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COI-based diversity of mahseer (*Tor* spp.) reveals divergent lineages across four foothill rivers of Mount Slamet, Central Java, Indonesia

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Abstract. *Suwarsito, Pratama I, Mustafidah H. 2026. COI-based diversity of Mahseer* (Tor *spp.*) *reveals divergent lineages across four foothill rivers of Mount Slamet, Central Java, Indonesia. Biodiversitas 27 (5): d270505. https://doi.org/10.13057/biodiv/d270505*. DNA barcoding has become an essential tool for resolving taxonomic ambiguity in freshwater fishes, including the economically and ecologically important mahseer (*Tor* spp.). This study employed mitochondrial Cytochrome C Oxidase subunit I (COI) barcodes (~700 bp) to identify species, assess genetic diversity, and infer phylogenetic relationships among 14 individuals collected from four upstream rivers at the foothills of Mount Slamet, Indonesia (Comal, Welo, Tutung Gunung, and Pelus rivers). Basic Local Alignment Search Tool (BLAST) and Barcode of Life Data System (BOLD) analyses assigned sequences to *Tor tambroides* (99.68% to 99.84% similarity) and *Tor tambra* (100% similarity). Analysis of Kimura 2-Parameter (K2P) genetic distances by species revealed extremely low intraspecific divergence within *T. tambroides* (0.0000-0.0035) and generally low divergence within *T. tambra* (0.0000-0.0266). In contrast, interspecific divergence between *T. tambroides* and *T. tambra* ranged from 0.0285 to 0.0303, forming a clear barcode gap. Maximum likelihood phylogenetic analysis placed all samples within the Southeast Asian *Tor* clade, with clustering concordant with species-level identification. However, given the limited sample size (n = 14) and reliance on a single mitochondrial marker, population-level interpretations remain preliminary. These findings demonstrate that COI barcoding is highly effective for discriminating closely related *Tor* species and provide important genetic baseline data for biodiversity assessment. The presence of a clear barcode gap reinforces the reliability of molecular identification, while the observed genetic patterns highlight the need for broader sampling and the application of multilocus or genomic approaches. This study contributes to conservation-oriented management by improving species resolution and supporting future efforts to delineate population structure in Indonesian freshwater ecosystems.

Keyword: Central Java, COI barcoding, genetic divergence, *Tor tambra*, *Tor tambroides*

INTRODUCTION

Mahseer of the genus *Tor* are among the most culturally and economically important cyprinids in South and Southeast Asia, supporting inland fisheries, aquaculture, and traditional practices. However, many *Tor* species have experienced pronounced population declines driven by overexploitation, habitat alteration, pollution, and fragmentation of river systems (Atifah et al. 2021; Dwirastina and Wibowo 2022). The International Union for Conservation of Nature lists several *Tor* taxa as threatened (IUCN 2018), reflecting pressures associated with deforestation, land-use change, watershed degradation, and river modification (Larashati et al. 2022). Broad syntheses highlight these factors as major drivers of freshwater biodiversity loss at regional and global scales (Dudgeon 2020; Zarri et al. 2022). At local and basin scales in Indonesia, similar pressures have been documented to affect habitat quality and fish assemblages in river systems where *Tor* occurs (Roesma et al. 2016; Desrita et al. 2019). In the context of mahseer specifically, Jaafar et al. (2021) emphasized that such cumulative impacts can restrict dispersal, disrupt demographic connectivity, and elevate extinction risks in longitudinally connected rivers.

Specifically, in Indonesia, *Tor tambroides* (Bleeker, 1854) and *Tor tambra* (Valenciennes, 1842) represent key species of management concern; however, their taxonomic resolution is often complicated by overlapping morphological traits, phenotypic plasticity, and ontogenetic variation (Walton et al. 2017; Nazifa et al. 2026). To address these identification challenges, mitochondrial DNA barcoding, particularly the COI gene, has been widely applied to delimit species boundaries and assess lineage diversity in freshwater fishes, including mahseer (Esa and Rahim 2013; Bingpeng et al. 2018). When matched with voucher-verified reference libraries, COI barcodes provide strong discriminatory power for detecting cryptic species and characterizing genetic variation (Ivanova et al. 2007; Rahayu et al. 2022). In parallel, population genetic studies of riverine fishes emphasize that geographical isolation and anthropogenic fragmentation, such as dams and flow alterations, can enhance genetic subdivision among populations (Imron et al. 2024; Phanklam et al. 2026). Accordingly, integrating DNA barcoding with population genetic perspectives provides a robust framework for biodiversity assessment and conservation planning (Larashati et al. 2022).

Across the range of *Tor*, phylogeographic studies have revealed a deep genetic division between South Asian and

Southeast Asian lineages, reflecting long-term geological separation and the independent histories of major river basins (Walton et al. 2017; Nazifa et al. 2026). This pattern is supported by regional case studies, including documentation of intraspecific variation in *Tor putitora* (Hamilton, 1822) from Pakistan (Shafi et al. 2016) and syntheses of distributional and molecular evidence for Southeast Asian mahseer (Jaafar et al. 2021). More recently, phylogeographic analyses of *T. tambra* in Thailand have demonstrated cryptic diversity shaped by historical drainage evolution and contemporary catchment fragmentation (Phanklam et al. 2026).

Despite increasing molecular studies on *Tor* spp. in Indonesia and neighboring regions, Central Java, particularly the foothill river systems of Mount Slamet, remains largely absent from genetic datasets and barcode reference libraries. This represents a critical knowledge gap, as these rivers are subject to anthropogenic pressures such as land-use change, habitat modification, and potential fragmentation, which may influence population connectivity and genetic structure. Moreover, it remains unclear whether *Tor* populations in this region exhibit genetic differentiation consistent with river separation or instead show evidence of connectivity through shared haplotypes.

Addressing this gap is essential to determine whether river systems in Central Java function as isolated population units or as a connected metapopulation, which has direct implications for conservation and management. Therefore, molecular characterization of *Tor* populations in this region is essential to support biodiversity assessment and conservation planning. Here, we use COI sequences to (i) identify mahseer collected from four upstream rivers (Comal, Welo, Tutung Gunung, and Pelus rivers) , (ii) quantify within- and among-river genetic divergence, and (iii) place local haplotypes within a broader phylogenetic context.

This study hypothesizes that COI-based DNA barcoding can reliably discriminate *T. tambra* and *T. tambroides* across river systems in Central Java by exhibiting low intraspecific divergence, high interspecific divergence, and a distinct barcode gap. Specifically, the study tests whether genetic distance (K2P), sequence similarity (BLAST/BOLD), and phylogenetic reconstruction (maximum likelihood) converge to support consistent species delimitation and reveal the extent of phylogeographic structuring among populations.

MATERIALS AND METHODS

Study area

The study was conducted between July and December 2024 at four upstream river sites on the foothills of Mount Slamet, Central Java, Indonesia with coordinates points: Comal River (7°12′18.832″ S, 109°14′42.431″ E, Pemalang District), Welo River (7°9′23.795″ S, 109°44′48.072″ E, Pekalongan District), Tutung Gunung River (7°13′17.903″ S, 109°20′46.947″ E, Purbalingga District), and Pelus River (7°18′24.907″ S, 109°14′32.282″ E, Banyumas District) (Figure 1).

Fish sampling and tissue preservation

Fish were captured approximately every two weeks using hand nets and rods with assistance from local fishers. Standard length (cm) and body weight (g) were recorded. A provisional identification of collected fish specimens was carried out based on external morphometric features and meristic counts according to standard taxonomic surveys for the genus *Tor*. To explore the relationships between species, we conducted a morphological study based on observations including body coloration, shape of the body and head profile (frontal outline), mouth position (inferior/subterminal), presence/development of median and lateral lobes on lower lip, barbel number and length (rostral/barbels maxillary) to look for similarities (Desrita et al. 2018; Jaafar et al. 2021).

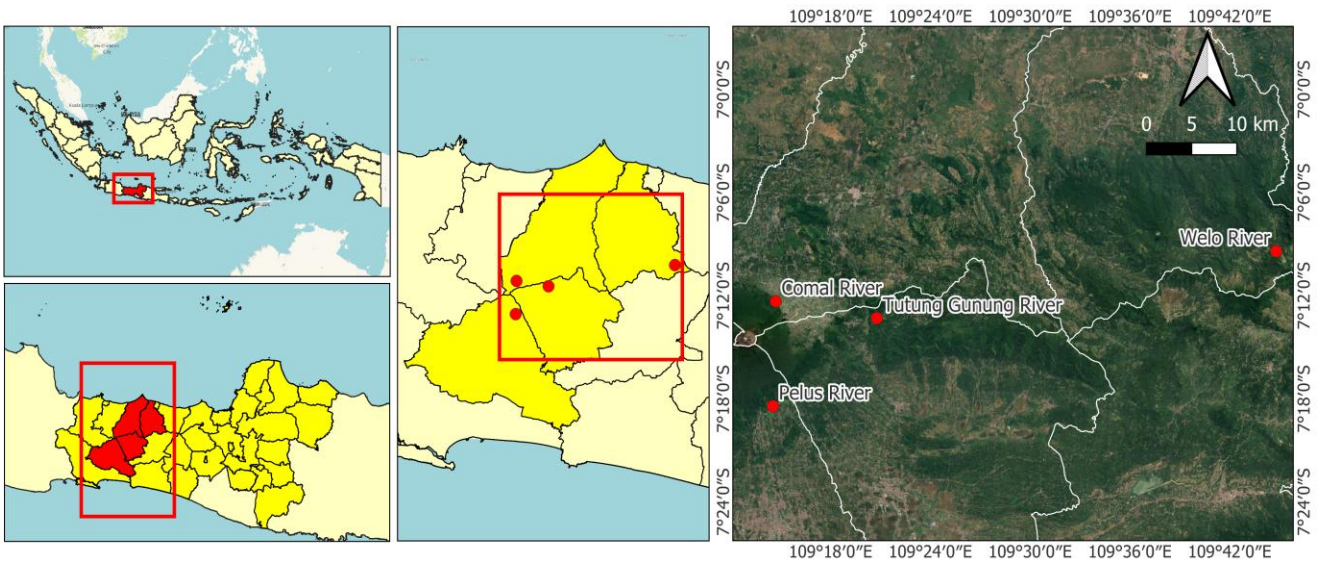

Figure 1. Map showing the four upstream river research sites located at the foothills of Mount Slamet, Central Java, Indonesia: Comal River, Welo River, Tutung Gunung River, and Pelus River. The map provides the geographical context for the collection of mahseer (*Tor* spp.) specimens analyzed in this study

Characters examined were the number of dorsal fin rays, pectoral fin rays, pelvic fin rays, anal fin rays, and lateral line scales. Due to the high morphological similarity among species within the genus *Tor*, the identification was considered tentative and further validated using molecular analysis. A small portion of the dorsal muscle and a fin clip were preserved in 70% ethanol in labeled microtubes and stored at room temperature prior to laboratory analysis. Fourteen individuals were selected for COI analysis (Pemalang 1-3, Purbalingga 1-6, Banyumas 1-2, Pekalongan 1-3). We note the limited number of individuals per river and treat inferences as preliminary.

Ethical statement and permits

All sampling and laboratory procedures were conducted under ethical approval and supervision granted by the Institute for Research and Community Service (LPPM), Universitas Muhammadiyah Purwokerto, Indonesia (Approval No. A11.III/190 S.Ket./KEP LPPM/VII/2024).

DNA extraction

Approximately 25 mg of tissue from each specimen was placed into a 1.5 mL microcentrifuge tube for genomic DNA extraction. Genomic DNA was isolated using the Zymo Research DNA Purification Kit following the manufacturer's protocol, as described by Isuosuo et al. (2024). Each tissue sample was homogenized with 95 µL of nuclease-free water, 95 µL of Solid Tissue Buffer (Blue), and 10 µL of Proteinase K using a vortex mixer, followed by brief centrifugation, and incubated at 55°C for 1-3 hours with vortexing every 5 minutes to ensure complete tissue lysis. Subsequently, 200 µL of Genomic Binding Buffer was added, and the mixture was vortexed and centrifuged for 10-15 seconds before being transferred to a Zymo-Spin™ IIC-XLR (Original Manufacturer, USA) column placed in a collection tube and centrifuged at 12,000 rpm for 1 minute. The column was washed sequentially with 400 µL of DNA Pre-Wash Buffer and 700 µL of g-DNA Wash Buffer, each followed by centrifugation at 12,000 rpm for 1 minute, and a final wash was performed using 200 µL of g-DNA Wash Buffer under the same conditions. Genomic DNA was eluted with 50 µL of DNA Elution Buffer and stored at -20°C until further analysis. After extraction, DNA quantity and purity were assessed using spectrophotometric measurements (A260/280), and only high-quality DNA was used for PCR amplification.

PCR and DNA barcoding

PCR amplification followed the protocol described by Ivanova et al. (2007) using M13-tailed universal COI primers, including the forward primers VF2_t1 (5′-TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC-3′) and FishF2_t1 (5′-TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC-3′), as well as the reverse primers FishR2_t1 (5′-CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA-3′) and FR1d_t1 (5′-CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA-3′). PT Genetika Sciences Indonesia synthesized all primers. PCR amplification was performed following the Bioline protocol using MyTaq™ HS Red Mix (2×) (Meridian Life Science, Memphis, UK). PCR was performed in a total volume of 50 µL containing 2 µL of DNA template, 0.5 µL each of forward and reverse primers, 25 µL of MyTaq™ HS Red Mix, and 21 µL of nuclease-free water. Thermal cycling conditions consisted of an initial denaturation at 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 15 seconds, with a final extension at 72°C for 5 minutes. PCR products were visualized on 1.5% agarose gels using a 100 bp DNA ladder as a size marker. Successfully amplified products were sent to PT Genetika Sciences Indonesia for sequencing.

Molecular identification was conducted using a DNA barcoding approach targeting the mitochondrial COI gene, which yielded sequence fragments of approximately 670-707 bp. Sequence quality was validated by comparing electropherogram profiles with the corresponding nucleotide sequences generated in this study (Damaso et al. 2024). Reverse sequences were generated through reverse complementation to ensure accurate bidirectional sequence assembly, following the procedure described by Mahrus et al. (2025).

Data analysis

The DNA sequences of *Tor* spp. obtained from PCR amplification were edited and aligned using the ClustalW algorithm implemented in MEGA version 12 (Kumar et al. 2024). The aligned sequences were subsequently compared with nine reference sequences available in the GenBank database using BLAST provided by the National Center for Biotechnology Information (NCBI). Sequence similarity analyses were performed to assess genetic variation among samples from different river locations. In addition, the sequences were compared with those in the Barcode of Life Data System (BOLD) to determine species identity based on similarity percentages (Bingpeng et al. 2018; Zhou et al. 2025).

Genetic divergence among the fourteen individuals was evaluated by calculating pairwise genetic distances using the Kimura 2-Parameter (K2P) model (Bingpeng et al. 2018; Rahayu et al. 2022). This model estimates evolutionary distances by accounting for both transition and transversion substitutions in the COI gene sequences and is commonly applied in DNA barcoding studies (Hutama et al. 2017).

Phylogenetic relationships were inferred using the maximum likelihood method with 1,000 bootstrap replications (Muyassar et al. 2025), also implemented in MEGA version 12. *Barbodes binotatus* (Valenciennes, 1842) (GenBank accession number: PP352538) was used as an outgroup. The phylogenetic tree was constructed to visualize the clustering of local samples (Pemalang, Purbalingga, Banyumas, and Pekalongan) relative to reference *Tor* species, including *T. tambra*, *T. tambroides*, *Tor tor* (Hamilton, 1822), *Tor mosal* (Hamilton, 1822), *Tor barakae* (Arunkumar & Basudha, 2003), *T. putitora*, *Tor douronensis* (Valenciennes, 1842), *Tor mussullah* (Sykes, 1839), and *Tor malabaricus* (Jerdon, 1849). Node support was assessed using bootstrap values, which are indicated at

the corresponding branches. The resulting tree facilitated comparison of the genetic profiles of the studied *Tor* populations with those of related species available in GenBank, providing insights into their taxonomic status and genetic divergence.

RESULTS AND DISCUSSION

Species identification and genetic similarity

The individual abundance at each sampling site varied in terms of individual count, body length, and body weight. Representative fish specimens collected from the four study locations are presented in Figure 2, while the size distribution of the captured specimens is summarized in Table 1.

Table 1 presents the number of sequenced individuals, body size variation, and species composition of *Tor* spp. collected from four rivers in Central Java: the Comal, Welo, Tutung Gunung, and Pelus rivers. A total of two species were identified, *T. tambroides* and *T. tambra*, with varying distribution patterns and size ranges across sampling locations.

Initial speciesb identification of Central Javan mahseer was first undertaken using external characters that are routinely applied to delimit *Tor*, namely body coloration, the distribution and luster of cycloid scales, fin coloration, and meristic traits such as lateral line scale counts, dorsal and anal fin ray counts , and head-body proportions, following regional taxonomic references for Southeast Asian mahseer (Jaafar et al. 2021; Akmal et al. 2022). Across sampling sites, a consistent set of phenotypes, characterized by a golden bronze body with metallic sheen, large cycloid scales, robust fusiform profile, reddish to orange caudal and pelvic fins, a moderately arched dorsal profile, and a relatively elongated caudal peduncle, corresponded closely with diagnostic descriptions of *T. tambroides* (Arif et al. 2025; Damayanti et al. 2025). In contrast, several individuals , predominantly from the Comal, Welo, and Pelus rivers, exhibited deeper bodies, darker dorsal pigmentation, and more intense red coloration on median fins. These individuals also showed slightly higher lateral line scale counts and subtle shifts in head depth and snout slope. Such characteristics are consistent with published diagnostic criteria for *T. tambra*, including recent studies from Indonesia, with some specimens additionally exhibiting a more falcate dorsal fin and a pronounced anterior lobe (Marnis et al. 2024).

Although both species share broadly similar phenotypes characteristics, the combined assessment of fin coloration, scale patterning, and meristic variation enabled reliable preliminary assignment of specimens to either *T. tambroides* or *T. tambra* prior to molecular confirmation. This integrative approach is widely recommended in regional syntheses and Indonesian case studies. The occurrence of both morphotypes within certain rivers, particularly the Pelus River, is consistent with previous reports of sympatry and overlapping diagnostic traits in Southeast Asian *Tor.* This finding further underscores the importance of integrating morphological and molecular approaches for accurate species delimitation (Jaafar et al. 2021; Muchlisin et al. 2022). These morphology-based assignments were subsequently validated using COI-based molecular identification approaches.

Table 1. Number of sequenced *Tor* spp. individuals from Comal, Welo, Tutung Gunung, and Pelus Rivers, Central Java, Indonesia

River	Species	Number of sequenced individuals (n)	Weight range (g)	Length range (cm)
Comal River	*Tor tambroides*	1	42.52	16.50
	Tor tambra	2	0.69-348.01	3.70-29.20
Tutung Gunung River	*Tor tambroides*	6	0.80-88.42	4.20-22.10
Pelus River	*Tor tambroides*	1	2.09	6.2
	Tor tambra	1	23.95	13.9
Welo River	*Tor tambra*	3	22.5-234.2	13.5-27.7

Figure 2. Representative mahseer specimens (*Tor tambroides* and *Tor tambra*) were collected from the four study rivers in Central Java, Indonesia. The images illustrate general external morphology and size variation among specimens observed during sampling and are provided for visual reference

The Tutung Gunung River exhibited the highest number of sequenced individuals, particularly for *T. tambroides* (n = 6), with body weights ranging from 0.80 to 88.42 g and lengths from 4.20 to 22.10 cm. This pattern may indicate that Tutung Gunung River provides relatively favorable habitat conditions for *T. tambroides*, potentially related to flow regime , substrate composition, or food availability. Similar patterns of habitat-specific dominance have been reported in mahseer populations, where ecological preferences influence species distribution at local scales. However, given the limited sample size, this interpretation should be considered preliminary and does not constitute conclusive evidence of habitat suitability or population stability.

In the Comal River, both of *T. tambra* (n = 2) and *T. tambroides* (n = 1) were recorded during the sampling period. The *T. tambra* individuals exhibited a relatively wide body size range (0.69-348.01 g, 3.70-29.20 cm). Nevertheless, due to the small number of specimens collected from this site, these observations should be interpreted cautiously and are not intended to reflect relative abundance, population structure, or habitat function. Instead, they represent preliminary, site-specific observations requiring validation through more extensive sampling.

In the Pelus River, both *T. tambroides* and *T. tambra* were recorded, with one individual per species (Table 1). The size range observed in this river was relatively narrow, reflecting limited representation in the dataset. In contrast, the Welo River was characterized exclusively by *T. tambra* (n = 3), with body weights ranging from 22.5 to 234.2 g and total lengths from 13.5 to 27.7 cm. Although multiple size classes were observed in the Comal River, the limited number of specimens precludes robust inference regarding population structure, recruitment, or nursery function.

Overall, variation in species composition and size distribution across rivers indicates spatial heterogeneity in mahseer populations in Central Java. The presence of both juvenile and adult individuals, particularly in the Comal and Welo rivers, may suggest ongoing recruitment and population sustainability. Conversely, uneven species representation among rivers may reflect ecological differences or anthropogenic pressures shaping local population structure. These findings provide important baseline data for understanding population dynamics, habitat preferences, and conservation management of *Tor* spp. in the region.

To strengthen the reliability of species identification and support the observed population patterns, molecular validation using standardized DNA barcode reference systems is essential. The BOLD provides an Identification Engine and Barcode Index Number (BIN) system that clusters COI sequences into species-level units (Ratnasingham and Hebert 2013; Bingpeng et al. 2018). High sequence similarity to voucher-linked references is generally considered reliable for species identification in fishes, especially when supported by BIN concordance (O'Mara et al. 2024; Zhou et al. 2025). Table 2 presents the comparison of sequence data with the BOLD reference system, showing that all fourteen sequences were successfully identified to the species level.

Sequence similarity analysis using the BOLD successfully identified all 14 COI sequences generated in this study to the species level (Table 2). Based on these results, the specimens were classified into two species: *T. tambroides* (n = 8) and *T. tambra* (n = 6). All *T. tambroides* specimens (Pemalang 2, Banyumas 1, and Purbalingga 1–6) showed high sequence similarity ranging from 99.68% to 99.84% relative to reference sequences in BOLD. Similarly, all *T. tambra* specimens (Pemalang 1, Pemalang 3, Pekalongan 1-3, and Banyumas 2) exhibited 100% sequence similarity to reference sequences. . Each COI sequence was deposited in GenBank under accession numbers PZ306284–PZ306297, ensuring traceability and reproducibility of the molecular identification results (Table 2).

The consistently high COI similarity values obtained from BOLD analysis support the effectiveness of DNA barcoding for species identification of mahseer from Central Java. Similarity values exceeding 99.5% are Similarity values exceeding 99.5% are generally regarded as indicative of reliable species-level assignments in fishes, particularly when reference databases are taxonomically curated and supported by voucher specimens (Jaafar et al. 2021; Lim et al. 2021). Consistent with this framework, numerous comparative studies have demonstrated that COI-based DNA barcoding provides high resolution for fish species discrimination due to its universality and strong discriminatory power (Alam et al. 2024; Nazifa et al. 2026).

The clear separation of specimens into *T. tambroides* (99.68-99.84%) and *T. tambra* (100%) based on COI similarity further corroborates the morphological identification and phylogenetic results obtained in this study. Importantly, specimens showing relatively higher genetic distances in the K2P analysis, including those from Purbalingga, were consistently assigned to *T. tambroides* by the BOLD system. This finding helps resolve potential ambiguities arising from morphological similarity and reinforces the robustness of integrative taxonomic approaches combining morphology and molecular data.

Table 2. Comparison of the sequence results with the BOLD system

Specimen code	GenBank accession number	Search result of similarity	Percentage (%)
Pemalang 2	PZ306285	*Tor tambroides*	99.84
Purbalingga 1	PZ306286	*Tor tambroides*	99.68
Purbalingga 2	PZ306287	*Tor tambroides*	99.84
Purbalingga 3	PZ306288	*Tor tambroides*	99.84
Purbalingga 4	PZ306289	*Tor tambroides*	99.84
Purbalingga 5	PZ306290	*Tor tambroides*	99.84
Purbalingga 6	PZ306291	*Tor tambroides*	99.68
Banyumas 1	PZ306284	*Tor tambroides*	99.84
Pemalang 1	PZ306296	*Tor tambra*	100
Pemalang 3	PZ306297	*Tor tambra*	100
Pekalongan 1	PZ306293	*Tor tambra*	100
Pekalongan 2	PZ306294	*Tor tambra*	100
Pekalongan 3	PZ306295	*Tor tambra*	100
Banyumas 2	PZ306292	*Tor tambra*	100

The slightly lower similarity values observed within *T. tambroides* compared to the uniform 100% similarity in *T. tambra* may indicate higher intraspecific mitochondrial variation in *T. tambroides*. This pattern is consistent with findings from previous studies on mahseer and may be associated with a broader geographic distribution, larger effective population size, or a longer evolutionary history within river systems (Kasayev and Arisuryanti 2022).

The concordance among COI similarity, species-based genetic distance analysis, and phylogenetic clustering strengthens confidence in the taxonomic assignments presented in this study. Collectively, these results indicate that the observed genetic divergence patterns reflect true interspecific differentiation rather than misidentification or analytical artifacts. This strengthens the robustness of the molecular dataset and supports its application in biodiversity assessment and conservation of aquatic ecosystems (Shi et al. 2025). Furthermore, the availability and integration of comprehensive barcode reference libraries, such as GenBank and BOLD, enhance species identification accuracy and facilitate cross-study comparability (Alam et al. 2024).

Taken together, these findings highlight the effectiveness of integrative molecular approaches in resolving taxonomic ambiguity and provide a strong foundation for future population-level and conservation studies of mahseer in Indonesia. Further support for species delimitation was obtained from genetic distance (K2P) analysis and phylogenetic reconstruction, as described in the following sections.

Genetic distance matrix

A total of 14 mahseer specimens (*Tor* spp.) from four river systems in Central Java, Comal (n = 3), Welo (n = 3), Tutung Gunung (n = 6), and Pelus (n = 2) were successfully analyzed using COI gene sequences. One Cyprinid species, *B. binotatus*, was included as an outgroup. K2P genetic distance analysis among *Tor* specimens revealed values ranging from 0.0000 to 0.0303 (Table 3).

Most pairwise comparisons among *Tor* specimens revealed very low K2P genetic divergence, ranging from 0.0000 to 0.0017. Particularly low divergence values were observed among specimens from the Pelus and Tutung Gunung rivers, as well as between those from the Welo and Comal rivers. In contrast, genetic distances between two distinct groups of *Tor* specimens were consistently higher, ranging from 0.0285 to 0.0303. Genetic divergence between all *Tor* specimens and the outgroup *B. binotatus* was substantially greater, with values ranging from 0.1652 to 0.1724. Based on COI sequence identification, specimen Purbalingga 6 was assigned to *T. tambroides*.

Accordingly, intraspecific K2P genetic distances within *T. tambroides* (n = 8) ranged from 0.0000 to 0.0035, while those within *T. tambra* (n = 6) were near zero. In contrast, interspecific genetic distances between *T. tambroides* and *T. tambra* consistently ranged from 0.0285 to 0.0303, with no overlap observed between intra- and interspecific values. Overall, Table 3 presents detailed pairwise genetic distance values that support species-level differentiation, while further interpretation is addressed in the Discussion.

The low intraspecific K2P genetic distances observed among *Tor* specimens from different river systems indicate limited mitochondrial differentiation within the sampled populations. Such low divergence is consistent with patterns commonly reported in freshwater fishes, where intraspecific COI genetic distances typically remain below 1-2% (Muhala et al. 2024; Li et al. 2025). This pattern may reflect relatively recent common ancestry, historical connectivity among river systems, or past gene flow associated with drainage rearrangements (Esa and Rahim 2013; Shen et al. 2016). In riverine environments, this genetic homogeneity is often associated with historical hydrological connectivity, downstream dispersal, and migration during flood events (Hopley and Byrne 2019; Neel et al. 2025).

In contrast, interspecific K2P genetic distances between *T. tambra* and *T. tambroides* were consistently higher, ranging from 0.0285 to 0.0303, falling within the expected range for interspecific divergence in fishes, which typically exceeds 2-3% in COI-based analyses (Muhala et al. 2024). The clear separation between low intraspecific and higher interspecific genetic distances indicates the presence of a barcode gap, a key criterion for reliable species discrimination in DNA barcoding studies (Shen et al. 2016). This genetic discontinuity is congruent with species delimitations inferred from COI similarity analysis and phylogenetic reconstruction, confirming that the COI marker effectively resolves these two closely related *Tor* species (Nazifa et al. 2026). Similar barcode gaps between *T. tambra* and *T. tambroides* have been reported in Malaysia (Esa and Rahim 2013), Indonesia (Kasayev and Arisuryanti 2022), and Thailand (Phanklam et al. 2026), including regions where both species occur sympatrically.

The predominance of near-zero K2P genetic distances observed among samples from the Tutung Gunung River (n = 6) indicates minimal mitochondrial variation within these individuals. While such patterns are often associated with homogeneous mitochondrial lineages, the present dataset does not allow direct inference regarding habitat stability or demographic processes. Slightly higher but still low divergence values (up to 3.03%) observed among specimens from different rivers may suggest limited spatial differentiation; however, interpretation should be made cautiously. Such variation may be associated with geographic separation or anthropogenic factors (Jiang et al. 2019), although similar patterns can also persist under fragmented conditions without indicating active population subdivision (Forgiarini et al. 2025). Therefore, these factors are presented as a conceptual context rather than demonstrated processes, particularly given the small sample size and reliance on a single mitochondrial marker. Within this scope, COI-based analyses remain most appropriate for resolving species-level boundaries in mahseer, as widely demonstrated in studies addressing morphologically convergent taxa (Esa and Rahim 2013; Phanklam et al. 2026).

Importantly, mitochondrial homogeneity should not be interpreted as definitive evidence of contemporary connectivity. Previous studies have shown that COI markers may underestimate fine-scale population structure, especially when nuclear markers reveal significant

differentiation among river basins (Esa and Rahim 2013; Nazifa et al. 2026). Accordingly, the present results provide a robust taxonomic baseline but remain insufficient for drawing strong conclusions regarding population connectivity or management units.

The presence of identical haplotypes across geographically distinct rivers may indicate historical hydrological connectivity within the Mount Slamet river system. In freshwater fishes, shared haplotypes across locations are often associated with dispersal through connected waterways, which can reduce genetic structuring and promote population homogeneity (Fullerton et al. 2010; Bingpeng et al. 2018). Similar patterns of low haplotype divergence have been reported in *Tor* populations from Aceh and Jambi (Muchlisin et al. 2022; Nazifa et al. 2026), supporting the observations in this study. More broadly, comparable levels of low intraspecific divergence have been documented in other cyprinid fishes, where average K2P values are typically below 0.5% (Lakra et al. 2015), suggesting that such genetic patterns may be common in freshwater taxa inhabiting interconnected systems.

The substantial genetic distances observed between all *Tor* samples and the outgroup *B. binotatus* (0.1652-0.1724) confirm deep intergeneric divergence. Such values are typical for comparisons across genera, where genetic distances often exceed 10% (Muhala et al 2024). This confirms that *B. binotatus* represents a suitable outgroup for phylogenetic reconstruction (Pinheiro et al. 2025). Comparable levels of divergence have also been reported in other regional studies on *Tor* (Esa and Rahim 2013; Haque et al. 2023).

Although the overall genetic divergence observed in this study was low, it is important to acknowledge the limitations of mitochondrial markers such as COI, which may underestimate fine-scale population structure due to maternal inheritance and relatively conserved evolutionary rates. Previous studies have shown that COI-based analyses may fail to detect subtle genetic differentiation, particularly in recently diverged or highly connected populations (Li et al. 2025). The observed low divergence may therefore reflect high connectivity, but alternative explanations, including cryptic diversity, historical isolation due to geomorphological processes, or stocking-related introductions, cannot be excluded (Imron et al. 2024; Nazifa et al. 2026).

Despite the clear distinction between intra- and interspecific patterns, the relatively small sample size (n = 14) and reliance on a single mitochondrial locus limit the robustness of population-level inference. While sufficient for detecting species boundaries and barcode gaps, this dataset remains inadequate for resolving fine-scale population structure or defining management units. Future studies should expand sampling coverage and incorporate nuclear markers to provide a more comprehensive understanding of genetic differentiation in *Tor* populations in Central Java. Integrating genomic data with geological history will be critical for disentangling the influence of historical drainage evolution and recent anthropogenic fragmentation. Overall, these findings highlight the importance of combining mitochondrial and nuclear approaches to improve the reliability of population genetic inference and to support evidence-based conservation strategies.

Phylogenetic tree

The phylogenetic tree reconstructed from mitochondrial COI gene sequences revealed a clear genetic structure among the 14 mahseer specimens (*Tor* spp.) and reference sequences retrieved from GenBank (Figure 3). *B. binotatus* was included as an outgroup and formed a distinct lineagen with the greatest genetic distance relative to all *Tor* taxa, confirming deep intergeneric divergence. All mahseer specimens clustered within a well-supported monophyletic clade, indicating close genetic relationships among individuals sampled from different river systems in Central Java.

The phylogenetic reconstruction showed a clear separation into two major clades corresponding to *T. tambra* and *T. tambroides*. Specimens of *T. tambra* from Comal and Welo rivers clustered consistently with reference sequences from GenBank, supported by high bootstrap values (≥97), indicating strong phylogenetic stability and concordance with molecular identification. In contrast, the *T. tambroides* clade consists predominantly of specimens from Tutung Gunung and Pelus rivers, which clustered closely with conspecific reference sequences. Branches lengths within this clade were generally short and supported by moderate to high bootstrap values, indicating high genetic homogeneity among individuals across sampling locations. This clear separation confirms the effectiveness of COI barcoding in distinguishing closely related mahseer species despite known morphological overlap (Jaafar et al. 2021; Lim et al. 2021). The outgroup (*B. binotatus*) formed a distinct lineage with long branch lengths, reflecting substantial intergeneric divergence and supporting the robustness of the phylogenetic reconstruction (Pinder et al. 2019; Pinheiro et al. 2025). Such patterns are expected for species from different genera within Cyprinidae, which typically exhibit high mitochondrial divergence.

Several reference sequences from other *Tor* species (e.g., *T. tor*, *T. mosal*, *T. barakae*, *T. putitora*, *T. douronensis*, *T. mussullah*, and *T. malabaricus*) formed separate branches outside the two principal clades, with variable bootstrap support. This pattern reflects their more distant phylogenetic relationships and confirms that the Mount Slamet specimens are not closely related to these taxa. Such separation is consistent with previous molecular phylogenetic studies showing that *Tor* species often exhibit deep genetic divergence associated with geographic distribution and distinct evolutionary histories (Walton et al. 2017; Jaafar et al. 2021).

Table 3. Pairwise genetic distance matrix based on the K2P model calculated from mitochondrial COI sequences of *Tor* spp. collected from four foothill rivers of Mount Slamet, Central Java, Indonesia. The table presents intraspecific and interspecific genetic distances among 14 specimens of *Tor tambroides* and *Tor tambra*, with *Barbodes binotatus* used as an outgroup. Intraspecific distances are generally very low, while higher values between species indicate clear species-level divergence

Specimen code	Species	BMS1	BMS2	PBG1	PBG2	PBG3	PBG4	PBG5	PBG6	PKL1	PKL2	PKL3	PML1	PML2	PML3	BB
BMS1	*Tor tambroides*	-	0.0285	0.0017	0.0000	0.0000	0.0000	0.0000	0.0017	0.0285	0.0285	0.0285	0.0285	0.0000	0.0285	0.1676
BMS2	*Tor tambra*	0.0285	-	0.0303	0.0285	0.0285	0.0285	0.0285	0.0266	0.0000	0.0000	0.0000	0.0000	0.0285	0.0000	0.1724
PBG1	*Tor tambroides*	0.0017	0.0303	-	0.0017	0.0017	0.0017	0.0017	0.0035	0.0303	0.0303	0.0303	0.0303	0.0017	0.0303	0.1697
PBG2	*Tor tambroides*	0.0000	0.0285	0.0017	-	0.0000	0.0000	0.0000	0.0017	0.0285	0.0285	0.0285	0.0285	0.0000	0.0285	0.1676
PBG3	*Tor tambroides*	0.0000	0.0285	0.0017	0.0000	-	0.0000	0.0000	0.0017	0.0285	0.0285	0.0285	0.0285	0.0000	0.0285	0.1676
PBG4	*Tor tambroides*	0.0000	0.0285	0.0017	0.0000	0.0000	-	0.0000	0.0017	0.0285	0.0285	0.0285	0.0285	0.0000	0.0285	0.1676
PBG5	*Tor tambroides*	0.0000	0.0285	0.0017	0.0000	0.0000	0.0000	-	0.0017	0.0285	0.0285	0.0285	0.0285	0.0000	0.0285	0.1676
PBG6	*Tor tambroides*	0.0017	0.0266	0.0035	0.0017	0.0017	0.0017	0.0017	-	0.0266	0.0266	0.0266	0.0266	0.0017	0.0266	0.1652
PKL1	*Tor tambra*	0.0285	0.0000	0.0303	0.0285	0.0285	0.0285	0.0285	0.0266	-	0.0000	0.0000	0.0000	0.0285	0.0000	0.1724
PKL2	*Tor tambra*	0.0285	0.0000	0.0303	0.0285	0.0285	0.0285	0.0285	0.0266	0.0000	-	0.0000	0.0000	0.0285	0.0000	0.1724
PKL3	*Tor tambra*	0.0285	0.0000	0.0303	0.0285	0.0285	0.0285	0.0285	0.0266	0.0000	0.0000	-	0.0000	0.0285	0.0000	0.1724
PML1	*Tor tambra*	0.0285	0.0000	0.0303	0.0285	0.0285	0.0285	0.0285	0.0266	0.0000	0.0000	0.0000	-	0.0285	0.0000	0.1724
PML2	*Tor tambroides*	0.0000	0.0285	0.0017	0.0000	0.0000	0.0000	0.0000	0.0017	0.0285	0.0285	0.0285	0.0285	-	0.0285	0.1676
PML3	*Tor tambra*	0.0285	0.0000	0.0303	0.0285	0.0285	0.0285	0.0285	0.0266	0.0000	0.0000	0.0000	0.0000	0.0285	-	0.1724
BB	*Barbodes binotatus*	0.1676	0.1724	0.1697	0.1676	0.1676	0.1676	0.1676	0.1652	0.1724	0.1724	0.1724	0.1724	0.1676	0.1724	-

Note: BB: *Barbodes binotatus* (outgroup), BMS: Banyumas, PBG: Purbalingga, PKL: Pekalongan, PML: Pemalang

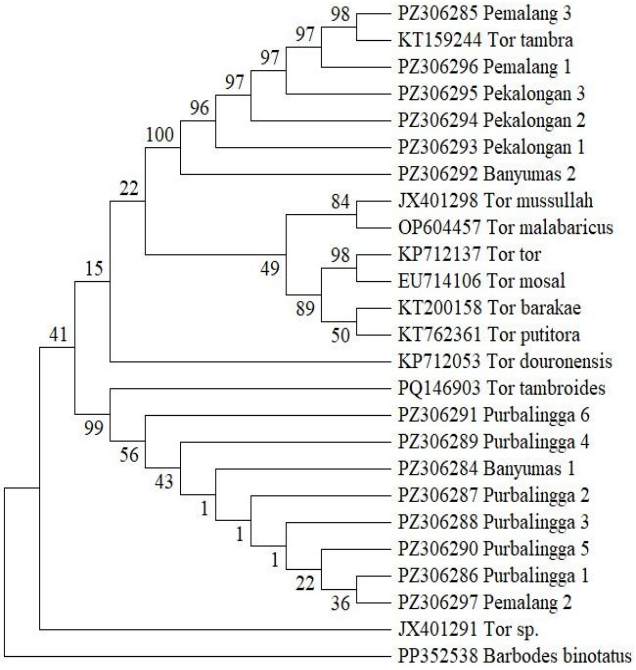

Figure 3. Maximum likelihood phylogenetic tree based on mitochondrial COI sequences (approximately 670-707 bp) showing relationships among 14 mahseer specimens (*Tor* spp.) from four foothill rivers of Mount Slamet, Central Java, Indonesia, and reference sequences obtained from GenBank. Two well-supported clades corresponding to *Tor tambroides* and *Tor tambra* are recovered, with *Barbodes binotatus* used as an outgroup. Bootstrap support values (≥50%) are shown at the nodes

In particular, analyses based on mitochondrial COI and *Cyt b* genes have repeatedly shown that several nominal *Tor* species form well-supported, independent lineages, rather than clustering within a single monophyletic group (Pavan-Kumar et al. 2016; Phanklam et al. 2026). Therefore, the distinct placement of these reference taxa further supports the genetic uniqueness of the Mount Slamet population and strengthens the inference that it represents a separate evolutionary lineage within the genus *Tor*.

Within each species-level clade , branch lengths were generally short, indicating low intraspecific genetic divergence, as phylogenetic branch length reflects accumulated nucleotide substitutions (Beck et al. 2022). This observation is consistent with the genetic distance analysis, which revealed minimal sequence variation and widespread haplotype sharing among individuals from different rivers. Such patterns are commonly associated with low mitochondrial divergence in freshwater fish populations (Shen et al. 2016). Similar trends have been reported in *Tor* species, where low intraspecific divergence reflects genetic connectivity among geographically separated populations (Jaafar et al. 2021; Phanklam et al. 2026).

Despite clear species-level separation, finer-scale geographic structuring among rivers was weak, as indicated by limited differentiation within each clade and stronger bootstrap support at higher taxonomic levels. This suggests that mitochondrial COI data alone provide limited resolution for detecting phylogeographic structure. While such patterns are often interpreted in relation to shared mitochondrial lineages or historical hydrological connectivity, the present dataset does not allow direct inference regarding contemporary gene flow or connectivity within the Mount Slamet river system. Comparable findings from Java and Malaysia have similarly interpreted low mitochondrial divergence cautiously, attributing it to recent common ancestry rather than definitive population structuring (Walton et al. 2017; Jaafar et al. 2021).

The close relationship between *T. tambra* and *T. tambroides* observed in this study is consistent with previous molecular evidence indicating that these species are closely related within the genus. Both phylogenomic and mitochondrial studies have demonstrated that low genetic differentiation at mitochondrial loci is common among *Tor* species, highlighting the limitations of single-marker approaches for resolving fine-scale evolutionary relationships (Lim et al. 2021; Surachat et al. 2022). In the present dataset, interspecific genetic distances (~2.8–3.0%) fall within the expected range for closely related mahseer species, supporting species-level discrimination while underscoring the limited resolution of mitochondrial markers for population analysis.

Overall, the molecular evidence derived from COI barcoding consistently supports species-level differentiation between *T. tambroides* and *T. tambra* across the foothill rivers of Mount Slamet. The combination of high sequence similarity, very low intraspecific K2P genetic distances, and markedly higher interspecific divergence demonstrates a clear and reproducible pattern of mitochondrial differentiation. These patterns are consistent across analytical approaches, including similarity analysis (Table 2), genetic distance matrix (Table 3), and phylogenetic reconstruction (Figure 3), supporting robust species delimitation based on COI barcoding (Hebert et al. 2003; Muchlisin et al. 2022).

At the same time, patterns of low intraspecific divergence and shared or near-identical haplotypes among rivers should be interpreted cautiously. While such patterns are often associated with shared mitochondrial lineages or historical connectivity, this study does not directly test the underlying processes. The limited sample size and reliance on a single mitochondrial marker constrain the resolution of fine-scale population structure and preclude strong inference regarding contemporary gene flow or demographic connectivity.

This study presents the first COI-based molecular assessment of mahseer (*Tor* spp.) from four foothill rivers of Mount Slamet, Central Java, Indonesia. Two species, *T. tambroides* (n = 8) and *T. tambra* (n = 6), based on COI sequences (~670-707 bp) from 14 individuals across four rivers in Central Java. Genetic analysis revealed very low intraspecific divergence (0.0000-0.0035) and clear interspecific divergence (0.0285-0.0303), forming a distinct barcode gap that supports robust species delimitation. Phylogenetic analysis further confirmed separation into two well-supported clades, with weak phylogeographic

structuring and shared haplotypes indicating limited mitochondrial differentiation among river systems. The congruence between genetic distance values and phylogenetic topology further supports the reliability of the molecular results obtained. Nevertheless, more robust inference of population structure and delineation of conservation units requires broader sampling and the incorporation of additional genetic markers. Future studies should expand sampling across river basins and incorporate nuclear or genomic markers to better resolve population connectivity, evolutionary history, and conservation units of *Tor* species in Central Java. Overall, this study provides an essential baseline for future research on mahseer diversity and conservation in Java.

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