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Integrative Taxonomic Approach for the Identification and Genetic Characterization of *Mystus singaringan* from the Serayu River Basin, Central Java, Indonesia

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ABSTRACT

Taxonomic ambiguity poses a major challenge for the sustainable management and conservation of freshwater fish biodiversity. This study identified and characterized *Mystus singaringan*, a cryptic species from the Serayu River Basin, Central Java, Indonesia, using an integrative taxonomic approach that combined morphological and molecular (DNA barcoding) analyses. Sampling was conducted from March to June 2024 at upstream, midstream, and downstream sites, including tributaries. Morphological characterization employed truss morphometrics and meristic counts, while molecular analysis targeted CO1, Cyt b, 12S rRNA, 16S rRNA, MiFish, and Fish primers. Six DNA sequences were analyzed using BLAST and BOLD Systems, showing 100% identity with *M. singaringan*. Phylogenetic analysis revealed a strongly supported monophyletic clade with zero genetic distance from *M. micracanthus* and *M. nigriceps*, suggesting possible synonymy. Haplotype network analysis indicated high intraspecific diversity, with 23 unique haplotypes. These results clarify the taxonomic status of *M. singaringan* and provide essential baseline data to support aquaculture broodstock management, conservation strategies, and restocking programs in the Serayu River Basin.

INTRODUCTION

Freshwater ecosystems in Southeast Asia are recognized as global biodiversity hotspots, supporting a wide variety of endemic fish species that provide significant ecological and socio-economic value. The Serayu River, one of the major river systems in Central Java, Indonesia, extends approximately 153km from Wonosobo Regency to Cilacap Regency **(Pramono *et al.*, 2025)**. Previous studies have reported considerable spatial variation in

freshwater fish diversity across the basin, with tributaries such as the Klawing River recording up to 23 species in a single site **(Pramono *et al.*, 2019)**.

Despite this relatively high species richness, taxonomic inconsistencies remain a persistent challenge. Several species have been reported under different scientific names across studies—for example, *Mystus micracanthus*, *Mystus nigriceps*, and *Mystus singaringan* all refer to the same "Senggaringan" catfish. Similar inconsistencies occur in other taxa, including *Barbonymus balleroides* vs. *Puntius orphoides*, and *Labiobarbus leptocheilus* vs. *Dangila cuvieri* **(Budhi *et al.*, 2019)**. Such discrepancies hinder effective conservation, management, and aquaculture development because species misidentification can distort biodiversity assessments and lead to inappropriate management decisions.

These problems reflect the broader "taxonomic crisis" identified by biodiversity experts, who estimate that up to 10 million species remain undescribed or poorly defined **(Al Asif & Nerurkar, 2024)**. Misidentification arises from multiple sources, including morphological similarity, intraspecific variation, geographical isolation, gene flow, and environmental influences **(Pramono *et al.*, 2019)**. The issue is particularly acute for cryptic (sibling) species—those that are morphologically similar but genetically distinct—as their misclassification can compromise biodiversity estimates, fisheries management, and conservation strategies **(Rosadi & Pratomo, 2021; Ahmed, 2022)**.

An integrative taxonomic approach, combining traditional morphological analysis with molecular techniques such as DNA barcoding, offers a robust solution to these challenges **(Utomo *et al.*, 2021)**. Morphometric and meristic analyses remain valuable for species identification, stock discrimination, and functional morphology studies, while DNA barcoding—especially using mitochondrial cytochrome c oxidase subunit 1 (CO1) sequences—has proven highly effective in resolving cryptic taxa and providing accurate identifications **(Pramono *et al.*, 2019)**.

In the context of the Serayu River, clarifying the taxonomic identity of target species such as *Mystus singaringan* is essential for sustainable fisheries management, aquaculture broodstock development, and conservation planning. This study applied an integrative taxonomy framework to characterize the habitat, morphology, and genetics of cryptic freshwater fishes in the Serayu River Basin, with particular focus on resolving ambiguities surrounding *M. singaringan*. The findings would provide critical baseline data to support biodiversity conservation and aquaculture development in Central Java.

MATERIALS AND METHODS

1. Study area and sampling period

This study was conducted in the Serayu River Basin (DAS Serayu), encompassing upstream, midstream, and downstream sections across five regencies: Wonosobo, Banjarnegara, Purbalingga, Banyumas, and Cilacap, Central Java, Indonesia. Fish sampling was carried out from March to June 2024. The sampling sites included both the main river channel and its tributaries (Fig. 1). Geographic coordinates of each station were recorded using a handheld GPS device (Garmin 5, Olathe KS, USA) (Table 1).

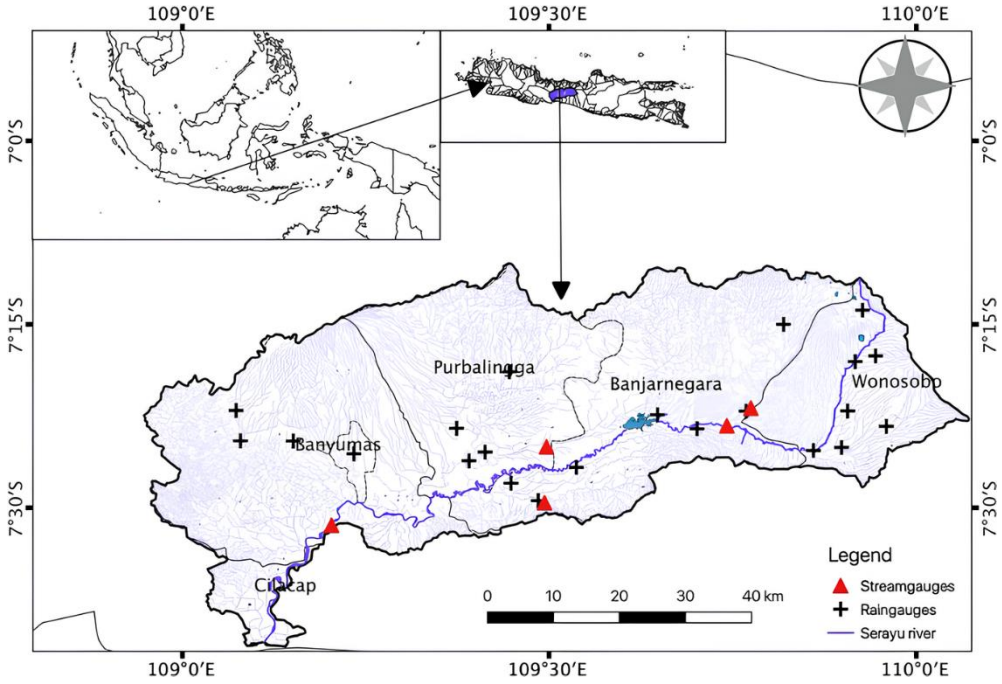

Fig. 1. Map of the Serayu River Basin (adapted from **Kusuma *et al.*, 2024**)

Table 1. Geographic coordinates of fish sampling locations in the Serayu River Basin

Sampling Site	Coordinates
Tulis River, Wonosobo Regency	7°24'35" S 109°15'15.6" E
Mrawu River, Wonosobo Regency	7°23'27.5" S 109°45'004" E
Kalisapi River, Wonosobo Regency	7°48'478" S 109°47'133" E
Danaraja Village, Purwonegoro, Banjarnegara Regency	7°26'379" S 109°31'911" E
Tapen Village, Wanadadi, Banjarnegara Regency	7°24'034" S 109°35'544" E
Mantrianom Village, Bawang, Banjarnegara Regency	7°23'305" S 109°36'484" E
Tlewang Village, Bawang	7°23'222" S 109°39'395" E
Rejasa Village, Madukara, Banjarnegara Regency	7°23'193" S 109°41'488" E
Bandingan Village, Sigaluh, Banjarnegara Regency	7°23'345" S 109°44'680" E
Congot Kedungbenda, Purbalingga Regency	(07°29.558’ S, 109°20.270’ E)

2. Fish sampling and preservation

Fish specimens were collected using gill nets operated in collaboration with local fishers. Thirty individuals representing cryptic species were obtained from the combined catch. Captured fish were immediately preserved in chilled conditions (4°C) in ice boxes for transport to the Fisheries and Marine Science Research Laboratory, Universitas Jenderal Soedirman. For genetic analysis, caudal fin tissue samples were excised from each specimen and were preserved in 95% ethanol until further processing.

3. Habitat characterization and water quality analysis

Physicochemical parameters of river water were measured *in situ* following the Indonesian National Water Quality Standards (PP 82/2001). Parameters and methods used are

listed in Table (2). Environmental conditions at each site were also recorded descriptively, including surrounding land use and anthropogenic influences.

Table 2. Water quality parameters and measurement methods used in the Serayu River Basin

No.	Parameter	Unit	Method/Instrument
1	Dissolved Oxygen (DO)	mg/L	Winkler Method
2	pH	unit	pH pen meter
3	BOD5	mg/L	Winkler Method
4	Nitrate (NO_3–N)	mg/L	Spectrophotometer/Brucine
5	Nitrite (NO_2–N)	mg/L	Sulfanilic Acid
6	Ammonia (NH_3–N)	mg/L	Spectrophotometer/Phenate
7	Alkalinity	mg $CaCO_3$/L	Digital Alkalinity Meter
8	Temperature	°C	Digital Thermometer
9	Current velocity	m/s	Floating method
10	Depth	m	Weighted rope measurement
11	Turbidity	NTU	Turbidimeter
12	Environmental profile	–	Descriptive

4. Morphological analysis

Morphological characterization involved both morphometric and meristic analyses **(Prakasham *et al.*, 2024)**. Morphometric measurements were taken using a digital caliper (±0.01 mm accuracy), including truss morphometrics based on 21 reference landmarks. Meristic counts were recorded for dorsal, anal, pectoral, caudal, and pelvic fin rays.

5. Genetic analysis (DNA barcoding)

Genetic characterization was conducted using DNA barcoding techniques with six primers (Table 3): CO1, Fish, Cyt b, 12S rRNA, 16S rRNA, and MiFish. Tissue samples were processed using the Quick DNA Tissue/Insect Miniprep Kit (Zymo Research). PCR amplification was carried out using a Toyobo KOD FX Neo thermal cycler under the following conditions: initial denaturation at 95°C for 3min; 35 cycles of denaturation at 98°C for 10s, annealing at 50°C for 30s, extension at 68°C for 1.0 min; and a final extension at 68°C for 5min. Amplicons were visualized via 1% agarose gel electrophoresis and sequenced bi-directionally by First Base Co. (Malaysia).

Table 3. Primer sequences used in genetic analysis

Primer	Sequence	Source
CO1 F1	TCA ACC AAC CAC AAA GAC ATT GCC AC	Genetika Science
CO1 R1	TAG ACT TCT GGG TGG CCA AAG AAT CA	Genetika Science
FISH F2	TCG ACT AAT CAT AAA GAT ATC GGC AC	Genetika Science
FISH R2	ACT TCA GGG TGA CCG AAG AAT CAG AA	Genetika Science
Cyt B Fw	CGT CAT TAC CAA CCT TTC	Genetika Science
Cyt B Rw	GCG TAT GCA AAT AGG AAG TAT CAC	Genetika Science
12S RNA-F	GCT TGG TCC TGA CTT TAG TA	Genetika Science
12S RNA-R	CTT ACC ATG TTA CGA CTT GC	Genetika Science
16S RNA-F	GAC CCT ATG GAG CTT TAG AC	Genetika Science
16S RNA-R	CGC TGT TAT CCC TAD RGT AAC T	Genetika Science

MiFISH-U-F	GTC GGT AAA ACT CGT GCC AGC	Genetika Science
MiFISH-U-R	CAT AGT GGG GTA TCT AAT CCC AGT TTG	Genetika Science

6. Data analysis

Habitat data from each sampling location were evaluated by comparing them with the Indonesian National Water Quality Standards (PP 82/2001) to determine whether environmental conditions met the requirements for the target species. Morphometric measurements were described and compared with published information on other *Mystus* species to support accurate identification. The general growth pattern and overall condition of the fish were assessed using standard fisheries biology indicators. For genetic data, the *dna* sequences obtained were edited and aligned using MEGA5 software, then matched with reference sequences in GenBank through BLAST searches and verified using the Barcode of Life Data Systems (BOLD). Phylogenetic trees were then generated using the neighbor-joining method to visualize the genetic relationships between the studied specimens and related species.

RESULTS

1. Morphometric and meristic characteristics

Morphometric data for *Mystus singaringan* obtained from FishBase (www.fishbase.se) reveals key characteristics of this species. The maximum reported total length (TL) is 15.0cm, with a common length of 10.0cm. The species is characterized by its elongated body and specific fin ray counts. It possesses 6-7 soft rays in the dorsal fin, 38-41 soft rays in the anal fin, 10-12 soft rays in the pectoral fin, and 6 soft rays in the pelvic fin (Table 4).

Table 4. Morphometric and meristic data of *Mystus singaringan*

Character	Value	Source
Morphometric Data		
Max. Total Length (TL)	22.83±1.54cm	
Common Length (TL)	19.78±1.14 cm	
Meristic Data		
Dorsal Fin Rays	6–7	FishBase
Anal Fin Rays	38–41	
Pectoral Fin Rays	10–12	
Pelvic Fin Rays	6	

2. Genetic identification and phylogenetic relationships

DNA barcoding using *co1* sequences from six specimens yielded 100% similarity with *Mystus singaringan* in both BOLD Systems and GenBank BLAST analyses. Phylogenetic reconstruction using the neighbor-joining method clustered all samples into a well-supported monophyletic clade, with a genetic distance of 0.00 among them. This clade showed close affinity with *M. micracanthus* and *M. nigriceps*, with genetic distances of 0.00 and 0.03, respectively, suggesting that these taxa are likely synonyms of *M. singaringan* (Figs. 2, 3). These findings address inconsistencies in previous reports from the Serayu River Basin, where the same morphotype had been referred to under different scientific names **(Pramono, 2017)**.

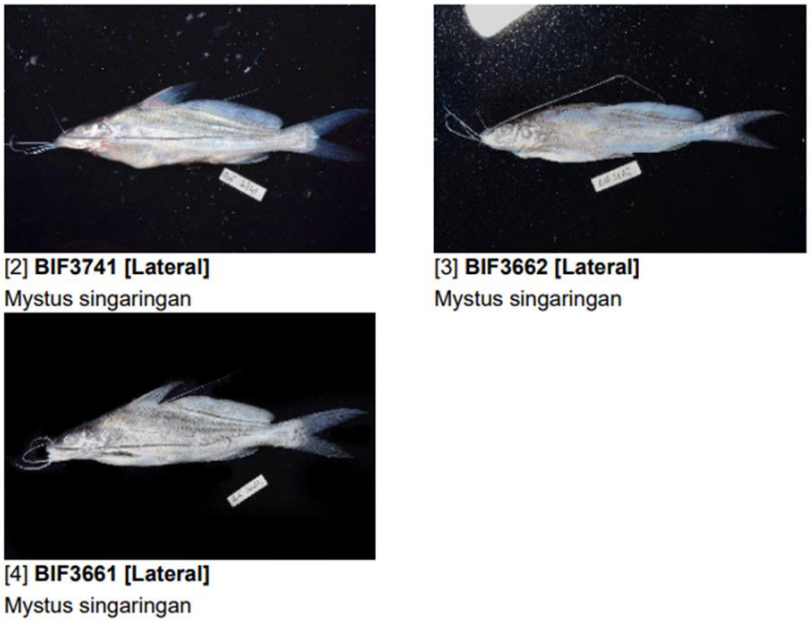

Fig. 2. Three lateral photographs of *Mystus singaringan* identified through BOLD Systems analysis. (2,3,4 are morphometric differences in samples of the same species). The codes [2] BIF3741, [3] BIF3662, and [4] BIF3661 represent unique specimen identifiers in the BOLD Systems dataset.

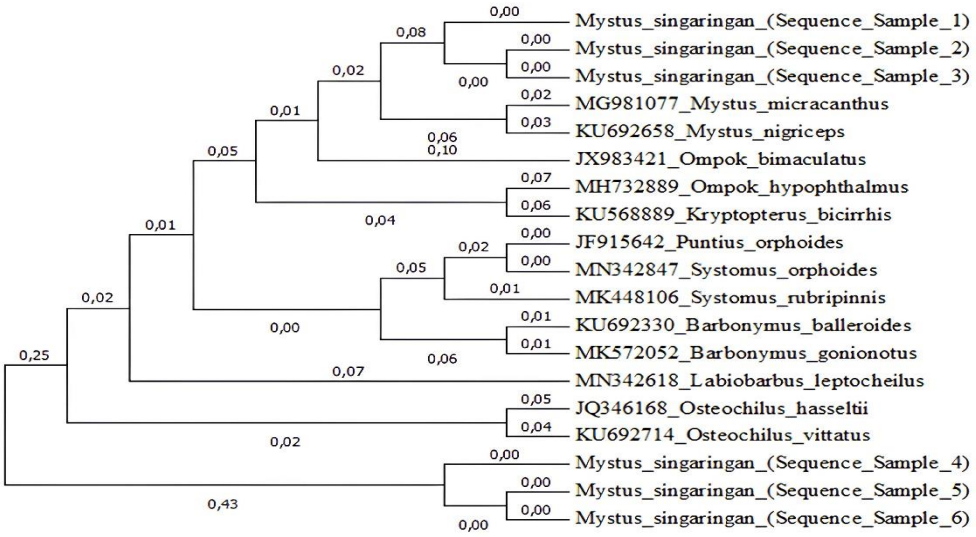

Fig. 3. Phylogenetic tree of *Mystus singaringan* and related species

3. Haplotype diversity

Haplotype analysis of the six *Mystus singaringan* samples from the Serayu River, combined with reference sequences, revealed 23 unique haplotypes, indicating a high level of intraspecific genetic diversity within the population (Fig. 4). The network, represented by black circles of varying sizes, indicated high genetic variation, with larger circles denoting common haplotypes and smaller ones representing rare or unique types. The lack of connecting lines or clear topology limited visualization of evolutionary relationships, but when compared with the phylogenetic tree—which confirmed a clear monophyletic clade—the haplotype network highlighted notable intraspecific genetic diversity and differentiation within the species.

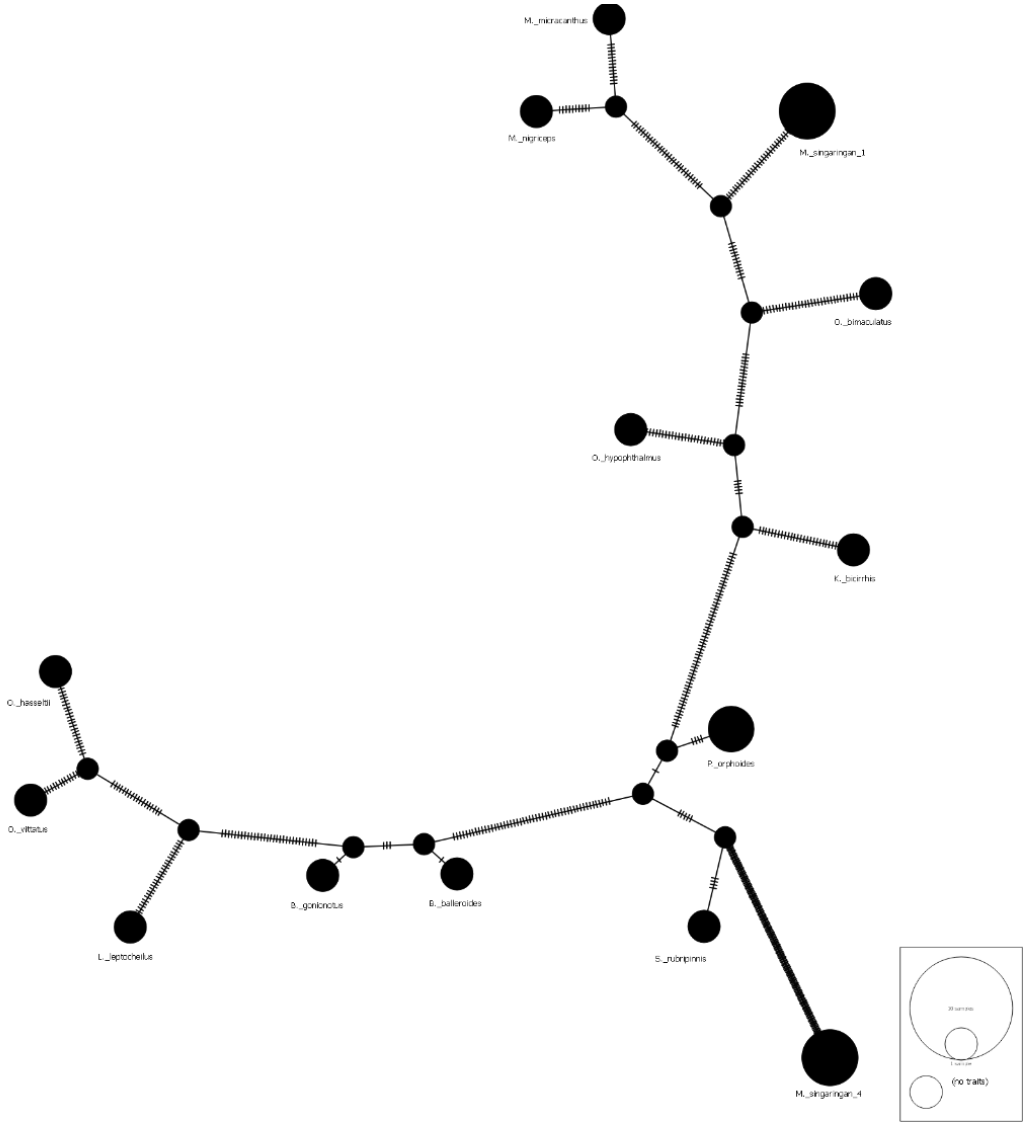

Fig. 4. Haplotype relationship of *Mystus singaringan* and related species

4. Implications for taxonomy, conservation, and aquaculture

The integration of morphological and molecular data in this study provides strong evidence for the accurate taxonomic identification of *M. singaringan* in the Serayu River Basin. Correct identification is essential for consistent biodiversity assessments, sustainable fisheries management, and the development of genetically robust broodstock for aquaculture. Genetic information on population structure also informs targeted conservation measures, such as habitat protection and restocking programs. Previous misidentifications may have led to inaccurate distribution records and misguided management actions; thus, this study establishes a standardized nomenclature and reliable reference for future ecological and aquaculture-related work on the species.

DISCUSSION

Morphometric and meristic data for *Mystus singaringan*—compiled from references such as **Kottelat (1998)** in Laos and **Vidthayanon (2002)** in Thailand—along with

measurements from 30 specimens collected in the Serayu River, provide a robust foundation for species identification and comparison. The Serayu samples attained a maximum total length of 22.83 ± 1.54cm and an average length of 19.78 ± 1.14cm. In contrast, previous reports list a maximum total length of 15.0cm and a common length of 10.0cm **(Kottelat, 1998; Vidthayanon, 2002)**, confirming that *M. singaringan* is a relatively small-sized species.

These size parameters are critical for aquaculture and fisheries management, as they inform decisions regarding stocking density, feeding regimes, and marketability. In addition, fin ray counts in the dorsal, anal, pectoral, and pelvic fins serve as valuable meristic markers for resolving taxonomic ambiguities. Such morphological "fingerprints," when corroborated with DNA barcoding, confirm the species' correct identity as *M. singaringan*.

Molecular identification and phylogenetic relationships

DNA barcoding using the mitochondrial CO1 gene has proven highly effective in confirming the taxonomic identity of *M. singaringan* **(Pramono *et al.*, 2017)**. BLAST and BOLD analyses indicated 100% sequence identity with reference samples, while phylogenetic reconstruction (Neighbor-joining method) placed the Serayu samples in a well-supported clade. Notably, the genetic distance to *M. micracanthus* and *M. nigriceps* was effectively zero, suggesting possible synonymy.

Similar applications of CO1 and cyt b genes have clarified species identities in other genera, such as *Osteochilus* **(Asiah *et al.*, 2020)**, *Ompok* **(Kasayev & Tuti, 2022)**, and *Barbonymus* **(Rahmawati *et al.*, 2023)**. Collectively, these results demonstrate the broader utility of CO1 barcoding for resolving taxonomic ambiguities and illuminating phylogenetic relationships among freshwater fishes in Indonesia.

Haplotype analysis and genetic diversity

Haplotype analysis, which identifies combinations of alleles inherited together on the same chromosome **(Sivabharathi *et al.*, 2024; Wang, 2024; Yang *et al.*, 2024)**, was applied to the Serayu samples. The analysis revealed 23 unique haplotypes, demonstrating a high degree of intraspecific genetic diversity.

From an aquaculture perspective, such diversity is advantageous, as it enhances resilience to environmental change, disease resistance, and growth potential. The haplotype network displayed several dominant haplotypes (larger nodes), likely representing well-established lineages, alongside rarer haplotypes (smaller nodes) that contribute to a broader genetic base. Both categories are important: dominant haplotypes may support immediate breeding programs, while rarer haplotypes safeguard long-term adaptability.

The fragmented structure of the haplotype network likely reflects the inclusion of distantly related taxa (e.g., *Mystus* vs. *Ompok*), which obscures intraspecific relationships. Haplotype networks are most informative when applied within species or among closely related taxa. Thus, future studies should focus specifically on *M. singaringan* and its congeners to clarify population structure and gene flow.

Implications for fisheries and aquaculture

The combination of morphometric, meristic, and molecular evidence confirms the identity of *M. singaringan* and highlights its considerable intraspecific genetic diversity. This

provides a strong baseline for conservation, aquaculture broodstock management, and restocking programs in the Serayu River. To maintain sustainability, breeding strategies should aim to conserve both common and rare haplotypes, thereby preventing genetic erosion and preserving traits essential for long-term adaptability.

CONCLUSION

This study successfully identified and confirmed *Mystus singaringan* from the Serayu River Basin through an integrative taxonomic approach combining morphological and genetic analyses. Morphometric and meristic measurements provided consistent morphological traits for species differentiation, while DNA barcoding using *co1* and other genetic markers achieved 100% confidence in species identification. The phylogenetic analysis demonstrated close genetic relationships with *Mystus micracanthus* and *Mystus nigriceps*, supporting the hypothesis of synonymy. Haplotype analysis revealed considerable genetic diversity, underscoring the species' adaptive potential and importance for sustainable aquaculture and conservation. Standardizing the taxonomic identity of *M. singaringan* will enhance the effectiveness of fisheries management, broodstock development, and biodiversity preservation in the Serayu River Basin.

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