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DNA Barcoding Reveals the Genetic Identity of Licorice Gourami *Parosphromenus deissneri*, an Endangered Species Threatened by Tin Mining in Bangka

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ABSTRACT

Parosphromenus deissneri, an endangered ornamental freshwater fish endemic to Bangka Island, Indonesia, has experienced a drastic decline in population due to habitat degradation, particularly from tin mining activities. Accurate species identification is crucial in supporting conservation efforts, especially in taxonomic groups that are complex or morphologically similar (cryptic). This study aimed to characterize *P. deissneri* molecularly using the mitochondrial *cytochrome c oxidase subunit 1* (*co1*) gene. A *co1* fragment of approximately 650 base pairs was successfully amplified and sequenced. BLAST and BOLD analyses revealed high similarity (>98%) with the reference sequence of *P. deissneri*. Genetic distance analysis using the Kimura 2-parameter model indicated low intra-species divergence (0.000–0.008), while inter-species distances were significantly higher: 0.183–0.197 with *P. quindecim* and 0.121–0.142 with *P. nagyi*. Phylogenetic reconstruction using the neighbor-joining and maximum likelihood methods yielded similar topologies, with all specimens clustering within the monophyletic clade of *P. deissneri*. These results confirm the validity of *co1*-based DNA barcoding for species identification and provide molecular insights into evolutionary patterns within the genus *Parosphromenus*. Further research is recommended to use additional genetic markers and a broader geographical coverage to evaluate the potential for local population variation and support more precise conservation strategies.

INTRODUCTION

The freshwater region of Southeast Asia is recognized as one of the world's biodiversity hotspots **(Valen *et al.*, 2020; Hasan *et al.*, 2023a; Robin *et al.*, 2023a; Samara *et al.*, 2024)**, with many endemic fish species **(Serdiati *et al.*, 2023)** now facing serious environmental pressures. The Bangka Belitung Islands—particularly Bangka Island—are part of this biologically rich region and harbor high freshwater fish diversity

(Hasan *et al.*, 2023b; Pramono *et al.*, 2025), including species from genera such as *Betta* and *Parosphromenus* **(Valen *et al.*, 2023a)**. Many of these fishes are endemic or restricted to specific habitats, making them highly susceptible to environmental changes **(Valen *et al.*, 2024b)**. This ichthyofaunal richness is largely supported by the presence of unique freshwater ecosystems such as blackwater peat swamps, lowland streams, and swamp forests.

However, this exceptional biodiversity is under serious threat, particularly from widespread environmental degradation caused by tin mining **(Hasan *et al.*, 2023b)**. Bangka Island is historically one of the world's leading tin-producing regions **(Rachman *et al.*, 2019)**, and decades of both legal and illegal tin mining—especially open-pit and river-based extraction—have led to severe ecological consequences **(Hasan *et al.*, 2023c; Kusumah *et al.*, 2023; Syarif *et al.*, 2023a)**. These activities cause habitat fragmentation, sedimentation, water pollution, and hydrological changes, all of which directly affect freshwater ecosystems **(Yusof *et al.*, 2001; Vendrell-Puigmitja *et al.*, 2020)**. As a result, fish populations in affected areas have declined sharply, with several species becoming increasingly rare or even locally extinct.

In addition to habitat degradation, the introduction of invasive species poses a growing threat to native and endemic fish populations in Bangka Belitung **(Valen *et al.*, 2023; Insani *et al.*, 2024; Ramadhanu *et al.*, 2024; Islamy *et al.*, 2025a; Syarif *et al.*, 2025b)**. Non-native species such as *Oreochromis niloticus* (the Nile tilapia) and *Clarias gariepinus* (African catfish), often introduced for aquaculture, have been reported in several freshwater habitats on the island **(Serdiati *et al.*, 2021; Insani *et al.*, 2025)**. These invasive species can outcompete native fishes for food and habitat, disrupt ecological balances, and even prey on smaller endemic species **(Jatayu *et al.*, 2023; Jerikho *et al.*, 2023; Robin *et al.*, 2023; Islamy *et al.*, 2025b)**. Such biotic interactions further increase the vulnerability of localized species already stressed by environmental degradation.

One such threatened endemic species is *Parosphromenus deissneri*, a small member of the family Osphronemidae, commonly known as the licorice gourami **(Shi *et al.*, 2021)**. This species is restricted to Bangka Island and is currently categorized as threatened due to ongoing habitat degradation **(Valen *et al.*, 2024a)**. However, its taxonomic status remains controversial. Morphological similarities among species within the genus *Parosphromenus*, combined with historical misidentification, hinder accurate classification using traditional methods. High levels of intraspecific variation and the presence of cryptic species further complicate identification based solely on morphology.

Molecular techniques such as DNA barcoding—based on a segment of the mitochondrial cytochrome c oxidase subunit I (CO1) gene—have proven effective in resolving taxonomic ambiguities and uncovering hidden genetic diversity **(Hasan *et al.*, 2021; Insani *et al.*, 2022; Syarif *et al.*, 2023a; Maulidya *et al.*, 2024; Valen *et al.*, 2024c)**. This method has been widely adopted in freshwater fish research to delineate

species boundaries where morphological traits are unreliable **(Valen *et al.*, 2023a; Syarif *et al.*, 2025a)**.

This study aimed to genetically identify *Parosphromenus deissneri* from Bangka Island using DNA barcoding. By comparing CO1 gene sequences from field-collected specimens with reference data and conducting phylogenetic analyses **(Austerlitz *et al.*, 2009)**, we sought to verify the taxonomic status of this species and assess its genetic distinctiveness. The results not only clarify systematics within the genus *Parosphromenus* but also highlight the urgent need for conservation measures to protect this unique lineage. Moreover, they emphasize the broader importance of conserving the freshwater fish diversity of Bangka Belitung, which continues to decline under mounting pressures from unsustainable tin mining, habitat loss, and biological invasions.

MATERIALS AND METHODS

1. Study area and sampling Sites

Field sampling was conducted in May 2025 within freshwater stream habitats in Tugang Village, Kelapa Subdistrict, Bangka Regency, Bangka Island, Indonesia. This location represents a characteristic lowland peat swamp ecosystem, which serves as a natural refuge for various ichthyofauna, including the endangered species *Parosphromenus deissneri*. The stream exhibited typical blackwater conditions, characterized by dense canopy coverage and slow-flowing currents, forming specialized microhabitats conducive to the persistence of this species.

A total of five *P. deissneri* individuals were collected using passive fish traps. To preserve the integrity of genetic material for downstream molecular analyses, specimens were immediately fixed in 96% ethanol on-site **(Valen *et al.*, 2022)**. The samples were then transferred to the Basic Biology Laboratory, Faculty of Agriculture, Fisheries, and Marine Sciences, Universitas Bangka Belitung. Prior to molecular examination, standard morphometric and meristic evaluations were conducted to confirm species identification and assess phenotypic variation. Photographic documentation of live specimens was also carried out to support species verification and field records (Fig. 1).

Fig. 1. Live specimen of *Parosphromenus deissneri* (BBIM 005)

2. DNA isolation

DNA extraction was performed using the Geneaid™ DNA Extraction Kit, following the manufacturer's protocol, which includes lysis, binding, washing, and elution steps. Approximately 20– 25mg of tissue was homogenized in 200µL of lysis buffer, followed by the addition of 20µL of Proteinase K. Samples were incubated at 60 °C for 30–45 minutes to ensure complete cell lysis and protein digestion. After centrifugation, the supernatant was mixed with GSB buffer and absolute ethanol, then transferred to a spin column to facilitate DNA binding. The column was subsequently washed with W1 and W2 buffers. Residual ethanol was removed by an additional centrifugation step. Finally, DNA was eluted with 100µL of elution buffer, yielding purified DNA suitable for PCR amplification and sequencing.

DNA amplification

Polymerase chain reaction (PCR) was conducted using primers targeting the mitochondrial cytochrome c oxidase subunit I (*co1*) gene, which is widely used in fish barcoding. Primer stocks were diluted to working concentrations by mixing 10 µL of each primer with 90 µL of double-distilled water (ddH_2O). The primer pair used consisted of FishF2 (5′-TCGACTAATCATAAAGATATCGGCAC-3′) and FishR2 (5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′) **(Ward *et al.*, 2005)**. Each PCR reaction had a total volume of 50µL, including 25µL of PCR master mix, 15µL of nuclease-free water, 5µL of DNA template, and 2.5µL of each primer.

Thermal cycling was conducted using a thermocycler under the following conditions: Initial denaturation at 94°C for 1 minute; 30 cycles of denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute and 30 seconds, and extension at 72°C for 1 minute

(Syarif *et al.*, 2023c). PCR products were then prepared for electrophoresis and subsequent sequencing.

DNA electrophoresis

To evaluate PCR amplification success, electrophoresis was performed using a 1% agarose gel **(Syarif *et al.*, 2023c)**. Each well was loaded with 5µL of PCR product and 5µL of a 100 bp DNA ladder. The gel was run in 1× TAE (Tris–Acetate–EDTA) buffer at a constant voltage of 100 V for 30 minutes. DNA bands were visualized under UV light using a transilluminator. A clear DNA band at approximately 650bp, corresponding to the expected *co1* fragment size, indicated successful amplification.

DNA sequencing and data analysis

PCR products with clear single bands were selected for sequencing. Amplicons were purified and submitted to 1st BASE DNA sequencing services (Selangor, Malaysia) for bidirectional Sanger sequencing using the same primers (FishF2 and FishR2). Chromatogram files were quality-checked, and forward and reverse reads were assembled into consensus sequences using BioEdit software.

Consensus sequences were aligned using ClustalW to ensure correct reading frames and resolve ambiguous base calls. Species identification was performed via BLAST searches against NCBI GenBank and the BOLD (Barcode of Life Data System) database. Sequences showing >98% similarity to reference *Parosphromenus deissneri* entries were considered positively identified.

Phylogenetic analysis was conducted using MEGA X software **(Kumar *et al.*, 2018)**. The neighbor-joining method with Kimura 2-parameter distances was employed to assess genetic relationships among Bangka Island specimens and related taxa. Node support was evaluated using bootstrap analysis with 1,000 replicates to determine the robustness of phylogenetic groupings.

RESULTS

1. DNA barcoding

The *co1* gene sequence (665 bp) of *Parosphromenus deissneri* from Bangka Island was successfully amplified and sequenced, producing a high-quality DNA barcode. This sequence falls within the standard range for fish DNA barcoding and serves as a reliable genetic marker for species identification (Table 1).

Table 1. DNA Barcoding of *Parosphromenus deissneri* Bangka

DNA barcoding of Barcoding of *Parosphromenus deissneri* Bangka
CTGAGCAGGCATAGTTGGAACTGCCCTAAGTCTACTTATTCGAGCAGAACTCAGCCAGCC GGGGGCCCTTCTTGGTAACGATCAGATTTATAATGTGTTAGTCACGGCCCATGCCTTCGT CATAATCTTCTTCATGGTAATGCCCGCCATGATCGGGGGTTTTGGGAACTGACTGGTGCC TTTGATAATCGGGGCCCCAGACATGGCCTTCCCTCGAATAAATAACATGAGCTTTTGACT TCTGCCTCCCTCTTTTCTCCTCCTATTGGCCTCTTCCGGCGTAGAGGCTGGAGCTGGGAC TGGCTGAACCGTATACCCCCCACTTGCCAGTAACCTGGCCCATGCAGGGGCCTCCGTTGA

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CTTAACCATCTTCTCCCTTCACTTAGCCGGCATCTCTTCAATCCTGGGTGCAATTAACTT
CATCACAACCATTATTAACATAAAACCCCCAGCAATTTCCCAATATCAGACCCCCCTATT
CGTGTGGTCTGTAATAATCACCGCTGTCCTTCTTCTTCTATCCCTTCCTGTGCTAGCCGC
AGGCATCACAATGCTCTTGACAGACCGAAACCTCAATACAACCTTTTTTGACCCCGCCGG
CGGGGGGGACCCCATCCTCTATCAGCACCTATTCTGATTCTTCGGTCACCCTGAAGTAAT
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2. Species Identification Based on the *co1* Gene

BLAST analysis showed >99% similarity to reference *P. deissneri* sequences (Table 2), confirming morphological identification.

Table 2. Sequence similarity of *Parosphromenus deissneri.* Bangka

Specimens	Query Coverage (%)	Simlarity GenBank (%)	Species Outcome	Accession Number (GenBank)
Parosphromenus deissneri	100%	0.0	99.53	MW021426.1

3. Genetic distances

The Bangka *P. deissneri* showed minimal genetic distance (0.008) from reference *P. deissneri,* confirming its identity. Higher distances with other species (e.g., *P. quindecim* 0.187) indicate clear interspecific divergence. Close values with *P. opallios* and *P. cf. bintan* suggest phylogenetic relatedness within the group (Table 3).

Tabel 3. Genetic distances of *Parosphromenus deissneri* Bangka

		1	2	3	4	5	6	7
1	*P. deissneri* Bangka							
2	*P. deissneri*	0,008						
3	*P. quindecim*	0,187	0,182					
4	*P. nagyi*	0,141	0,141	0,177				
5	*P. opallios*	0,076	0,071	0,175	0,118			
6	*P. cf. bintan*	0,078	0,076	0,192	0,118	0,072		
7	*P. tweediei*	0,085	0,085	0,207	0,111	0,076	0,044	
8	*P. rubrimontis*	0,089	0,090	0,215	0,119	0,084	0,048	0,016

4. Nucleotide Composition

The nucleotide composition of *P. deissneri* Bangka (T=28%, C=31%, A=21%, G=20%) is identical to the reference *P. deissneri*, supporting species-level consistency. Slight variations among other species reflect interspecific genetic differences, with C and T being the most abundant bases across all taxa (Table 4).

Table 4. Nucleotide composition of *Parosphromenus deissneri* Bangka

	T(U)	C	A	G	Total
P. deissneri Bangka	28	31	21	20	645
P. deissneri	28	31	21	20	645
P. quindecim	29	30	22	19	645
P. nagyi	28	31	21	20	645
P. opallios	30	29	21	20	645
P. cf. bintan	29	30	20	20	645
P. tweediei	29	31	20	21	645
P. rubrimontis	29	31	20	21	645

5. Molecular phylogeny

The phylogenetic tree shows that *P. deissneri* from Bangka forms a well-supported clade (bootstrap 100%) with reference *P. deissneri* (GBJNCI1439-20), confirming its genetic identity. This clade is distinct from other Parosphromenus species, such as *P. quindecim* and *P. nagyi*, which are placed in separate branches with high bootstrap values, indicating clear genetic divergence. The close relationship with *P. cf. bintan* and *P. opallios* suggests phylogenetic proximity within the species group (Fig. 2).

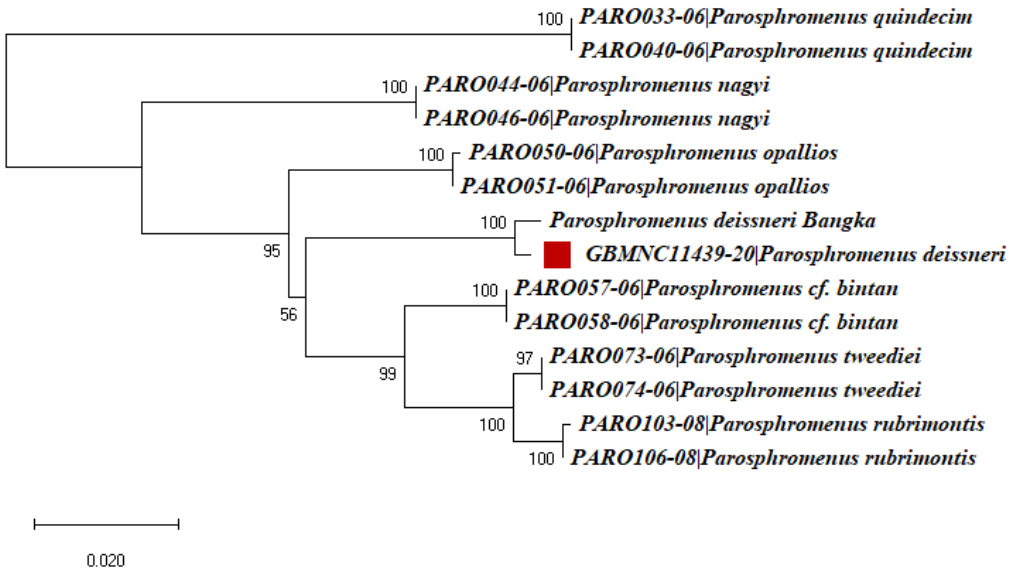

Fig. 2. Evolutionary tree of *Parosphromenus deissneri* Bangka (red square)

DISCUSSION

DNA barcoding is a widely recognized and accurate method for molecular-based species identification **(Falah *et al.*, 2023; Valen *et al.*, 2023b)**. In this study, the successful sequencing of the *co1* gene (665 bp) from *Parosphromenus deissneri* specimens collected from Bangka Island demonstrated up to 99% similarity to reference sequences. This confirms the utility of DNA barcoding in accurately distinguishing species within a genus **(Bolaji *et al.*, 2023)**. These findings align with numerous previous studies showing that the *co1* gene reliably differentiates organisms at the species level **(Seetapan *et al.*, 2024; Valen *et al.*, 2024d)**. In some cases, *co1* barcoding has even facilitated the discovery of previously unrecognized or cryptic species, indicating its effectiveness in revealing hidden biodiversity **(Nazran *et al.*, 2025; Syarif *et al.*, 2025a)**.

The *P. deissneri* DNA barcode obtained in this study was 665 base pairs in length, consistent with the standard fragment size required for effective species-level identification. According to **Hebert *et al.* (2003)**, *co1* sequences longer than 600 bp are considered sufficient for distinguishing species. This benchmark is further supported by studies on other Southeast Asian freshwater fishes, such as *Sicyopus* species from Sulawesi **(Nurjirana *et al.*, 2022)**, *Barbodes lateristriga* from Bangka Island **(Robin *et al.*, 2022)**, and *Esomus metallicus* from West Sumatra **(Robin *et al.*, 2023b)**, which showed over 99% similarity with their respective conspecifics.

BLAST analysis revealed that the *co1* sequence from the Bangka *P. deissneri* specimen exhibited 99.53% similarity and 100% query coverage with a reference sequence in GenBank (accession number MW021426.1), strongly supporting its identification at the species level. The genetic distance between the Bangka specimen and the reference was calculated at 0.008 (0.8%), well below the commonly accepted interspecific divergence threshold of 2–3% for *co1* barcoding **(Hebert *et al.*, 2003; Ge *et al.*, 2021; Kathirvelpandian *et al.*, 2022; Li *et al.*, 2025)**. This low level of divergence suggests intraspecific variation, likely attributable to localized genetic structuring or adaptation to specific microhabitats, rather than the presence of a distinct or cryptic taxon **(Robert *et al.*, 2019)**.

In contrast, genetic distances between the Bangka specimen and other congeneric species were substantially higher: 0.187 (18.7%) with *P. quindecim*, 0.141 (14.1%) with *P. nagyi*, and 0.089 (8.9%) with *P. rubrimontis*. These values indicate significant evolutionary divergence and support the distinct taxonomic status of these species. According to DNA barcoding standards, interspecific divergence exceeding 10% is strong evidence for species-level separation **(Valen *et al.*, 2024e)**.

Phylogenetic analysis further supported these results. The *P. deissneri* specimen from Bangka (highlighted in red) formed a strongly supported monophyletic clade with the *P. deissneri* reference sequence (MW021426.1). This grouping received a bootstrap value of 100%, indicating maximal statistical confidence. In phylogenetic studies, bootstrap values above 70% are considered robust, while values at or near 100% reflect strong support **(Soltis & Soltis, 2003; Hellwig *et al.*, 2022)**.

These findings confirm the identity of the Bangka Island population as *Parosphromenus deissneri*. Other congeneric species—*P. quindecim*, *P. nagyi*, *P. tweediei*, and *P. rubrimontis*—were resolved on separate, well-supported branches, illustrating significant evolutionary divergence. These relationships correspond to the interspecific genetic distances (0.14–0.21) presented in Table (3).

The successful identification of *P. deissneri* with 99.53% sequence similarity and full query coverage against the GenBank reference supports the interpretation that Bangka Island is part of the species' native range. However, *P. deissneri* is highly dependent on acidic blackwater habitats and peat swamp ecosystems, which are increasingly degraded by anthropogenic activities. Major threats in Bangka include tin mining and land conversion for oil palm plantations, leading to habitat fragmentation, loss of riparian vegetation, sedimentation, and heavy metal contamination. These peat swamp ecosystems are ecologically fragile, and the continued presence of *P. deissneri* in such environments highlights both the species' vulnerability and its dependence on undisturbed habitat conditions **(Posa *et al.*, 2011; Hasan *et al.*, 2023d)**.

These results emphasize the urgent need for both **in situ** and **ex situ** conservation strategies. *In situ* approaches should prioritize the protection of remaining natural habitats within the Bangka–Belitung region by establishing micro-reserves, enforcing anti-mining regulations, and restoring degraded swamp ecosystems. Complementary *ex situ* strategies include developing captive breeding programs and establishing genetic resource banks to preserve population-level diversity in the face of ongoing environmental threats **(Syarif *et al.*, 2023e; Budi *et al.*, 2024; Hasan *et al.*, 2024)**. The implementation of these programs can contribute to both conservation and sustainable aquaculture initiatives **(Priyadi *et al.*, 2024; Budi *et al.*, 2025)**.

Future research should explore the use of *P. deissneri* and other native labyrinth fishes as bioindicators for monitoring ecosystem health **(Islamy *et al.*, 2025c, d)**. Additionally, investigations into their nutritional requirements and disease susceptibility under controlled conditions will be essential for developing effective captive management protocols and health monitoring systems **(Islamy *et al.*, 2025e)**.

CONCLUSION

This study successfully confirmed the genetic identity of *Parosphromenus deissneri* from Bangka Island through DNA barcoding using the mitochondrial *co1* gene. The amplified 665 bp *co1* sequence exhibited high similarity (>99%) with reference sequences in GenBank and BOLD, validating accurate species-level identification. Genetic distance analysis revealed minimal divergence (0.008) between the Bangka specimen and the reference population, further confirming their conspecific status. Phylogenetic reconstruction placed the Bangka population within a strongly supported monophyletic clade alongside other *P. deissneri* samples, while remaining distinct from closely related taxa such as *P. cf. bintan* and *P. opallios*. Additionally, nucleotide composition patterns were consistent across populations, indicating genetic homogeneity within the species. These findings demonstrate the effectiveness of DNA barcoding as a molecular tool for species identification, particularly within taxonomically complex groups like *Parosphromenus*. The confirmed presence of *P. deissneri*, a species listed as endangered by the IUCN, underscores the ecological significance of Bangka Island's blackwater habitats and highlights the urgent need for conservation measures. Given the ongoing environmental degradation from tin mining and land-use change, molecular approaches such as DNA barcoding provide critical support for species monitoring, habitat management, and biodiversity preservation. The genetic data generated in this study serve as a vital reference for future ecological, taxonomic, and conservation-related research. They can also facilitate the development of non-invasive monitoring tools, such as environmental DNA (eDNA) assays, to track species distribution and population health. Overall, the study emphasizes the importance of integrating molecular evidence into conservation strategies and advocates for immediate action to protect the remaining habitats of *P. deissneri* on Bangka Island.

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