally shorter compared to congeners (e.g., 53.5–56.1% in *O. feae*, 53.0–56.5% in *O. neilli*, 55.8–56.1% in *O. belangeri*, 51.2–52.2% in *O. cotio*, 51.8–56.9% in *O. vigorsii* in this study) or may represent inaccurate data. Notably, Shangningam et al. [21] did not examine any specimens from the Godavari River Basin. Rath et al. [22] identified *O. tikarpadaensis* from an old collection from the Erai River, Chandrapur District, Maharashtra. However, discrepancies in body morphometry, including the pre-dorsal distance, that were reported by Rath et al. [22] compared closely to the descriptions of *O. tikarpadaensis*. The body morphometrics of *O. tikarpadaensis* from the Godavari River in this study, along with a reproduction of the data from Shangningam et al. [21], are presented to facilitate a comprehensive understanding (Table 3).

Table 3. Morphometric data of *O. tikarpadaensis* from the Godavari River (current study) and from Shangningam et al. [21].

Parameters	**Range**	
Standard Length	101.0–102.0 mm	Shangningam et al. [21]
In % SL	Specimens from Godavari River	
Head length	26.6–26.6	24.5–28.8
Head depth	11.7–12.7	16.4–18.6
Head width	11.0–12.0	13.2–14.4
Mouth width	5.2–5.7	5.6–7.1
Body depth	32.3–35.1	34.5–39.5
Body width	9.7–10.8	9.3–11.7
Pre-dorsal length	50.0–53.2	37.8–40.4
Pre-anal length	53.2–59.7	60.0–61.7
Pre-pelvic length	40.5–41.6	39.9–43.1
Pre-pectoral length	26.0–26.6	24.7–26.6
Pelvic–anal distance	13.9–15.6	19.7–21.3
Dorsal fin base length	11.7–12.0	13–14.2
Anal fin base length	27.8–28.6	29.5–32
Caudal peduncle length	12.0–15.6	14.5–15.6
Caudal peduncle depth	10.1–11.0	10.3–12.2
Snout length	7.1–7.6	7.1–8.3
Eye diameter	6.5–7.0	6.7–8.3
Inter-orbital distance	7.8–8.2	8.7–10.0
Inter-narial space	5.2–5.7	5.0–6.0
Dorsal fin height	27.2–27.3	24.6–29.4
Pectoral fin length	18.8–19.0	19.2–21.2
Anal fin height	13.0–13.3	29.7–31.7
Pelvic fin length	17.1–18.8	17.6–18.9

3.3. Genetic Inferences

The estimated genetic divergence (K2P) between the groups (genera) ranged from 17.3% to 18.4%. In both ML and BA phylogenetic trees (Figures 4 and S2), the de novo sequences of *O. vigorsii* from the Krishna River, including four database sequences labelled *O. cotio*, constitute a cohesive cluster with pairwise genetic distances (K2P) ranging from 0.00 to 0.77%. By analyzing COI data, we confirm that the four database sequences (KX946745 to KX946748) collected from Kolhapur, Maharashtra, likely from the Dhamna River, a tributary of the Krishna River, are indeed conspecific with *O. vigorsii*. The de novo sequence of *O. cotio* from Maharashtra State is placed in a distinct cluster, previously identified as *O. cotio* in an earlier study, and comprises conspecific sequences from the Brahmaputra and Meghna Rivers [20]. Rahman et al. [20] demonstrated that COI sequences identified as *O. cotio* from the Narmada River Basin, used in studies by Khedkar et al. [10] and Singh et al. [39], exhibited greater genetic distance from *O. cotio* from the Barak and Brahmaputra River Basins. We identified *O. peninsularis* from the Godavari River Basin, maintaining a 5.4% K2P genetic divergence with *O. cotio* from the Barak and Brahmaputra River Basins. The de novo sequence of *O. peninsularis* formed a distinct cluster with database sequences (KF550101 to KF550103) with pairwise genetic distances ranging from 0.0 to 0.62% and maintaining 5.28 to 5.68% genetic distances within the cluster of *O. cotio*. Sequences (KF550101 to KF550103) previously misidentified as *O. cotio* by Khedkar et al. [10] and Singh et al. [39] aligned with one of the subclades of Clade A, as referred to in Rahman et al. [20]. Similarly, the de novo sequence of *O. neilli* formed a cohesive cluster with one database sequence of *Osteobrama* sp. sampled from Kolhapur, Maharashtra, maintaining only a 0.8% genetic divergence within the group.

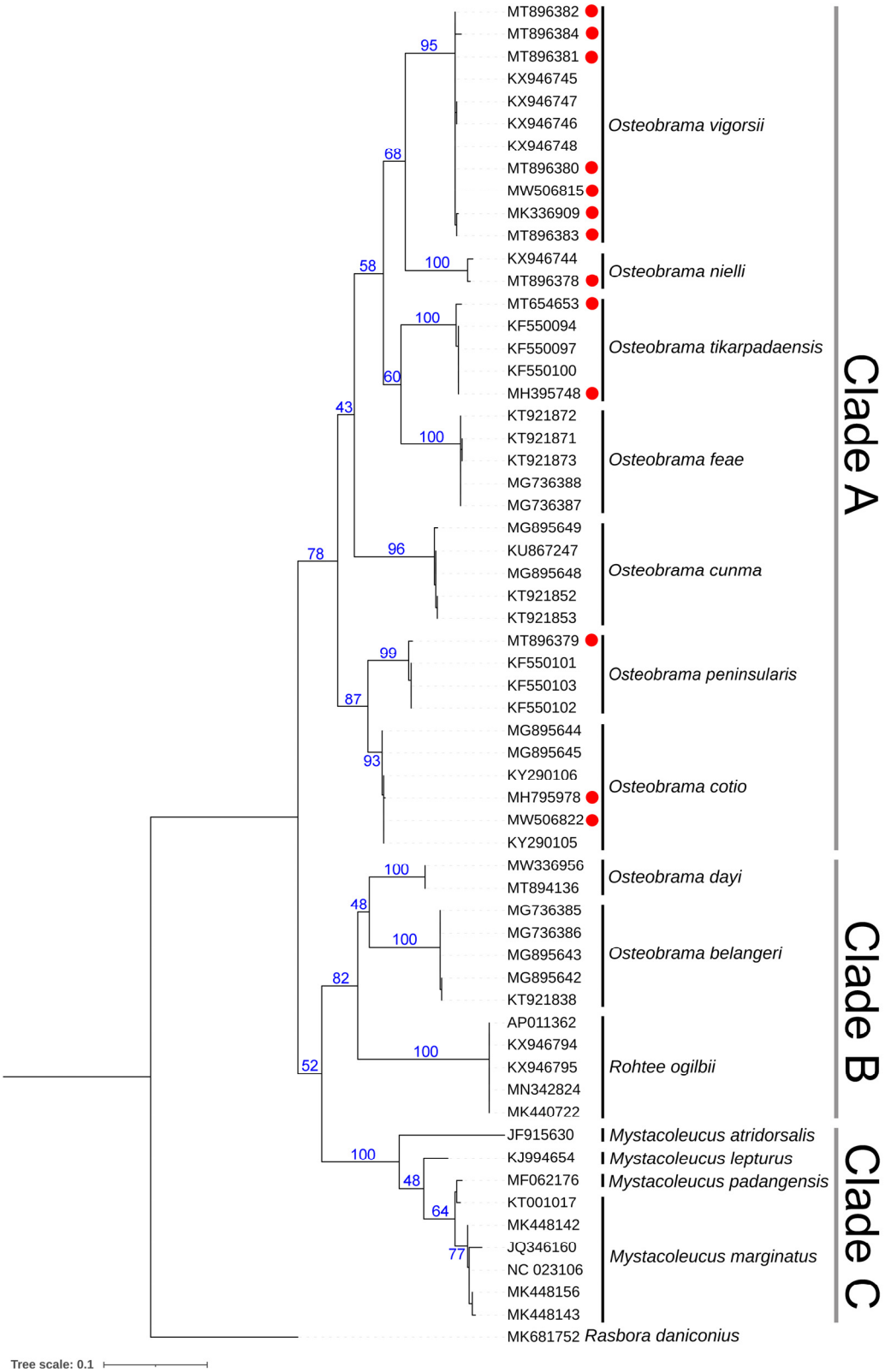

Figure 4. Maximum likelihood phylogeny of *Osteobrama* congeners, utilizing mtCOI data, distinctly separating *O. vigorsii* and *O. tikarpadaensis*. The sequences generated in this study are highlighted with red dots, and the species names with their corresponding GenBank accession numbers are indicated on the tree. Bootstrap support values are indicated at each node in blue font.

O. vigorsii from the Krishna River exhibits characteristics that are distinct from its congeners, as indicated by a K2P genetic divergences ranging from 9.31% to 17.50% in the partial COI gene sequence (Table 4). Its lowest genetic divergence (9.31%) was observed with *O. neilli*, while the highest (17.50%) was with *O. belangeri*. The sequences of *O. vigorsii* form a well-defined cluster, which also incorporated four database sequences with locality information in India, specifically Maharashtra, Kolhapur, the Northern Western Ghats of Kolhapur, and Gavashi, likely from the Dhamana River, a tributary of the Krishna River, situated at coordinates 16.605 N 73.987 E. Among the generated sequences of *Osteobrama*, two specimens from the Godavari River formed a cohesive cluster with certain database sequences identified as *O. vigorsii* but lacking locality information. These specimens maintained a significant interspecies genetic distance (10.64 to 12.35%) from sequences of *O. vigorsii* from the Krishna River Basin. Notably, the specimens from the Godavari River, characterized by having two pairs of barbels, represented a distinct species identified as *O. tikarpadaensis* based on morphological traits (Figure 2A,B).

Table 4. The estimated K2P genetic distance among the respective *Osteobrama* congeners.

Species	Genetic Distance (K2P)								
	Between Species (%)								Within Species (%)
O. cotio									0.2
O. peninsularis	5.28–5.68								0.3
O. cunma	10.64–11.23	10.46–11.25							0.3
O. feae	12.23–13.23	12.58–13.88	13.34–14.94						0.1
O. tikarpadaensis	12.54–13.43	13.90–15.17	13.33–14.33	9.25–9.77					0.3
O. neilli	12.14–12.75	12.50–12.96	11.91–12.58	11.48–12.23	11.53–11.80				0.8
O. vigorsii	10.95–11.95	12.91–14.47	13.88–15.31	10.96–11.97	10.64–12.35	9.31–10.26			0.3
O. belangeri	13.50–14.37	15.22–16.16	15.33–16.24	18.92–20.08	16.48–17.73	16.98–17.60	15.32–17.50		0.1
O. dayi	16.2–16.5	16.7–16.9	15.5–15.8	17.6–17.8	16.5–17.3	16.3–16.5	17.5–18.5	11.1–11.4	0.0

The current phylogenetic analysis robustly distinguished the two targeted species, *O. vigorsii* and *O. tikarpadaensis*, with high bootstrap support. However, among the congeners, the phylogenetic analysis revealed inconsistent clustering in certain instances. Differently named sequences occasionally exhibited conspecific clustering, as discussed in the preceding section. Although the genus *Rohtee* is presently regarded as monotypic [18,23], *Rohtee ogilbii* Sykes 1839 exhibited a cohesive clustering with *O. dayi* and *O. belangeri* in the current phylogeny (Clade B), irrespective of the presence or absence of barrels. These three species in Clade B shared similarities as deep-bodied and large-growing, surpassing the sizes of any species in Clade A. Both Clade A and Clade B species exhibited similarities in possessing a long anal fin with more than 11 branched anal fin rays, distinguishing them from species in *Mystacoleucus* (Clade C) due to the length of the anal fin. *R. ogilbii*, while showing similarity to *Mystacoleucus* species through the presence of a procumbent pre-dorsal spine, formed a separate clade with a mean genetic divergence of 17.3%. The procumbent pre-dorsal spine in *R. ogilbii* is reduced and somewhat concealed by scales compared to other *Mystacoleucus* species. However, additional sampling with multiple gene markers would be necessary to assess the potential merging of the two genera, *Rohtee* and *Osteobrama*.

Taxonomic investigations involving an ample number of specimens from diverse taxonomic lineages, along with their DNA data, have proven effective in illuminating the diversity and phylogeographic structure associated with biogeography [40,41]. The results from this comprehensive research indicate that *O. vigorsii* may have a limited distribution primarily within the Krishna River system in southern India, whereas *O. tikarpadaensis* might potentially have a larger range from the Mahanadi River in Central India to the Godavari River in southern India (Figure 5). Further, considering the present phylogeny and genetic distances among *Osteobrama* congeners, this study underscores the need for additional

genetic data from various riverine systems in the Indian subcontinent to unravel the true species diversity of these cyprinids, thereby informing future conservation implications.

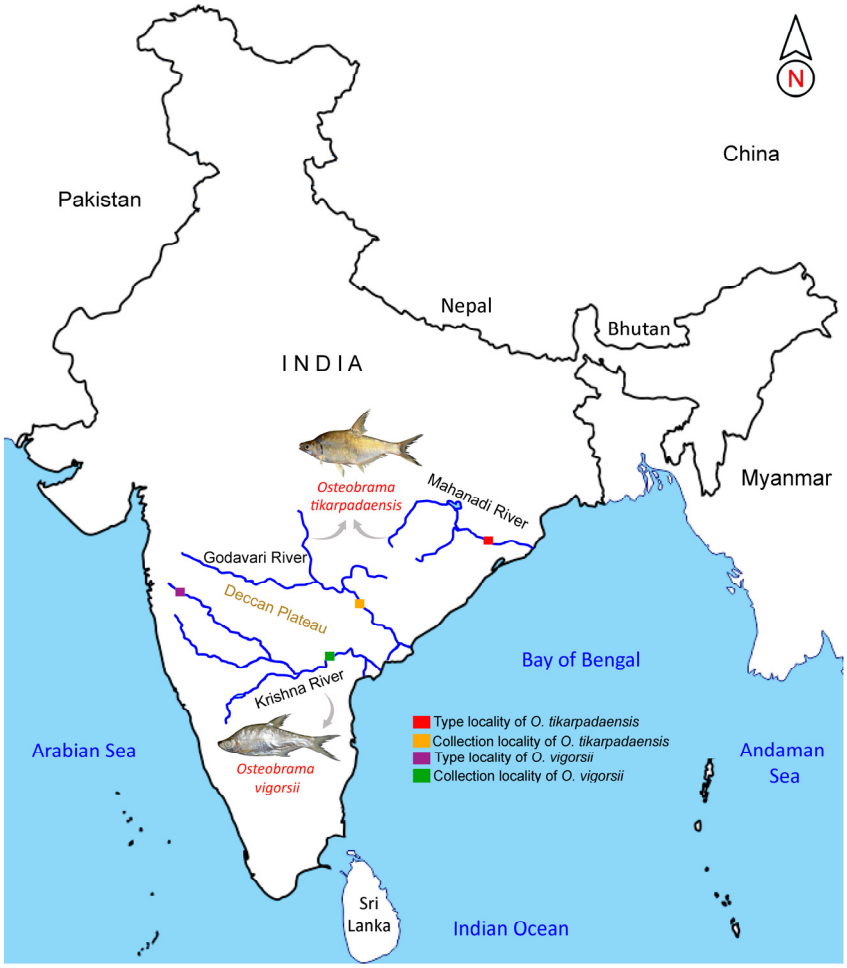

Figure 5. The true distributions of *O. vigorsii* and *O. tikarpadaensis* across diverse riverine systems in India.

3.4. Revised Key to Species of the Genus Osteobrama

1. Barbels absent	2.
- Barbels present	5.
2. Lateral line scales 42–63, pre-dorsal scales 21–30	3.
- Lateral line scales 71–76, pre-dorsal scales 30–32	*O. belangeri.*
3. Branched pectoral fin rays 14–15, lateral line scales 55–63	4.
- Branched pectoral fin rays 12, lateral line scales 42–53	*O. cunma.*
4. Lateral-line scales 55–60	*O. peninsularis.*
- Lateral-line scales 62–63	*O. cotio.*
5. Both rostral and maxillary barbels present	6.
- Only maxillary barbels present, branched anal fin rays 16–18, lateral line scales 68–70	*O. dayi.*
6. Barbels prominent	7.
- Barbels minute	9.
7. Branched anal fin rays 11–18	8.
- Branched anal fin rays 22–27, pre-dorsal scales 34–38, branched pectoral fin rays 14	*O. feae.*
8. Pre-dorsal scales 15, lateral line scales 44, branched anal fin ray 11	*O. bakeri.*
- Pre-dorsal scales 19–22, lateral line scales 52–57, branched anal fin rays 16–18	*O. nielli.*
9. Branched anal fin rays 25–27, branched pectoral fin rays 15–16, presence of oblique black streak on the body immediately posterior to the operculum, lateral line scales 59–71	*O. tikarpadaensis.*
- Branched anal fin rays 21–23, branched pectoral fin rays 13–14, lateral line scales 74–84, no oblique black streak on the body	*O. vigorsii.*

4. Conclusions

Prior to this investigation, the systematic and phylogenetic relationships between two *Osteobrama* species, *O. vigorsii* and *O. tikarpadaensis*, presented challenges to ichthyologists, causing confusion. Proposed amendments to the morphological characters of

both *O. vigorsii* and *O. tikarpadaensis*, accompanied by revised taxonomic keys for distinguishing *Osteobrama* congeners, aim to address these challenges. Although the urohyal bone structure offers insights into these two *Osteobrama* species, vertebra and rib counts are expected to provide more informative data for future investigations. The inter-species genetic divergence and maximum likelihood phylogeny distinctly differentiate *O. vigorsii* and *O. tikarpadaensis*. The study's findings indicate that *O. vigorsii* may have a restricted distribution in the Krishna River system in southern India, while *O. tikarpadaensis* could potentially extend from the Mahanadi River in central India to the Godavari River in southern India. The genetic diversity information obtained from various riverine systems for *Osteobrama* species will be pivotal in guiding aquaculture practices and formulating effective conservation action plans. A similar integrated approach with morphological and molecular data provides a resource for future investigations of cyprinids in India and neighboring regions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes9030087/s1, Figure S1: (A) *Osteobrama cotio*, reproduced from Hamilton (1822) plate 207; (B) *Osteobrama neilli*, FBRC/ZSI/F/3548, 68.0 mm SL; India: Telangana, Nagarkurnool District, Krishna River: near Somasila; (C) *Osteobrama peninsularis*, FBRC/ZSI/F/3549, India; Telangana, Wyra lake, Khamam District; (D) *Osteobrama dayi*, FBRC-ZSI-F 3795, India: Telangana, Godavari River. Photo credit @Boni Amin Laskar; Figure S2: Bayesian phylogeny of *Osteobrama* congeners distinctly separating *O. vigorsii* and *O. tikarpadaensis*. The sequences generated in this study are highlighted with red dots, and the species names with their corresponding GenBank accession numbers are indicated on the tree. Posterior probability values are indicated at each node.

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Institutional Review Board Statement: Given that the specimens examined in this article are edible freshwater fishes typically captured from unprotected water bodies, the fish specimens are exempt from any provisions of animal ethics. Consequently, the authors declare that the study does not involve any act of animal ethics.

Informed Consent Statement: Not applicable.

Data Availability Statement: The DNA sequence data that support the findings of this study are available in NCBI GenBank (https://www.ncbi.nlm.nih.gov/nuccore) and BOLD systems (https://www.boldsystems.org/), under the accession numbers and BOLD-IDs presented in Table 1.

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