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Original Article

Unveiling the identity of three *Parapsilorhynchus* species from the Eastern Ghats: an integrative taxonomic approach

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Abstract
The identities of *Parapsilorhynchus odishaensis*, Mahanadhi minnow *P. swaini*, and *P. alluriensis* have been elucidated through a comprehensive integration of morphological and molecular data, specifically utilizing mitochondrial cytochrome oxidase subunit 1 (COI) sequences. Specimens collected from the type localities of *P. swaini* and *P. odishaensis*, closely resembling their initial descriptions, underwent molecular analysis and were compared to the holotype sequence of *P. alluriensis*. The genetic distances (K2P) observed among these three species ranged from 0.0% to 2.1%. Notably, the K2P distance between *P. swaini* and *P. odishaensis* ranged from 0.2% to 0.3%, while between *P. odishaensis* and *P. alluriensis* (holotype) is 1%, and between *P. swaini* and *P. alluriensis* (holotype), there was a 0.7% difference. The intraspecific variations among the *P. alluriensis* sequences, uploaded from the nearby area of the type locality are 0.0 to 0.8%. Despite minor variations in morphological traits such as snout tubercles, anal fin black bar, and inter-orbital space, molecular analysis revealed no significant genetic differentiation among these species. These morphological variations are likely a result of habitat influences. Considering the overlapping morphological features supported by ML tree and ASAP analysis, *P. odishaensis*, *P. alluriensis*, and *P. swaini* (described from the Eastern Ghats) are suggested to be synonymous. As *P. odishaensis* was the first species described, the other two should be regarded as junior synonyms of *P. odishaensis*.

Keywords: molecular analysis; morphology; *Parapsilorhynchus alluriensis*; *P. odishaensis*; *P. swaini*; synonyms

1 | INTRODUCTION

The mountain carps of the genus *Psilorhynchus* McClelland, 1838 are currently classified under the family Psilorhynchidae, primarily due to their distinct morphological features such as a reduced swim bladder, fleshy lips, narrow gill openings, horizontally oriented pectoral fins, and absence of barbels (Nelson *et al*. 2016). However, Annandale (1919) reported a species from the Western Ghats, *Psilorhynchus tentaculatus*, which possessed a pair of rostral barbels. Subsequently, Hora (1921) established the genus *Parapsilorhynchus*, differentiating it from *Psilorhynchus* based on the presence of barbels, a larger

swim bladder, and a fringed labial fold, and designated *P. tentaculatus* as its type species. The genus *Parapsilorhynchus* (Cyprinidae: Labeoninae) is characterized by several features, such as small scales, two obtuse rostral barbels originating from the snout, an inferior mouth with an upper lip fringed along its edges, numerous minute tubercles dotting the snout, a distinct lower lip with an underdeveloped callous pad at its posterior boundary, dorsal fin insertion anterior to the pelvic-fin origin, and two or more simple pectoral fin rays (Jayaram 2010).

Currently, this genus comprises seven distinct species: Khandalla minnow *Parapsilorhynchus tentaculatus* (Annandale 1919); Ratnagiri minnow *Parapsilorhynchus discophorus* Hora 1921; Deolali minnow *Parapsilorhynchus prateri* Hora and Misra, 1938; *Parapsilorhynchus elongatus* Singh 1994; *Parapsilorhynchus odishaensis* Baliarsingh, Kosygin and Swain 2017; Mahanadhi minnow *Parapsilorhynchus swaini* Baliarsingh and Kosygin 2017; and *Parapsilorhynchus alluriensis* Jadhav, Karuthapandi, Chandra, Jaiswal, Dinesh and Narahari 2020. Of these, only three species, viz., *P. odishaensis*, *P. swaini*, and *P. alluriensis* have been described from the Eastern Ghats (Baliarsingh *et al.* 2017; Jadhav *et al*. 2020). *Parapsilorhynchus odishaensis* was described from Mahendra Tanaya River (Bansadhara riverine system) and Rushikulya Riverine system (Paratype location), whereas, *P. swaini* described from Harishankar (Mahanadi riverine system) without molecular characterization. *Parapsilorhynchus alluriensis*, described from Dharamattam water fall in the Alluri Forest of Visakhapatnam, Andhra Pradesh was accompanied by a COI gene sequence (Jadhav *et al*. 2020). These three species primarily differ in the nature of tubercles on the snout, interorbital space, presence or absence of a black bar on the anal fin, and their body depth (Baliarsingh and Kosygin 2017; Baliarsingh *et al.* 2017 Jadhav *et al*. 2020). All these three species were described from different riverine systems along the Eastern Ghats.

As part of an effort to explore the lotic ichthyofauna of the Harishankar Hill Stream, Bolangir District, Odisha, under a research project, the first author (RKP) initially identified a fish specimen belonging to the genus *Parapsilorhynchus*. Subsequently, eight additional specimens of *Parapsilorhynchus* were collected from the same locality. Of these, five specimens unambiguously conformed to the diagnostic features of *P. swaini*, as Harishankar Hill Stream represents the type locality of the species. However, the remaining four specimens exhibited diagnostic characters consistent with the recently described *P. alluriensis*, notably the absence of tubercles on the snout and the absence of a black bar on the anal fin. The presence of overlapping morphological characters, coupled with the lack of molecular data, resulted in taxonomic ambiguity, necessitating molecular investigation.

In the course of further sampling, the authors collected eight specimens of *Parapsilorhynchus* from Baghua Nala, Ganjam District, Odisha—the type locality of *P. odishaensis*—and six specimens from the Mahendra Tanaya Stream, Ganjam District, Odisha. Specimens from Baghua Nala clearly exhibited diagnostic features of *P. odishaensis*, including well-developed and prominent tubercles, whereas specimens from the Mahendra Tanaya Stream possessed a nearly smooth snout with poorly developed tubercles, closely resembling *P. alluriensis*. DNA barcode sequences (cytochrome c oxidase subunit I, COI) of *P. odishaensis* and *P. swaini* were generated for the first time and compared with the available COI sequence of the *P. alluriensis* holotype. The present study integrates molecular evidence with detailed morphometric analyses to evaluate the taxonomic validity and distinctness of these three species.

2 | METHODOLOGY

2.1 Sample collection

A total of 23 specimens of *Parapsilorhynchus* fishes were collected from 03 different localities (09 specimens from Harishankar hill stream, Bolangir, Odisha during April, 2023; 08 specimens from Baghua Nala, Ganjam, Odisha in July, 2023 and 06 specimens from Mahendra Tanaya stream, Mahendragiri, Gajapati, Odisha in January, 2023) (Table 1).

TABLE 1 Coordinates of collection localities and generated sequences details.

Topotypes	Type Locality	Coordinates	NCBI accession no.
P. swaini	Harishankar hill stream	20°51′16″N 82°51′42″E	OR453529 (632 bps) OR453530 (649 bps)
P. odishaensis	Baghua Nala	19°29′09″N 84°23′31″E	OR458581 (630 bps) OR458596 (626 bps) OR458573 (633 bps) OR459627 (549 bps)
P. alluriensis	Mahendra Tanaya stream	19°00′53″N 84°22′17″E	OQ455716 (590 bps) OQ433919 (620 bps)

The detailed map of the sites of the collection along with the type locality of *P. alluriensis* is provided in Figure 1. Fresh tissue samples from the specimens of above mentioned the localities were kept in a –20°C deep freez-

er (24 hours) for molecular studies immediately after bringing them into the laboratory. Subsequently, the specimens were preserved in 10% formalin.

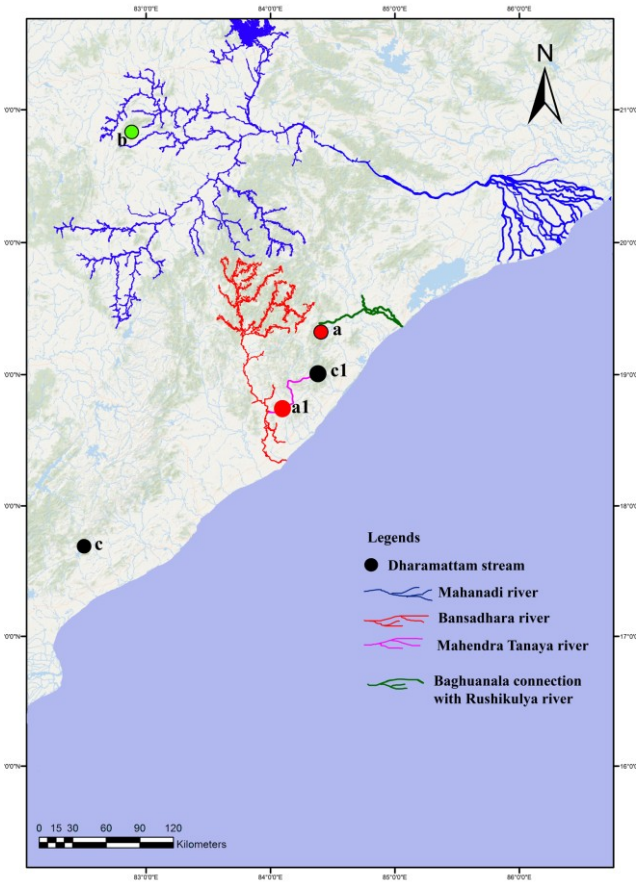

FIGURE 1 Distribution map of *Parapsilorhynchus odishaensis* (a. Baghua Nala - Paratype collection locality of *P. odishaensis* and present study collection; a1. Mahendra Tanaya stream - Holotype locality of *P. odishaensis*; b. Harishankar Hill Stream- Holotype, Paratype and present study's collection locality of specimens resembling *P. swaini*; c. Dharamattam stream - *P. alluriensis* holotype and paratype collection locality; c1. Mahendra Tanaya stream - Present study's collection locality of specimens resembling *P. alluriensis*).

For this study, the specimens were measured on their left side, with measurements taken up to the nearest 0.1 mm using digital callipers, accompanied by their respective scales. These measurements were then expressed as percentages relative to standard length (SL) and head length (HL). Fin-ray counts, distinguishing between simple and branched fin rays, were conducted utilizing a binocular stereo-zoom microscope (Leica S9i). The morphometric measurements and fin-ray counts adhered to the methodology outlined by Jadhav *et al*. (2020). The terminology used for describing body features and pigmentation patterns follows the guidelines established by Jayaram (2010) and Jadhav *et al*. (2020). The distribution map was constructed using ArcMap version 10.8.

2.2 Genomic DNA extraction, PCR amplification and DNA sequencing

After collecting specimens from various localities, tissue samples weighing 30 mg were obtained from eight specimens. Among these, two were collected from the Harishankar stream, four from the type locality of *P. odishaensis*, and the remaining two from the Mahendra Tanaya River for molecular analysis. Genomic DNA extraction was carried out according to the manufacturer's protocol using the HiPurA® Insect DNA Purification Kit, which serves for both extraction and purification. The concentration of genomic DNA in each sample was quantified using a Qubit 4 Fluorometer, yielding concentrations ranging from 32.6 to 46.4 ng μL^{-1}. Subsequently, the isolated DNA samples were stored at –20°C until further use.

The amplification of the COI gene was conducted using the iGene Labserve PCR machine, with a final reaction volume of 25 µL. This volume comprised 9 µL of Nuclease-free water, 12.5 µL of 2X Hi G9 Taq PCR Master Mix, 0.5 µL of the forward primer Fish F1 (5'TCAACCAACCACAAAGACATTGGCAC-3'), 0.5 µL of the reverse primer Fish R1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') (Ward *et al*. 2005), and 2.5 µL of the extracted DNA template. The thermo-cyclic conditions for PCR included an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min, followed by indefinite hold at 4°C (Mohapatra *et al*. 2022).

The eight PCR products obtained were subjected to sequencing through the Sanger Sequencing method. Sequence assembly was conducted using BioEdit version 7.2. Subsequently, the COI gene sequences were submitted to NCBI (Table 1). Multiple alignments of 23 similar COI gene sequences retrieved from the NCBI database were performed utilizing ClustalW multiple alignments (Thompson *et al*. 2002). Nucleotide distances and maximum likelihood (ML) tree analysis were conducted using the best-fit model HKY+G (Hasegawa *et al.* 1985) with MEGA 11 software (Kumar *et al*. 2018). Bootstrap analysis, comprising 1000 replications, was executed to comprehend internal node support.

3 | RESULTS

3.1 Morphological comparison

Morphometric and meristic data are provided in Table 2, while visual representations of the specimens are presented in Figure 2 and 3. A detailed morphological examination, coupled with morphometric measurements, revealed striking similarities between the specimens from Baghua Nala, Ganjam, Odisha (the type locality of *P. odishaensis*), and *P. odishaensis* itself. These resemblances

encompass key characteristics such as body depth (17.36–22.91% SL vs. 19.6–25.0% SL), inter-orbital space (50.63–57% HL vs. 53.3–64.3% HL), lateral line scales (33–34 vs. 33–35), as well as the presence of horny tubercles on the snout and the absence of a black bar on the anal fin. Likewise, the morphometric measurements of all nine specimens collected from Harishankar Hill Stream (the type locality of *P. swaini*) go with *P. swaini*, including body depth (16.36–18.7% SL vs.16.9–18.7% SL), inter-orbital space (46.0–50.0% HL vs. 46.6–50.0% HL), lateral line scales (33–34 vs. 34). However, four specimens exhibited morphological features of *P. alluriensis* such as smooth snout and absence of black bar on anal fin and five specimens displayed morphological characteristics of *P. swaini* such as weakly developed tubercles along with faint black bar on anal fin.

TABLE 2 Morphometric and meristic data of *Parapsilorhynchus odishaensis*. SL, Standard length; HL, head length; a, Present study.

Morphometric and meristic data	***P. odishaensis*** (Baliarsingh *et al.* 2017)	**Specimens resembling *P. odishaensis* from type locality Baghua Nala, Ganjam, Odisha (*n*=8)[a]**		***P. swaini*** (Baliarsingh and Kosygin 2017)	**Specimens resembling *P. swaini* from type locality Harishankar Hill Stream, Bolangir, Odisha (*n*=9)[a]**		***P. alluriensis*** (Jadhav *et al.* 2020)	**Specimens resembling *P. alluriensis* Mahendra Tanaya Hill Stream, Mahendragiri, Gajapati, Odisha (*n*=6)[a]**		**TOTAL RANGE**
		Range	**Mean ±SD**		**Range**	**Mean ±SD**		**Range**	**Mean ±SD**	
SL (mm)	26.0–36.0	22.5–33.5		32–42	24.1–44.3		21.7–35.4	21.5–35		
HL (mm)		5.8–7.7			6.3–9.7			6–9		
	%SL									
Body depth	19.6–25.0	17.36–22.91	19.94 ±2.54	16.9–18.7	16.36–18.7	17.48 ±0.99	17.3–21.7	18.2–21.2	19.3 ±1.17	16.4–22.3
Pre dorsal length	51.5–57.1	44.59–64.80	52.71 ±7.15	50.0–53.5	49.4 – 53.9	51.88 ±1.17	46.2–56.0	48.6–54.9	52.9 ±1.07	44.59–64.80
	%HL									
Head height at occiput	57.2–75.0	50.25–72.60	59.27 ±7.36	40.0–50.0	39.7–50.5	43.74 ±2.63	47.3–72.3	46.7–50	49.2 ±1.30	39.7–72.6
Head width	71.4–94.1	65.65–86.40	73.88 ±8.68	60–68.7	67.5–69.0	68.25 ±0.42	62.7–73.8	64.3–70	66.7 ±2.06	64.3 – 86.4
Eye diameter	18.7–28.6	22.24–36.50	28.75 ±5.34	25–31.2	24.29–28.57	25.96 ±1.32	28.4–44.1	28.6–33.3	31.6 ±2.06	22.24–28.57
Inter orbital space	53.3–64.3	50.63–57.00	53.07 ±2.53	46.6–50.0	46.0–50.0	47.88 ±1.67	33.9–43.2	39.8–47.4	42.2 ±3.31	39.8–57.00
Mouth width	23.5–28.6	26.88–37.80	34.02 ±3.59	26–31.2	27.0–32.0	30.13 ±1.88	27.3–32.8	28.7–35.6	32.9 ±2.88	26.88–37.8
Lateral line scale	33–35	33–34		33–34	34		33–34	32–34		32–35
Pectoral fin rays	iii–iv, 9–11	iii,12		iii,10 i–iii,11, i	iii–iv, 11		iii,11	iii–iv, 10–11		iii–iv, 9–12
Horny tubercles on snout	Well developed	Well developed		Poorly developed	Poorly developed (n=5); absent/poorly developed (*n*=4)		Poorly developed	Poorly developed		
Visibility of eye from ventral side of head	Visible	Visible		Visible	Visible		Visible	Visible		
Pectoral fin length	Equal or longer than head length	Equal or longer than head length		Longer than head length	Longer than head length		Shorter than head length	Shorter than head length		
Callous pad	Poorly developed	Poorly developed		Poorly developed	Poorly developed		Poorly developed	Poorly developed		
Black bar on anal fin	Absent	Absent		Present	Present(n=05); Absent (n=04)		Absent	Absent		

The fish specimens obtained from Mahendra Tanaya Hill Stream, Mahendragiri, Gajapati, Odisha, exhibited notable similarities to *P. alluriensis*, as evidenced by shared characteristics including body depth (18.2–21.2%

SL vs. 17.3–21.7% SL), inter-orbital space (35.7–43.9% HL vs. 33.9–43.2% HL), lateral line scale count, poorly developed tubercles on the snout, and the absence of a black bar on the anal fin.

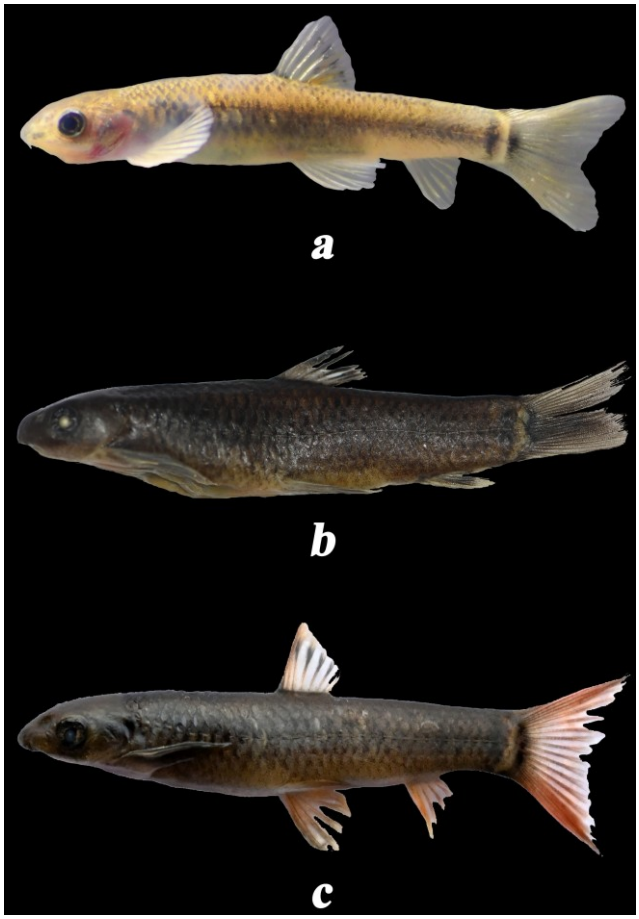

FIGURE 2 *Parapsilorhynchus odishaensis* (a. from *P. odishaensis* type locality; b. from *P. swaini* type locality; c. from Mahendra Tanaya River).

3.2 Molecular analysis

The dataset comprises a total of 31 COI gene sequences representing the *Parapsilorhynchus* genus, with an additional outgroup sequence from the sister genus *Psilorhynchus* included in the ML tree (Figure 4). Of these sequences, eight were generated for this study. Among these, two sequences originated from specimens collected from Harishankar stream, exhibiting poorly developed tubercles and featuring a black bar on the anal fin, aligning morphologically with *P. swaini*. Four sequences were derived from specimens collected from Baghua Nala (type locality of *P. odishaensis*), displaying well-developed tubercles without a black bar on the anal fin, consistent with the morphological traits of *P. odishaensis*. Lastly, two sequences were obtained from specimens collected from Mahendra Tanaya stream, resembling *P. alluriensis*. The remaining sequences were sourced from NCBI and BOLD for comparative analysis. These sequences were juxtaposed with the holotype sequence of *P. alluriensis*. Notably, the COI gene sequences of the previously described species, namely *P. swaini*, *P. odishaensis*, and *P. alluriensis*, exhibited similarities with nucleotide differences ranging from 0.0% to 2.1%. Specifically, sequences of *P. odishaensis* diverged from *P. swaini* by a K2P distance of 0.2 – 0.3% and from *P. alluriensis* by 0.5 – 1.5%. Sequences of *P. swaini* varied from *P. alluriensis* by a K2P distance of 0.5 – 1.2% (Table S1).

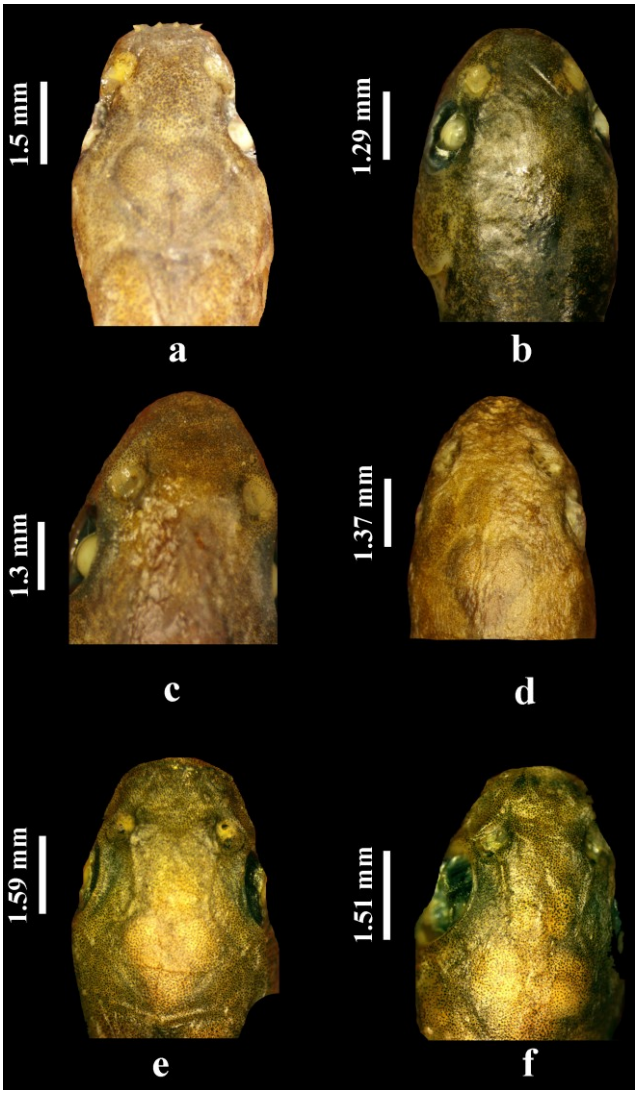

Figure 3 Head of *Parapsilorhynchus odishaensis* (a, b. from type locality of *P. odishaensis*; c, d. from type locality of *P. swaini*; e, f. from Mahendra Tanaya River).

The ASAP species delimitation analysis revealed clustering of *P. alluriensis*, *P. odishaensis*, and *P. swaini* together in a single group with a threshold value of less than 0.1, whereas *P. prateri* formed a distinct group. Thus, genetically, it is evident that *P. alluriensis*, *P. odishaensis*, and *P. swaini* constitute the same species (Figure 5).

Molecular analysis shows that *P. odishaensis*, *P. swaini*, and *P. alluriensis* have nucleotide distances rang-

ing from 0–2.1%. The highest intraspecific distance for *P. alluriensis* from Andhra Pradesh is 0.8%. The nucleotide distance between *P. swaini* and *P. alluriensis* from Andhra Pradesh (including both morphs: no tubercles and poorly developed tubercles) ranges from 0.7–1.2%, with the lower end matching the highest intraspecific distance of *P. alluriensis*. Therefore, *P. alluriensis* might be the same as *P. swaini*. The distance between *P. swaini* and *P. odishaensis* (with both morphs: poorly developed and well-developed tubercles) is only 0.2–0.3%, suggesting that *P. swaini* could be *P. odishaensis*. Additionally, the nucleotide distance between *P. odishaensis* and *P. alluriensis* from Andhra Pradesh is 0.9–1.5%, which is also minimal. Overall, *P. swaini* and *P. alluriensis* may be considered as *P. odishaensis*.

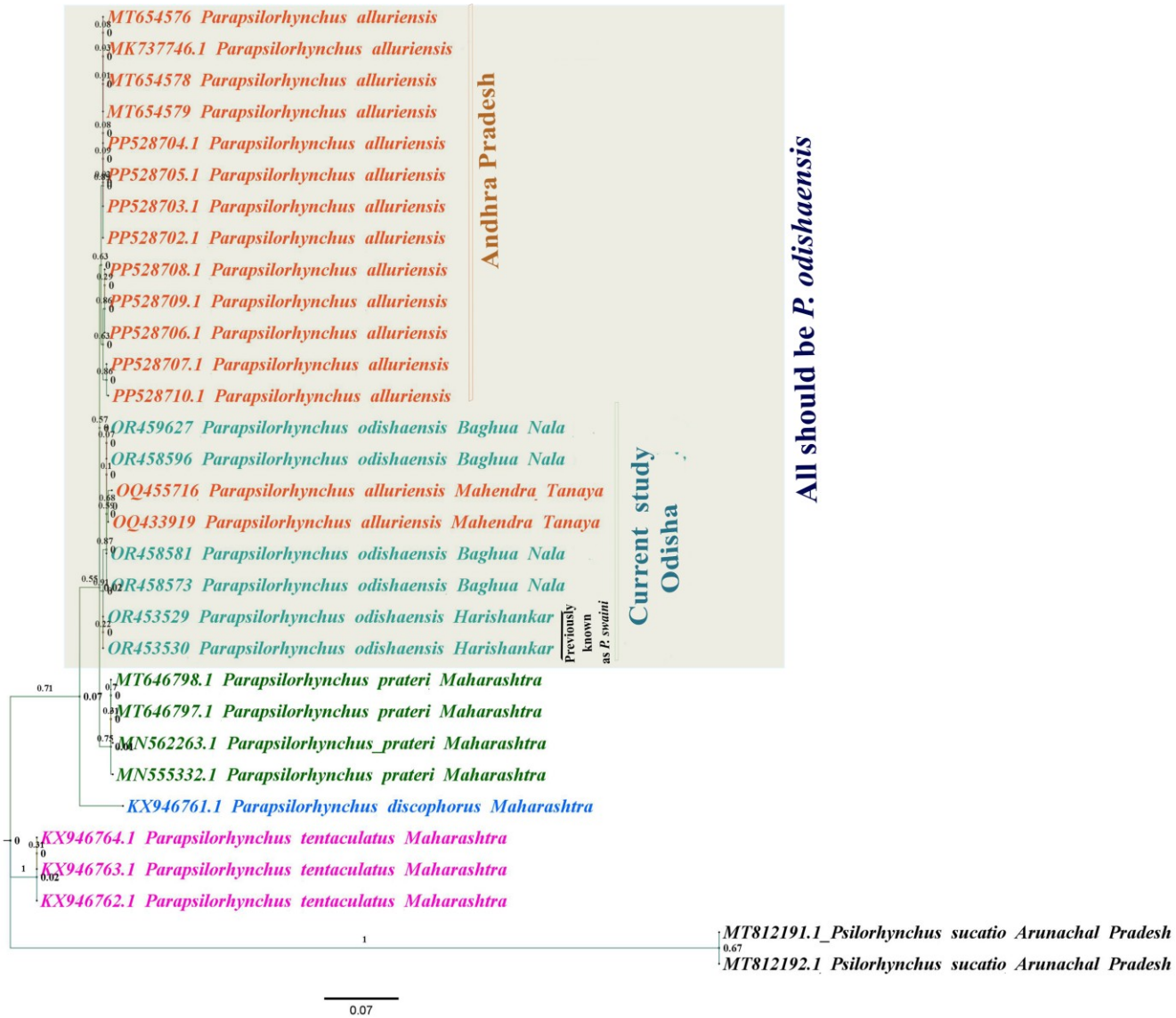

FIGURE 4 Maximum likelihood tree showing the phylogenetic relationships between currently studied *Parapsilorhynchus odishaensis* (Collected from the type locality of *P. odishaensis* and *P. swaini*) with previously known species of *P. alluriensis* (holotype).

4 | DISCUSSION

Most of the specimens from the type locality of *P. odishaensis* showed poorly developed tubercles, similar to those of *P. swaini*. This indicates that tubercles may not be a reliable taxonomic character, as molecular analysis revealed no significant genetic differences. Additionally, some specimens from the type locality of *P. swaini* displayed characteristics of *P. alluriensis*, such as the absence of tubercles, with no significant genetic variation between the two varieties.

The initial description of *P. alluriensis* by Jadhav *et al*. (2020) raised clarity concerns, as it primarily relied on the ratio of inter-orbital space to head length and the presence or absence of a black bar on the anal fin to dif-

ferentiate *P. alluriensis* from *P. swaini*. However, molecular analysis using COI gene barcoding revealed no significant differences between these two variants.

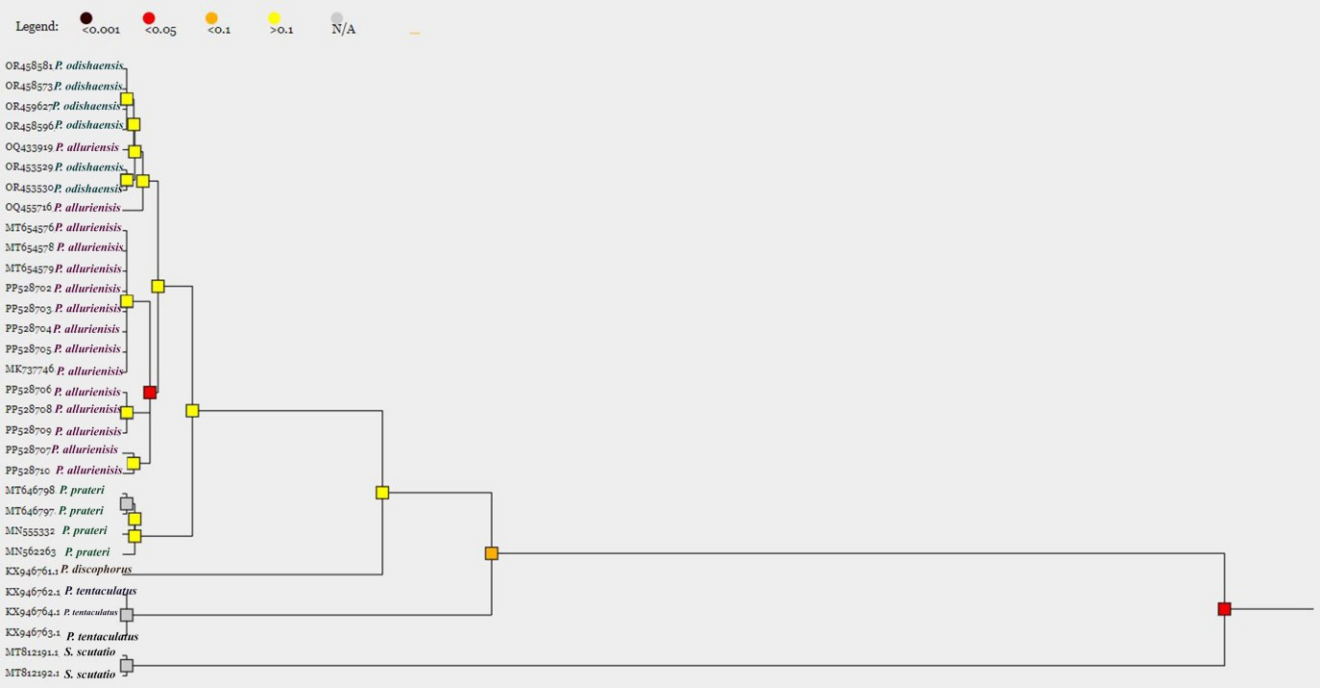

FIGURE 5 Species delimitation analysis of all the species included in the phylogenetic analysis.

Additionally, *P. odishaensis* can be distinguished from both *P. alluriensis* and *P. swaini* by the presence of well-developed horny tubercles on the snout, contrasting with the poorly developed tubercles found in the other two species (Jadhav *et al*. 2020). Inter-orbital width, expressed as a percentage of head length, also served as a key distinguishing feature: 53.3 – 64.3% HL in *P. odishaensis*, 46.6 – 50.0% HL in *P. swaini*, and 33.9 – 43.2% HL in *P. alluriensis* (Jadhav *et al*. 2020). Analysis of COI sequences revealed insignificant genetic distances among *P. alluriensis*, *P. odishaensis*, *P. swaini*, and *P. prateri* species, with nucleotide distances ranging from 0% to 2.1% for the former three and from 1.4% to 2.3% for *P. prateri*. Despite *P. prateri* exhibiting less than 2.5% nucleotide distance from the aforementioned three species, the authors suggest synonymizing *P. alluriensis* and *P. swaini* with *P. odishaensis* due to their clustering in the same clade in phylogenetic and species delimitation analyses, while *P. prateri* forms a distinct clade with distinctly larger branch length than *P. alluriensis* and *P. odishaensis*. Moreover, *P. prateri* displays distinct morphological characteristics, including scale morphology, lateral line scale counts (43 – 47 vs. 32 – 35), and transverse scale rows (12 vs. 9 – 10) (Jayaram 2010). Although COI gene sequences of *P. alluriensis* from its type locality form a separate clade in the ML tree and ASAP analysis, those collected from Mahendra Tanaya streams of Odisha cluster with *P. odishaensis*. Specimens resembling *P. alluriensis* from Mahendra Tanaya streams lack prominent tubercles and morphologically match the type description of *P. alluriensis*, thus supporting the synonymization of *P. alluriensis* with *P. odishaensis* in this study. Despite *P. prateri* not exhibiting significant genetic distance, it is treated as a separate species based on morphological and molecular characteristics.

Hence, considering the overlapping morphological features supported by molecular evidence from both the ML tree and ASAP analysis, it is reasonable to conclude that *P. odishaensis*, *P. alluriensis*, and *P. swaini* constitute a single species. Conversely, *P. prateri* displays distinct morphological dissimilarities, indicating that it should not be merged within the same group.

Furthermore, the variability observed in interorbital space appeared to correlate with species size and preservation condition, casting doubt on its reliability as a defining characteristic for species identification. The discrepancies noted in the original descriptions likely stem from limited data sets. Similarly, the structure of tubercles seemed to be a consequence of environmental influences, transitioning from poorly developed to well-developed under different conditions (Hu *et al*. 2024). Consequently, it is logical to consider the three previously delineated species described from the streams of Eastern Ghats as a single entity. Hence, *P. alluriensis* Jadhav *et al*. 2020 and *P. swaini* Baliarsingh and Kosygin, 2017 (published on 2018-03-14, date sourced from the journal's website) should be recognized as junior synonyms of *P. odishaensis* Baliarsingh *et al*. 2017 (published on 2017-09-

08).

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTION

RKP, TT: Specimen collection, photography, identification, manuscript preparation; SA: Molecular analysis, manuscript preparation; SS, SKM: Specimen collection and photography; LS, JKS, SSM, AM: species identification confirmation, overall designing and critical analysis of manuscript.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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