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Morphology and Molecular Identification of Cyprinidae from the Sumber Umbulan Ngenep, Malang Regency, East Java

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ABSTRACT

The genus Tor comprises freshwater fish belonging to the Cyprinidae family, recognized for their remarkable diversity and broad distribution across Southeast Asia, including Indonesia. Despite this, data on Tor species in certain regions are limited, highlighting the need for additional research to confirm their presence. Therefore, this study aims to examine the genetic diversity and phylogenetic reconstruction of the species observed in Sumber Umbulan Ngenep using the COI gene as a DNA barcode marker. The research was conducting using the Purposive Random Sampling method and fin tissues were preserved in 96% ethanol for DNA analysis. The species found at Sumber Umbulan Ngenep were observed morphologically using a phenetic approach. Fins from each fish sample were collected and subjected to PCR, sequence analysis using the BOLD System, phylogenetic tree reconstruction, ABGD analysis, and QR barcoding. The results of morphological, genetic, and phylogenetic tree reconstruction based on the COI gene indicate that three species have been identified at Sumber Umbulan Ngenep, namely *Tor douronensis*, *Tor tambra*, and *Barbonymus gonionotus*. Based on the phenetic approach, three apomorphies and five automorphies were identified, indicating the relatedness of the observed species with the reference species. The phylogenetic tree topology shows an unambiguous branching pattern for the observed sample cluster with *Poecilia reticulata* as the outgroup. This study is the first to identify the species present at Sumber Umbulan Ngenep, Malang Regency, East Java, Indonesia. The findings provide new information about the existence of these species and can serve as a basis for future conservation and management strategies.

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INTRODUCTION

The Tor fish has various local names in different regions of Indonesia, such as ikan dewa, sengkaring, semah, and kancra. This fish was listed as endangered on the IUCN Red List in 2018. Tor sp. in Indonesia can also be used as an icon or identity for several tourist attractions, such as springs. Utilizing this fauna increases conservation value and tourist appeal while supporting environmental conservation. The Tor sp. fish can also serve as a bioindicator due to its ability to survive in high-salinity water, indicating good water quality. The Tor sp. fish is commonly found in clear rivers with rocky areas, sandy riverbeds, high oxygen levels, and fast-flowing water (Dwirastina & Wibowo, 2022; Subagja & Marson, 2017).

Genus Tor is one of the most highly valued genera in the Cyprinidae Family. However, its natural populations are under threat due to environmental degradation. Its morphological similarity to other *Tor* species often leads to identification challenges, resulting in limited data availability. These difficulties in accurate species identification hinder effective conservation initiatives. To mitigate population decline, both habitat preservation and species-specific conservation measures are essential.

In freshwater fish, adaptations to ensure survival can be observed morphologically and physiologically. Specifically in Tor fish, Barus et al. (2023) found genetic and morphological variations in Tor fish corresponding to habitat differences. Morphological observations include meristic and morphometric measurements. Morphological variations in fish are measured using morphometric observations (Seno et al., 2024). Morphometric data are important for determining the suitability of fish samples and identifying and describing patterns of morphological variation and growth patterns, both between populations and between species. Morphometric-meristic characters are often used as a guide in identifying fish genera or species, providing information on the genetic status of a fish population based on similarities or differences in body shape (Ath-thar et al., 2018). Meanwhile, the use of DNA barcoding helps verify errors in taxonomic determination, as some fish species often experience ambiguity in their taxonomic classification.

Several studies have highlighted the effectiveness of DNA barcoding in species identification in fish populations in Indonesia. For example, Rahayu et al. (2009) used COI DNA barcode genetic markers to successfully identify three fish species in Telaga Sari. This was supported by Muchlisin et al. (2022) who reported that based on the COI gene, only two valid species of Tor were found in the waters of Aceh. Methods for conservation strategies using DNA barcoding are considered more accurate and effective for species identification techniques (Kusuma et al., 2021). The protein-coding gene used for species identification is the Cytochrome C Oxidase Subunit I (COI) gene. COI is a short gene sequence that aids in constructing phylogenetic trees and can serve as a marker gene during DNA barcoding (Afifah et al., 2021; Sulung et al., 2024). The COI gene is part of the mtDNA genome and is known to rarely undergo deletions or insertions in its sequence, making it suitable as a DNA barcode for identifying each species (Tindi et al. 2017). To facilitate species identification, the results of DNA barcoding are formatted into DNA QR barcodes.

Tor spp. fish can be found in Sumber Umbulan Ngenep, a clear, slightly bluish spring with a rocky and sandy bottom. Several other local fish species are also suspected to inhabit the spring. The characteristic of these fish living in groups and the high degree of morphological similarity among species within the group make species identification more difficult. The location of Sumber Umbulan, which is still preserved by local culture, limits the discovery of these fish to their known locations, and there is no inventory related to the taxonomic identification of each species. This is an important concern that requires immediate research on the taxonomic status of these fish through morphological and molecular observations.

This study aimed to preserve and introduce protected local fish by identifying fish species and characterizing the morphology, interspecific relationships, profiles, and haplotypes of fish found in Sumber Umbulan that have not yet been described. This study is the first to characterize the molecular sequence of partial mitochondrial DNA based on the COI gene from fish in East Java and describe the complete nucleotide base composition and its phylogenetic relationships. The results of this study align with SDG 14 (*Life Below Water*), as they support the conservation of the Tor fish which is a potential aquaculture species in East Java.

MATERIALS AND METHODS

Time and Location

Fish samples were collected from Sumber Umbulan Ngenep, Karangploso District, Malang Regency, East Java (Figure 1). Sampling was conducted using a fishing net to catch the fish. This study used the Purposive Random Sampling method, in which 20 individuals were selected from each identified species among all fish found in Sumber Umbulan for further analysis. The samples of fin tissues obtained were put into sample bottles filled with 96% absolute ethanol specifically for DNA.

Procedure

Morphological observation

Morphological observations were conducted by direct identification in the field. Morphological character measurements were performed on 21 characters, as shown in Figure 2. Using a ruler,

following the protocol of (Rahayu et al., 2013). At the same time, diagnostic characters of *Tor* spp., namely fin shape, were also observed.

DNA Extraction

Fish fin samples (20 mg) were ground using liquid nitrogen until fine, then placed into a 1.5 ml tube. A total of 200 µl GT1 buffer was added, and the sample was homogenized using a vortex. Next, 200 µl of GT2 buffer and 20 µl of Proteinase K were added, and the mixture was homogenised with a vortex. The mixture was then incubated at 56°C for 10 minutes, with the tube inverted every 5 minutes during incubation. Next, 200 µl absolute ethanol was added and briefly vortexed. The mixture was transferred to a spin column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded, and 500 µl buffer W1 was added to the spin column, followed by centrifugation at 13,000 rpm for 1 minute. After discarding the flow-through, 700 µl buffer W2 (prepared with ethanol) was added and centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded again, and the sample was centrifuged for an additional 2 minutes at 13,000 rpm. The isolated DNA was transferred to a new 1.5 ml tube on the Spin Column, adding 50–100 µl elution buffer, incubating at room temperature for 1 minute, and centrifuging at 13,000 rpm for 1 minute. The spin column was discarded, and the purified DNA was stored at –20 °C for short-term use or at –70 °C for long-term storage.

DNA amplification

DNA amplification was performed using a Biorad PCR machine in a 30 µl reaction mixture containing 15 µl Nexpro PCR Master Mix, 3 µl DNA template sample (100 ng/µl), 6 µl water, and 3 µl primer (10 pmol of each forward and reverse primer). The primers used were LCO1490 5'GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA3'. Amplification was performed with the following temperature settings: pre-denaturation at 94 °C for 1 minute, followed by 40 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 41°C for 45 seconds, and extension at 72 °C for 1 minute 30 seconds. Next, a post-elongation process was performed at 72°C for 10 minutes. The PCR results were then electrophoresed on 1% agarose. The PCR results were subsequently sequenced using 1stBASE Laboratories Sdn Bhd sequencing services.

Data analysis

Morphology

Based on the results of morphological observations, descriptions of morphometry, meristics, and diagnostic characters of each species found were obtained. The morphometric measurement data were tested using SPSS and showed that the data were not normally distributed (p-value < 0.05).

The next step was to conduct a nonparametric (Kruskal-Wallis) test to determine whether there were significant differences in morphometry. Meristic data were analyzed by forming a dendrogram to illustrate the degree of relatedness between species groups, indicated by percentage units (Sabran et al., 2021).

DNA barcode

This study obtained partial COI gene sequences from 3 samples of Tor douronensis, two samples of Tor tambra, and three samples of Barbonymus gonionotus from Sumber Umbulan Ngenep, Malang Regency, East Java, Indonesia, which constitute the final dataset. Each sequence was pseudogenes by translating it into an amino acid sequence following the protocol outlined by (Muhala et al., 2008; Slectova et al., 2021; Song et al., 2008). The next step was to use Finch TV software to analyze the chromatograms. The translated amino acid sequences were then verified through the ExPASy website (https://web.expasy.org/translate/). The sequences were compared with data in the GenBank database using the BOLD System website (https://id.boldsystems.org/) (Dailami et al., 2025). A phylogenetic tree reconstruction was performed using data from four sequences, where the ingroup and outgroup accessions were obtained from the GenBank database available at the National Centre for Biotechnology Information (NCBI). Sequence alignment was performed using Clustal X (Fauzi et al., 2021) and manually verified using BioEdit software. The partial COI gene sequences from the fish samples in this study were registered in GenBank with the corresponding accession numbers (Table 4).

Phylogenetic tree reconstruction

Phylogenetic reconstruction was performed to organize the grouping of different species with

related species. In-group and out-group gene sequences obtained from the National Centre for Biotechnology Information (NCBI) (Table 1) were downloaded and aligned using Clustal X 2.1 software. The alignment results were used to reconstruct the phylogenetic tree using MEGA XI.0 software (Kumar et al., 2018). Phylogenetic tree reconstruction was performed using the Neighbour-Joining (NJ) and Maximum Likelihood (ML) methods with a bootstrap value of 1000x. The genetic distance matrix was calculated using the Kimura 2-parameter model. The Kimura 2-parameter model was chosen in phylogenetics to determine the similarity between sequences. It has the advantages of being fast and easy to analyze a group of sequences (Rafsanjani et al., 2024). The similarity values between sequences were calculated using Microsoft Excel. Next, the similarity percentage was calculated using the formula proposed by (Purba & Sitorus, 2018), namely (1-dist) x 100%.

Deliminasi Spesies dengan ABGD

Species Delimitation with ABGD

Molecular approaches based on objective criteria that propose species delineation based on genetic data are commonly used to produce accurate results in species diversity studies. A technique that helps identify species boundaries by examining significant genetic distances among individuals is the Automatic Barcode Gap Discovery (ABGD) method (Alghamdi et al., 2021). Species boundaries were analyzed from the COI gene of the three identified fish species. In the initial stage, an uncorrected pairwise distance matrix was constructed using MEGA software, with ambiguous positions in sequence pairs excluded. This was then analyzed using the Jukes-Cantor and K2P models on an online website accessible at http://wwwabi.snv.jussieu.fr/public/abgd/. These models were used to calculate genetic distances, considering different assumptions about the nucleotide substitution rate (Ambarwati et al., 2025). This method was used to find the point at which the distribution of genetic distances showed a clear separation, indicating the presence of a potential species boundary.

DNA QR Barcode

QR Barcode DNA is very effective in reducing species identification errors and can be easily accessed by the public through digital devices, such as smartphones (Khan et al., 2017; Khrishna & Dugar, 2016). The nucleotide base sequences obtained from sequencing were stored and converted into QR codes using QR code creation software or an online generator (Naulia, 2015). After the QR code was successfully generated, verification was conducted using a QR code scanner, either through an app on a mobile device or via an online scanning platform. This process aims to ensure that the generated QR code can be read accurately and adequately displays the DNA sequence information or related data, without any errors in conversion or loss of information.

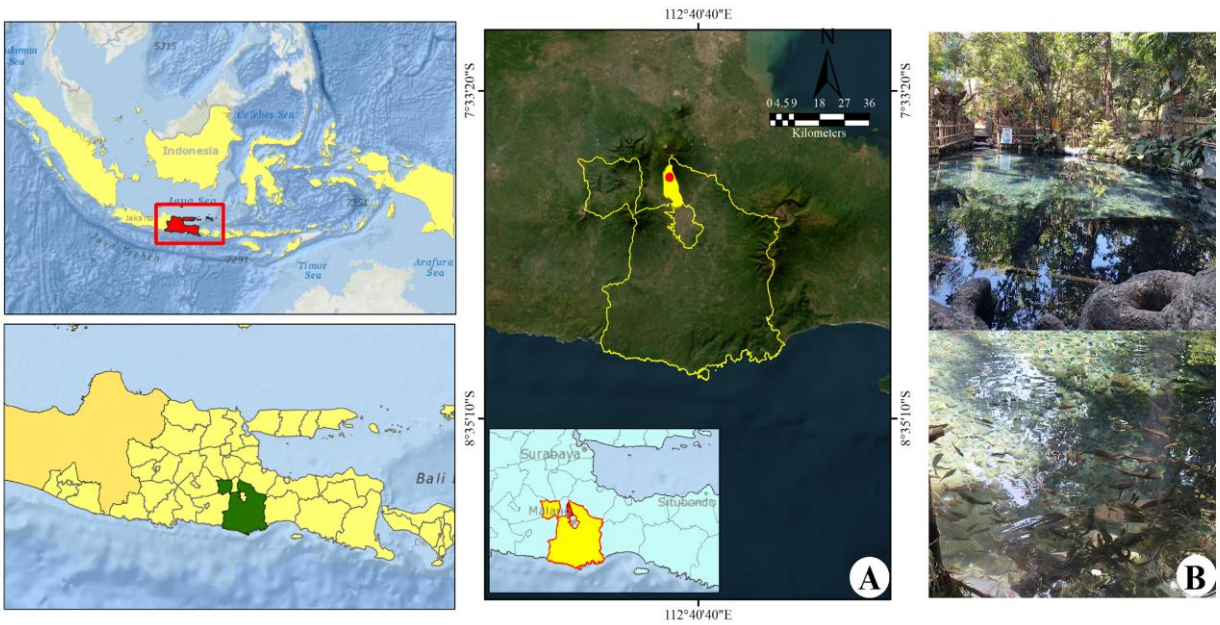

Figure 1. Sampling site A. Umbulan Ngenep in Karang Ploso District, Malang Regency, East Java, Indonesia, and B. Habitat showing the location of fish sampling

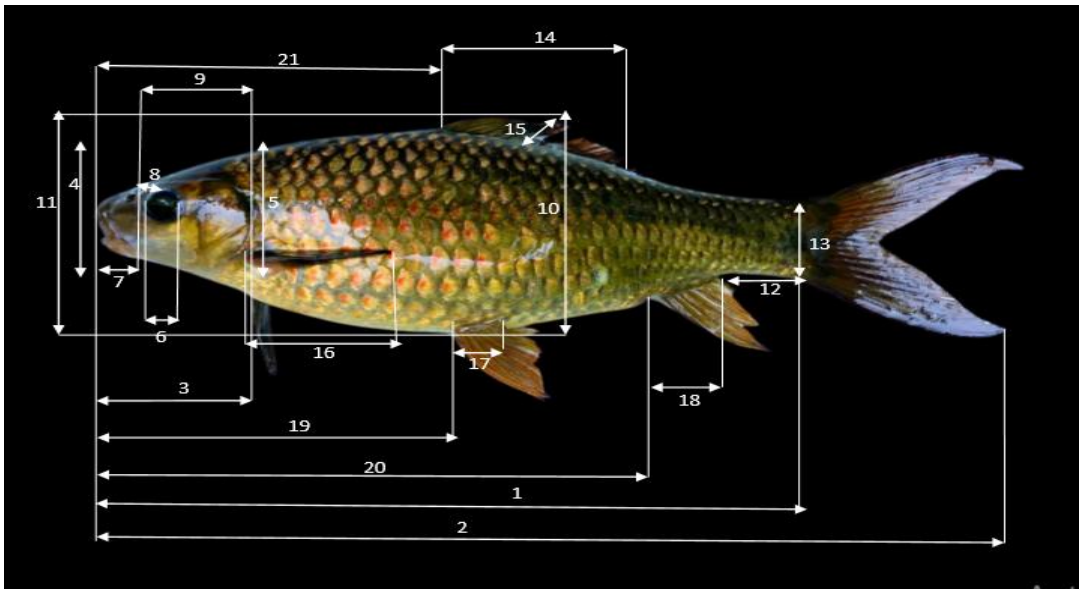

Figure 2. Morphometric Measurement Scheme for Fish at Sumber Umbulan Ngenep, East Java, Indonesia. Notes: 1. standard length (SL); 2. total length (TL); 3. head length (HL); 4. head width (HW); 5. head height (HD); 6. eye diameter (ED); 7. snout length (SNL); 8. interorbital distance (IW); 9. head length without snout (PKTM); 10. body height (BD); 11. body width (BW); 12. Caudal fin length (CPL); 13. Dorsal fin height (DFH); 16. Pectoral fin length (PL); 17. Length of ventral fin base (VBL); 18. Length of anal fin base (ABL); 19. Length before dorsal fin (PPL); 20. Length before anal fin (PAL); 21. Length before dorsal fin (PDL) (Rahayu et al., 2013)

Table 1. DNA Sequences from NCBI Genebank

Species	Acc number genbank	Location
Barbonymus gonionotus	PV570356	Sumber Umbulan Ngenep, Lawang
Barbonymus gonionotus	PV576115	Sumber Umbulan Ngenep, Lawang
Barbonymus gonionotus	PV576116	Sumber Umbulan Ngenep, Lawang
Barbonymus gonionotus	PQ469932.1	China
Tor douronensis	PV576247	Sumber Umbulan Ngenep, Lawang
Tor douronensis	PV576262	Sumber Umbulan Ngenep, Lawang
Tor douronensis	PV576263	Sumber Umbulan Ngenep, Lawang
Tor douronensis	DQ532812.1	Malaysia
Tor tambra	PV576316	Sumber Umbulan Ngenep, Lawang
Tor tambra	PV576512	Sumber Umbulan Ngenep, Lawang
Tor tambra	KT159244.1	Malaysia
Poecilia reticulata	JX968696.1	India

RESULTS AND DISCUSSION

The results showed the existence of a fish species with the local name as Sengkaring, Tambra, and Bader. The taxonomic status of the fish can be determined based on morphological characters supported by genetic analysis. The taxonomic status of these three fish can be determined based on morphological characteristics supported by genetic analysis. The main parameters used as the basis for morphological identification are

General morphological, morphometric, and meristic characteristics. It is suspected that fish belong to the genus Tor, so fin characteristics are important for identifying fish and the main parameters for identification. The taxonomic status of *Tor* spp. was clarified and confirmed with reference species from BPPBAT, Cijeruk, Bogor, Weber & Beaufort and Haryono.

***Tor douronensis* (Valenciennes, 1842)**

Class: Actinopterygii (Cope, 1871)
Order: Cypriniformes (Nelson, 1994)
Family: Cyprinidae (Cavender, 1991)
Genus: Tor (Karaman, 1997)
Species: *Tor douronensis*

Description of morphological characteristics was based on the identification key book by Weber & Beaufort, 1916 of the characteristics of Tor douronensis (Figure 3) found in Sumber Umbulan Ngenep: the dorsal fin has three hard rays and 9 soft rays (DIII.9); the anal fin has 3 hard rays and 6 soft rays (AIII.6); the pectoral fin has 1 hard ray and 16 soft rays (PI.16); the ventral fin has 2 hard rays and 8 soft rays (VII.8); there are 24–28 scales on the lateral line. Thick lips, continuous, with a long flap that does not reach the corner of the mouth. Dorsal fin concave; ventral fins rounded; anal fin angular; caudal fin forked; mouth subterminal; scales cycloid and large; caudal peduncle surrounded by 10 scales; body surrounded by 11 scales. The beginning of the dorsal fin is opposite the 7th-8th scales of the lateral line, and is located in front of the pelvic fin, 8-9 scales in front of the dorsal fin. The last dorsal fin rays are equal to or longer than the length of the head without the snout. The body colour is golden yellow.

Distribution of the fish Tor douronensis is quite widespread in Indonesia. It is found in Telaga Rambut Monte Blitar, Telaga Banyu Biru Pasuruan (Rahayu et al., 2014) and the Batang Ulakan protected area in Padang Pariaman District (Endryeni & Amrullah, 2018). The fish Tor douronensis is also commonly found in the waters of Malaysia and Thailand.

Body size of 20 individuals was found, standard length (SL) ranged from 16.3 to 19.2 cm, with an average of 17.54 cm. Total length (TL) ranged from 20.6 to 25.4 cm, averaging 21.64 cm.

***Tor tambra* (Valenciennes, 1842)**

Class: Actinopteri (Cope, 1871)
Order: Cypriniformes (Nelson, 1994)
Family: Cyprinidae (Cavender, 1991)
Genus: Tor (Karaman, 1997)
Species: *Tor tambra*

Description of the characteristics of tambra (Figure 4) found are dorsal fins with 4 hard rays and 8–9 soft rays (DIV.8–9); anal fins with 3 hard rays and 5–6 soft rays (AIII.5–6); the pectoral fin has 1 hard ray and 14-16 soft rays (PI.14-16); the ventral fin has 2 hard rays and 8 soft rays (VII.8); there are 22-24 scales on the lateral line. Thick lips, continuous, with short lobes that do not reach the corners of the mouth. Dorsal fin concave; ventral fins rounded; anal fin angular; caudal fin forked; subterminal mouth; cycloid scales, large; caudal peduncle surrounded by 8 scales; body surrounded by 12 scales. The beginning of the dorsal fin is opposite the eighth scale of the lateral line and is located in front of the pelvic fin, with eight scales in front of the dorsal fin. The last dorsal fin rays are shorter than the length of the head without the snout. The body colour is golden yellow.

Distribution The distribution of tambra fish is extensive. This fish is also found in Malaysia and Thailand. In Indonesia, the tambra fish can be found in Telaga Banyu Biru Pasuruan (Rahayu et al., 2014), waters in Aceh (Almunadiya et al., 2023), and West Java (Amanda et al., 2023). This species is also widely distributed in the waters of Sumatra, Malaysia, Burma, Thailand, and Indochina.

Body size of 20 individuals found, standard length (SL) ranged from 10.6 to 12.2 cm, with an average of 11.40 cm. Total length (TL) ranged from 14.6 to 16.5 cm, averaging 15.79 cm.

***Barbonymus gonionotus* (Bleeker, 1849)**

Class: Actinopterygii (Cope, 1871)
Order: Cypriniformes (Nelson, 1994)
Family: Cyprinidae (Cavender, 1991)
Genus: Barbonymus (Kottelat, 1999)
Species: *Barbonymus gonionotus*

Description of the characteristics of the bader (Figure 5) was based on field observations. The species is characterized by dorsal fin has 2 hard rays and 8-9 soft rays (DII.8-9); the anal fin has 1 hard ray and 6 soft rays (AI.6); the pectoral fin has 1 hard ray and 6–10 soft rays (PI.6–10); the ventral fin has 1 hard ray and 8 soft rays (VI.8); there are 27–29 scales on the lateral line. The lips are thick, continuous, and lack a lobe. Dorsal fin concave; ventral fins angular; anal fin angular; caudal fin forked; terminal mouth; cycloid scales, large; caudal peduncle surrounded by 10 scales; body surrounded by 18 scales. The base of the dorsal fin is opposite the ninth scale of the lateral line, and its position is before the pelvic fin, with 10 scales in front of the dorsal fin. The hard rays of the last dorsal fin are parallel to the length of the head, without the snout. The body colour is silvery, darker, and there is red colour on some tips of the fins.

Distribution The habitat characteristics of *Barbonymus gonionotus* include rocky bottoms,

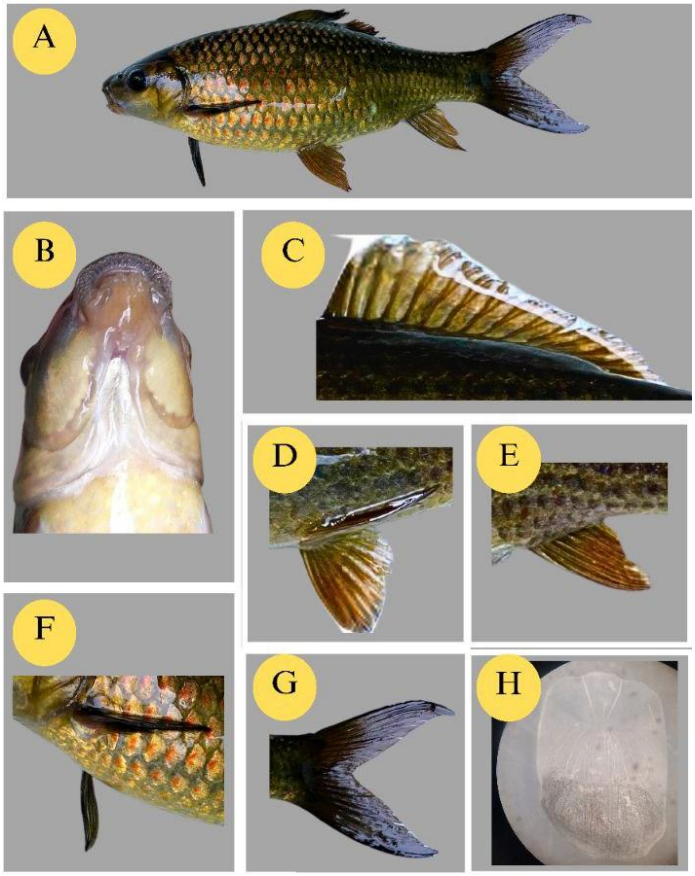

Figure 3. Morphological characteristics of the Tor douronensis fish: A. Side view, B. Lobe reaching the corner of the mouth, C. Dorsal fin, D. Ventral fin, E. Anal fin, F. Pectoral fin, G. Caudal fin, H. Cycloid scales.

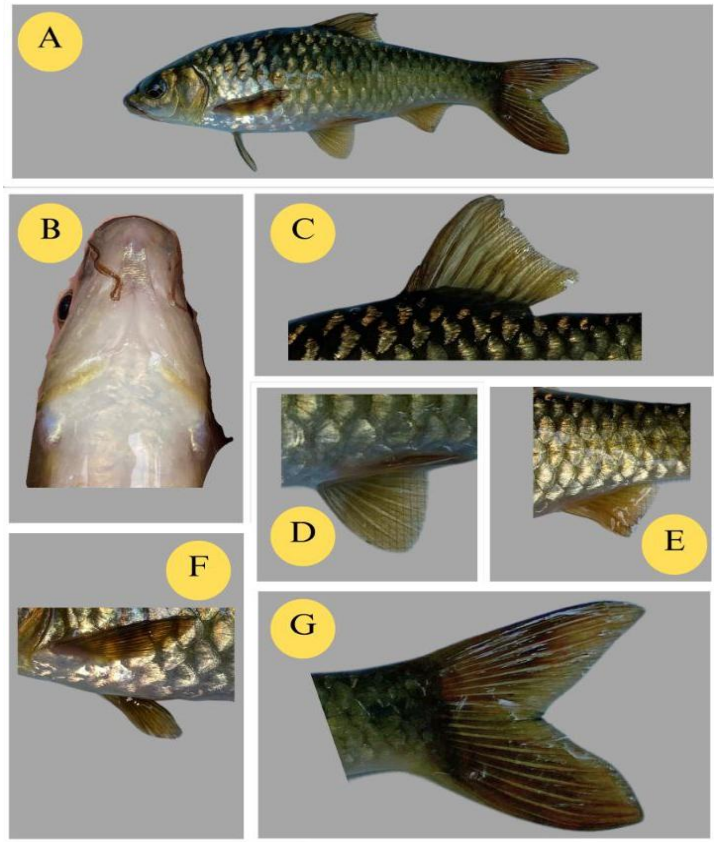

Figure 4. Morphological characteristics of the Tor tambra fish: A. Side view, B. Lips reaching the corner of the mouth, C. Dorsal fin, D. Ventral fin, E. Anal fin, F. Pectoral fin, G. Caudal fin.

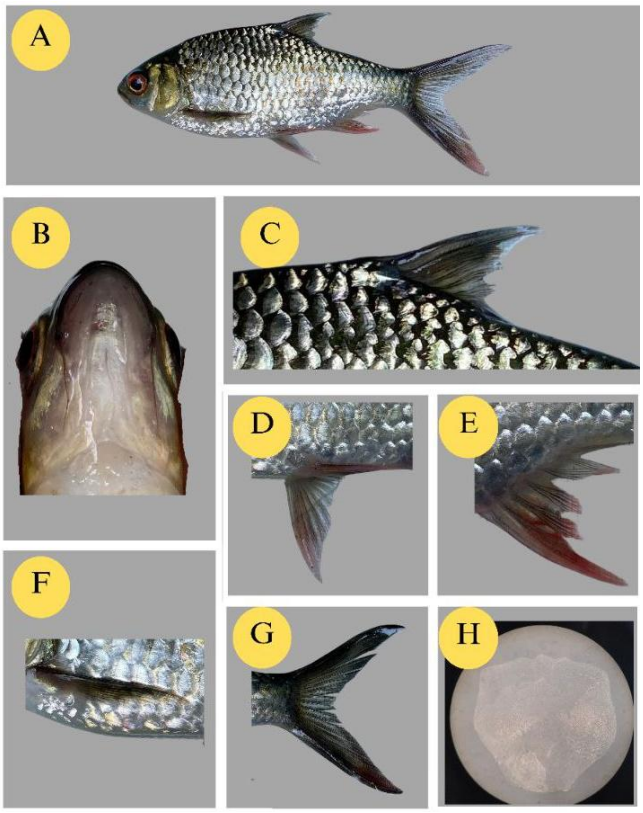

Figure 5. Morphological characteristics of the fish *Barbonymus gonionotus*, A. Side view, B. No fins, C. Dorsal fin, D. Ventral fin, E. Anal fin, F. Pectoral fin, G. Caudal fin, H. Cycloid scales

strong currents, a substrate mainly of sand and gravel, and relatively high dissolved oxygen content. This fish thrives in water temperatures ranging from 25 to 33°c (Nasution & Machrizal, 2021). The distribution range of this fish includes Sumatra, Sulawesi, Java, Malaysia, Kalimantan, the Philippines, Cambodia, Myanmar, Laos, Vietnam, Thailand, and northern Australia). In Java, this fish is found in several locations, including the Brantas River (Hayati et al., 2017), Serayu (Haryono et al., 2017), Surabaya (Lestari et al., 2021), and Semarang Regency (Adi et al., 2023).

Body size of 20 individuals was found, standard length (SL) ranged from 13.7 to 15.2 cm, with an average of 14.39 cm. Total length (TL) ranged from 15.0 to 20.0 cm, averaging 18.05 cm.

The fish observed were *Tor duoronensis* and *Tor tambra*. Both fish have fins, as an important characteristic of *Tor* fish. The differences between the two lies in the fin rays. The sengkaring fish samples share similarities with the reference species *Tor douronensis* from KKP, Cijeruk, Bogor, including fin shape, number of pectoral fin rays, number of dorsal fin rays, number of ventral fin rays, snout shape, number of scales on the lateral line opposite the base of the dorsal fin, number of scales in front of the dorsal fin, and a shorter snout compared to the upper jaw. The tambra fish resembles the reference species Tor tambra (Weber & Beaufort, 1916; Haryono & Tjakrawidjaja, 2006), including characteristics: fin shape that does not reach the corner of the mouth, the number of pectoral fin rays, the number of dorsal fin rays, the number of ventral fin rays, the last dorsal fin ray being shorter than the head length without the snout, the dorsal fin origin in front of the ventral fin, and the snout length being longer than the upper jaw.

Barbonymus gonionotus was previously classified under the genus Puntius. The scientific name of this genus has undergone several changes and has been revised to Barbonymus (Kottelat & Widjanarti, 2005). Based on its physical characteristics, this fish has a silver body and orange-red or red caudal fins. The observed bader fish shares the same characteristics as those reported by Haryono et al. (2017), including the following meristic characteristics of the brek fish: pectoral fin rays (I.14-16), ventral fin rays (I-2,8), number of scales on the lateral line (27-32), and number of scales before the dorsal fin (10-13). There were no spots on the base of the tail, nor were there any spots on

the gill cover, and there were no black lines on the edge of the tail fin.

Classification of *Tor duoronensis, Tor tambra,* and *Barbonymus gonionotus* with Reference Species Based on Morphological and Morphometric

Phenetic analysis was conducted using 45 morphological characters. The dendrogram resulting from the morphological analysis was analyzed using the UPGMA method with NTSYSpc V2.02 software. The analysis yielded a dendrogram with two clusters the clade between Tor spp. and the reference fish and *Barbonymus gonionotus* (Figure 6). Phenetic analysis revealed complex evolutionary relationships among the test species based on their morphology. Two groups of relationships were identified, ranging from the closest to the most distant, comprising three groups of apomorphy's and five groups of automorphy.

Apomorphy include species from the genus Tor that share specific characteristics in their fins. Apomorphy group B compares the reference species Tor tambraides with automorphy group B, with a similarity value of 60.00%. Meanwhile, apomorphy C compares Tor soro with automorphy group D, with a similarity value of 64.44%. The automorphic group A contains B. gonionotus from Sumber Umbulan Ngenep, forming its cluster. The similarity percentage of B. gonionotus with other species is also the lowest, at 51.85%. The automorphic group B includes T. tambra from Sumber Umbulan Ngenep with the reference T. tambra, with a similarity value of 86.67% (Table 2). In automorph D, there is T. douronensis from Sumber Umbulan Ngenep with the reference T. douronensis with a similarity value of 86.67%. Due to their high similarity values, the closest kinship relationship was found in automorph B and automorph D. This finding is consistent with the research by Ardiani & Jannah (2023), which states that the higher the similarity index, the closer the kinship relationship.

This study successfully identified the kinship of Tor spp. found in Sumber Umbulan Ngenep with the reference *Tor* spp. Based on the results of this study, it is known that the phenetic analysis method, which compares morphological characteristics, is successful in identifying the kinship of a species and that morphological characteristics are very influential in the accuracy of species identification. These data reinforce the urgency of detailed morphological observation as a support for genetic analysis in species classification, and serve as a basis for further research on species relationships and distribution before establishing conservation strategies.

Furthermore, to strengthen the taxonomic status of the fish found with reference species, morphometric character measurements were conducted using 21 morphometric characters. Among these characters, 15 unique characters distinguished the three observed species. The morphometric measurements of the three fish yielded data with a non-normal distribution, so a Kruskal-Walli's test was performed, with results as shown in Table 3. The Kruskal-Wallis test is generally used as an alternative to the ANOVA test when one or all data distributions are not normally distributed (Harefa & Widyastuti, 2023). The results of the Kruskal-Wallis test showed that 21 data points had $p<0.05$. It is known that differences in morphological characteristics exist. The physical conditions of a species' habitat can influence variations in its morphometric characteristics. These differences may arise due to genetic factors inherited from parents and diverse environmental factors at each location (Nugroho et al., 2023; Syarif et al., 2021). However, significant differences in morphometric ratio patterns are caused by genetic variation, habitat differences, behavioural patterns, and diet (Kusumaningrum et al., 2021). Differences in fish habitat can affect the availability and type of food sources, impacting morphological diversity. Additionally, diverse habitats and wide distribution lead to varied morphological characteristics in fish (Faradiana et al., 2018).

DNA Barcode Analysis

The visualization results of COI gene PCR amplification showed a single band with varying band thickness and no smear (Figure 7). This indicates that the annealing temperature was optimal for binding to the long DNA template resulting from PCR amplification. The varying band thickness is due to DNA molecule degradation during extraction, resulting in differences in DNA weight and quantity. Low DNA band intensity can be caused by inaccurate thermal parameters, such as PCR temperature and cycle duration, reaction composition, and template DNA concentration (Kusuma, 2022). Excessively high DNA concentrations can reduce amplification specificity and efficiency. Band thickness is directly proportional to the amount of isolated DNA, with thicker bands indicating higher DNA concentrations and vice versa.

The markers used in this study had a total size of 10,000 bp. The amplification bands from the samples of *Tor douronensis*, *Barbonymus gonionotus*, and *Tor tambra* using primers LCO1490 and HCO2198 were between 500-600 base pairs. The variation in the length of the DNA fragments produced is estimated to be caused by differences in molecular weight between samples (Ashar et al., 2025). The samples of Barbonymus gonionotus and Tor tambra reached electrophoresis running lengths of approximately 500 bp, as they had smaller molecular weights than Tor douronensis. This is consistent with the study by Larashati et al. 2022. on Tor spp., where mtDNA COI gene amplification produced bands around 650 bp. The study by Dahruddin et al. (2021) also supports the results of mtDNA COI amplification from *Barbonymus gonionotus*, where all sequences had lengths above 500 bp.

Table 2. Similarity values (%) of *Tor douronensis*, *Barbonymus gonionotus*, and *Tor tambra* with reference species based on morphological characters

Taxa	A	B	C	D	E	F	G
A	100						
B	51.85	100					
C	51.85	59.01	100				
D	51.85	59.01	76.67	100			
E	51.85	60.00	59.01	59.01	100		
F	51.85	59.01	64.44	64.44	59.01	100	
G	51.85	86.67	59.01	59.01	60.00	59.01	100

Note: A = Barbonymus gonionotus SUN, B = *Tor Tambra* SUN, C = *Tor duoronensis* SUN, D = *Tor duoronensis*, E = *Tor tambraides*, F = *Tor soro*, G= *Tor tambra*

Table 3. Kruskal-Wallis test results of morphometric characters

Characters	*Tor douronensis* N=20					*Tor tambra* N=20					*Barbonymus gonionotus* N=20				
	Min	Max	Mean	Std.error	p	Min	Max	Mean	Std.error	p	Min	Max	Mean	Std.error	p
Standard length	16.3	19.2	17.55	0.18	0	10.6	12.2	11.41	0.11	0	13.7	15.2	14.40	0.10	0
Total length	20.6	25.4	21.64	0.23	0	14.6	16.5	15.79	0.11	0	15	20	18.06	0.29	0
Head length	2.5	4.8	3.28	0.13	0	2	3	2.48	0.07	0	2	3.1	2.66	0.07	0
Head width	2.5	5.2	3.42	0.16	0	1.6	2.7	2.12	0.08	0	2.6	4.1	3.44	0.10	0
Head height	2.4	4.9	3.18	0.14	0	1.9	3	2.36	0.07	0	2.5	3.6	3.05	0.07	0
Eye diameter	0.4	1.2	0.68	0.05	0	0.5	1	0.74	0.03	0	0.5	1.1	0.85	0.04	0
Snout length	1.4	2.4	1.65	0.06	0	0.8	1.4	1.07	0.03	0	0.4	1	0.74	0.04	0
Body height	4.3	6.3	5.19	0.10	0	2.4	3.5	3.01	0.06	0	3.7	4.7	4.19	0.06	0
Body width	5.4	7	6.15	0.10	0	3.5	4.6	4.10	0.07	0	4.7	5.8	5.13	0.06	0
Tail length	2	4.7	3.14	0.12	0	0.4	1.6	1.21	0.06	0	1.2	2	1.63	0.05	0
Tail height	1.4	3.7	2.31	0.14	0	1.3	2.3	1.84	0.06	0	1.6	3.4	2.39	0.11	0
Dorsal fin base length	4.7	6.9	5.39	0.14	0	2.9	4.1	3.50	0.08	0	2.6	4	3.16	0.08	0
Pectoral fin length	1.2	3.8	20.75	0.14	0	1.9	2.7	2.35	0.05	0	1.9	3.7	2.79	0.10	0
Ventral fin length	0.4	1.4	8.55	0.06	0	1	1.7	1.31	0.06	0	1.6	2.9	2.18	0.08	0
Anal fin length	0.5	2	1.24	0.11	0	1.1	2.8	1.95	0.09	0	1.7	3	2.38	0.09	0

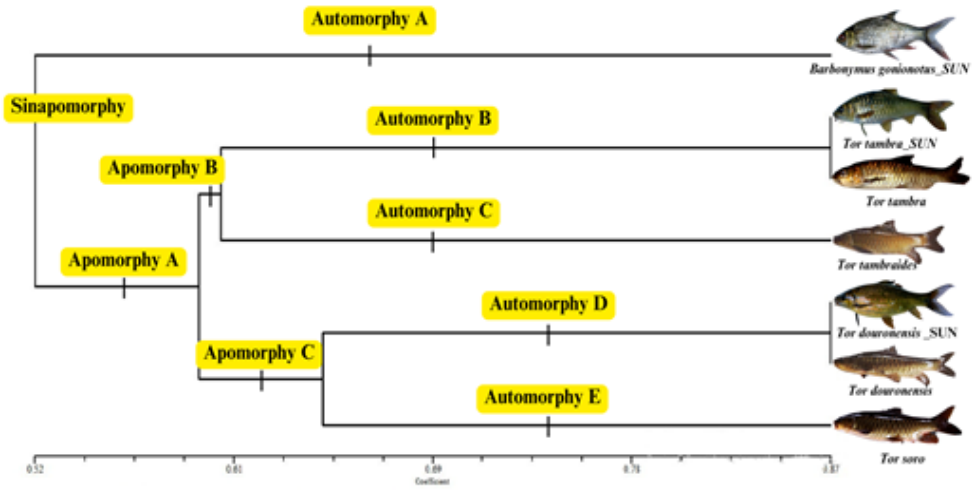

Figure 5. Dendogram analysis of *Tor duoronensis*, *Tor tambra*, and *Barbonymus gonionotus* with reference species based on morphological and morphometric

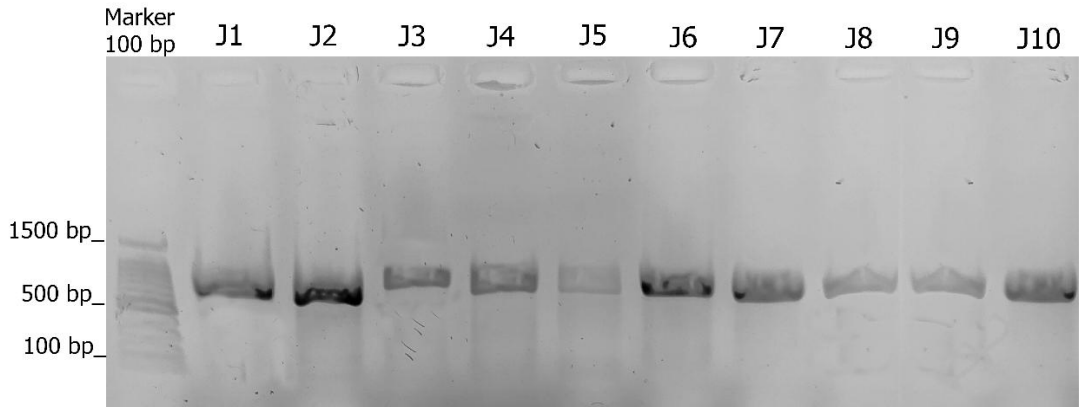

Figure 6. DNA amplification results of *Tor douronensis*, *Barbonymus gonionotus*, and *Tor tambra* with reference species on 1% agarose gel

In this study, the LCO1490/HCO2198 primer set was used and successfully produced precise amplification in all three fish species analyzed. These results indicate that the amplified gene is the target gene corresponding to the primers used. The concentration of the PCR product nucleotides was determined based on band intensity. Riyadini et al. (2020) stated that efficient primers are capable of identifying various taxa with a relatively small number of specimens. Thus, molecular analysis using the COI gene sequence was consistent with the results of morphological identification. The compatibility between DNA data and morphological characters was also evident in the BOLD System analysis, codon composition, and phylogenetic tree.

BOLD System Identification

The nucleotide bases of the three fish samples were further analyzed using the BOLD System (Table 4) to match the nucleotide bases of the samples with the reference data. Eight samples showed very high similarity values. The Tor douronensis sample had a similarity value of <99.35%, the *Tor tambra* sample had a similarity value of <99.78%, and the Barbonymus gonionotus sample had a value of <99.52%. According to Nuryanto et al. (2018), similarity values > 97% can help strengthen the taxonomic status of a species, thereby avoiding uncertainty in identifying its phylogenetic relationships based on BOLD results.

In the *Tor* spp. fish group, morphological variations between species are more difficult to distinguish. The identification approach using DNA barcoding validated by the BOLD System is an accurate and important step to obtain valid results. To date, no research has used the BOLD System analysis on *Tor* spp. Meanwhile, research by Salis et al. (2025) on *Barbonymus gonionotus* from Lake Singkara showed that the BOLD System successfully identified Cytochrome Oxidase-I gene sequences with a high similarity of 97%-99%.

Based on these results, using the BOLD System can contribute significantly to validating the genetic data of a species. This system enables more accurate and efficient matching of DNA sequences with reference databases, thereby simplifying the species identification process and ensuring its validity. The BOLD System accelerates the identification process and enhances the validity of results, which is crucial for future studies in taxonomy and biodiversity conservation.

The COI in mitochondrial DNA is known as the barcode region because it is commonly used as a molecular marker in DNA barcoding methods. This ±650 bp fragment has become the standard for species identification due to its ability to provide fast, accurate results and its usefulness in phylogenetic analysis (Dahruddin *et al.*, 2017; Fitriani *et al.*, 2022; Hebert *et al.*, 2003; Meliyana *et al.*, 2024). Several studies on the identification of Tor spp. The COI gene was used, and it has been conducted in several regions in Indonesia. Studies from Sumatra Island dominate research on the genetics of *Tor* spp. in Indonesia. This is evident in the results of studies by Larashati et al. (2022) in the waters of Samosir Regency, North Sumatra Province, and Marnis et al. (2024) in the waters of Jambi, which successfully identified *T. douronensis*.

Meanwhile, research on the Tor fish in Java is still minimal. In 2013, Rahayu et al. successfully identified *T. tambra* and *T. douronensis* from Pasuruan. In 2025, a recent study by Wibowo et al. successfully identified *T. tambra* larvae in B. gonionotus Sumber Umbulan Ngenep and B. gonionotus China with bootstrap values of 70-100. Higher bootstrap values in each species indicate higher genetic similarity (Simbolon & Aji, 2021). Therefore, this study's results could become the basis for adding new data to complete information on the genetic diversity of *Tor* spp. and *Barbonymus gonionotus* through molecular analysis in Indonesia. In addition, the results of this study can serve as supporting data for further molecular analysis related to relationship based on genetic variation.

Phylogenetic Tree Analysis

A phylogenetic tree reconstruction was performed for the three identified fish species. The reconstruction used 12 sequences from the ingroup and outgroup. The 12 sequences were obtained from the NCBI GenBank. The phylogenetic topology based on the Neighbour-Joining (NJ) method (Figure 8) showed two major clusters separating the ingroup and outgroup. The cladogram used the outgroup *Poecilia reticulata*, which separated the groups, indicating that the three fish samples belonging to the ingroup clustered within each clade based on nucleotide sequence similarity and morphological characters used as supporting evidence. The similarities and differences in characteristics between species were used to determine their relationship (Anafarida & Badruzsaufari, 2020). The ingroup cluster has 11 sequences from the clade *Tor douronensis*, *Barbonymus gonionotus*, and *Tor tambra*.

The bootstrap values between the Neighbour-Joining (NJ) and Maximum-Likelihood (ML) phylogenetic trees differ only in subclade 1a, which consists of *Tor tambra* from Sumber Umbulan Ngenep and *T. tambra* from Malaysia. The

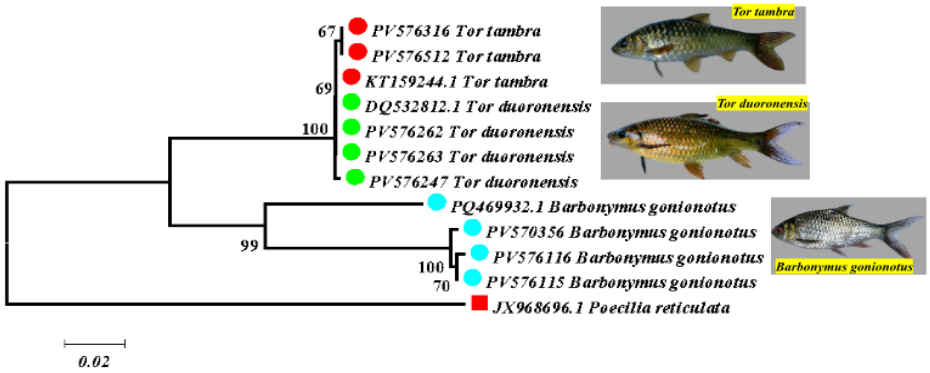

Figure 7. Neighbor-Joining (NJ) phylogenetic tree reconstruction of *Tor douronensis*, *Tor tambra*, and *Barbonymus gonionotus* based on the COI gene

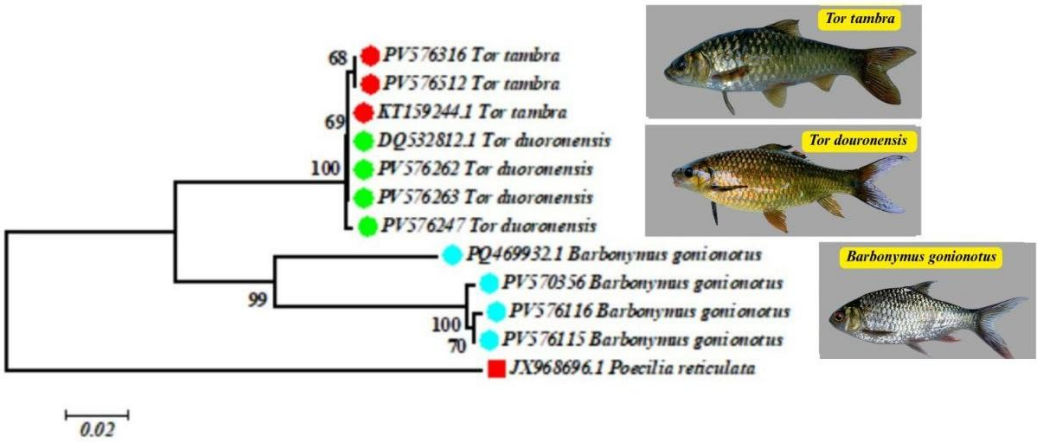

Figure 8. Maximum-Likelihood (ML) phylogenetic tree reconstruction of *Tor douronensis*, *Tor tambra*, and *Barbonymus gonionotus* based on the COI gene

Table 4. Characteristics of the partial COI gene sequence used for phylogenetic tree reconstruction and genetic distance analysis, including sequences from research samples and the GenBank/BOLD system (in group and out group)

Parameters	Position at codon			
	1st	2nd	3rd	Total
Thyrosine frequency	25.64 %	40%	21%	591 bp
Cytosine frequency	31.30%	30.1%	36.1%	591 bp
Adenine frequency	22.65%	16.5%	31.4%	591 bp
Guanine frequency	20.41%	13.0%	11.7%	591 bp
Frequency of invariable sites	59.352%			
Frequency of parsimony informative sites	22.646%			
Nucleotide diversity (Pi)	0.0891			
Haplotype diversity	0.782			
Number of haplotypes	12			
Polymorphic sites	132			
Variance of Haplotype diversity	0.00122			
ts/tv ratio (R)	2.167			
Gamma discrete distribution	0,4328			
Mean of evolutionary rate	0.00, 0.04, 0.09, 0.21, 0.27, 0.31, 0.42, 0.56, 0.63, 0.93, 1.20, 1.53, 2.01, and 2.79 substitutions per site			

Notes: The COI gene sequence characteristics were based on the 591 bp sequence length

phylogenetic tree using the Neighbour-Joining (NJ) method has a higher value than the Maximum-Likelihood (ML) method, at 68–69. Meanwhile, the Maximum-Likelihood (ML) tree has bootstrap values of 67–69. In subclade 1B, which consists of *Tor douronensis* from Sumber Umbulan Ngenep and *T. douronensis* from Malaysia, the bootstrap value is 100 and Clade 2 consists of *Barbonymus*.

Codon composition

Nucleotides are the basic units of genetic information. The basic structure of nucleotides consists of three main components: heterocyclic nitrogenous bases (nucleobases), pentose sugars, and phosphate groups. In DNA, four types of nitrogenous bases adenine (A), guanine (G), cytosine (C), and thymine (T), whose number and sequence determine the genetic information encoded. Based on 591 bp of partial COI sequences from *Tor douronensis*, *Tor tambra*, and *Barbonymus gonionotus*, the nucleotide base sequences differ as shown in Table 4.

The findings on codon composition based on Table 4 provide an overview of the evolutionary history and divergence patterns of the three fish samples studied from Sumber Umbulan Ngenep. Base composition analysis of the partial COI gene sequences showed a higher AT content (26.59%) compared to GC (24.13%), a significant finding consistent with previous observations in various Cyprinidae species across different regions. Base composition analysis of the COI gene indicates a

higher AT content than GC and Guanine. These results are consistent with previous studies by (Hakim et al., 2023 and Sajjad et al., 2023).

Species Delimination Using ABGD

In addition, ABGD method identified 6 groups specimens with the initial approach and the barcode gap threshold calculated by the ABGD analysis of the COI dataset (Figure 9A). The initial partition was obtained at prior maximal distance P = 0.001668, barcode gap distance = 0.100, and Kimura 2-parameter (K80) distance = 1.50. The ABGD method also identified a barcode gap centered around 1.8% of divergence between the available COI sequences. The analysis defined the existence of 4 to 6 hypothetical species in all recursive partitions with prior intraspecific genetic divergence values between 0.15% and 0.26%, a result we considered more likely than 3 or more species with intraspecific divergence values below 0.28% or as a single species with intraspecific divergence values greater than 2.15%. This is in accordance with the results of the ABGD grouping which divides the species into 6 groups (Figure 9B).

QR Barcode

DNA QR Barcode technology converts the nucleotide base sequence of a DNA barcode, which has been widely used for species identification. QR codes are the most efficient visual representation for conveying DNA barcode sequences. Online servers have also been developed to assist biologists in applying QR codes in practical applications.

There are 8 DNA QR Barcodes generated from the research sample sequences (Figure 9). The QR barcodes indicate that the three *Tor douronensis* samples have a nucleotide base length 632. The two Tor tambra samples have a nucleotide base length of 512. Furthermore, the *Barbonymus gonionotus* sample has varying nucleotide base lengths, namely 502, 514, and 562. The density of the generated QR Barcodes also varies. As the genetic information

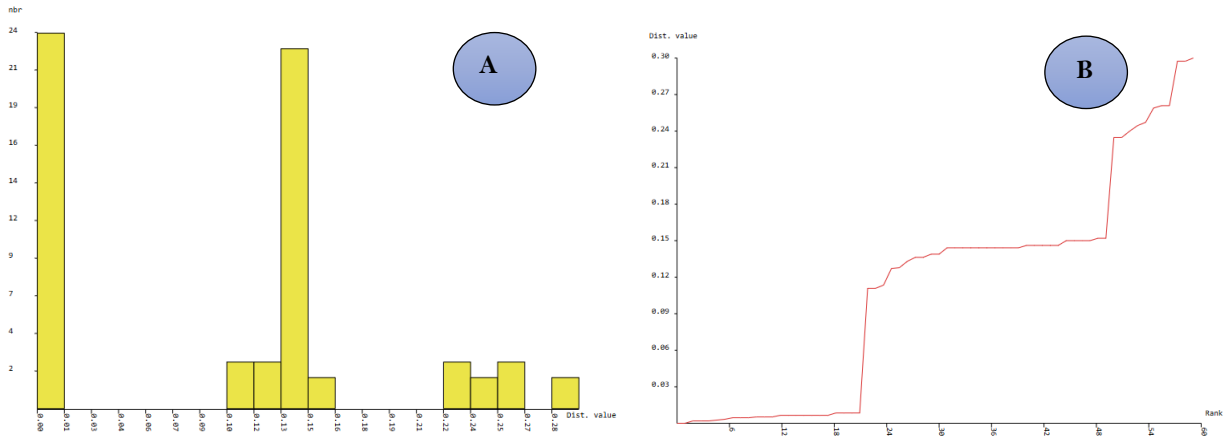

Figure 9. Barcode gap analysis generated by Automatic Barcode Discovery Gap Discovery. Distribution of K2P distances between each pair of specimens for the COI gene; A = Distance histogram; B = Ranked distances

encoded becomes more complex, the visual structure of the QR code becomes denser (Ghouri et al., 2020).

QR codes have undergone significant development and are now used to identify fauna at various taxonomic levels, including species. Various studies have successfully created DNA barcodes and converted them into QR codes with broader user benefits (Turhadi et al., 2025). Research on using QR codes in plants is generally easier to find than in animals. Meanwhile, the application of QR codes in animal studies, besides identifying fauna, is also used to validate and secure animals or animal products. For example, Naulia, 2015, successfully produced QR codes to ensure the meat content of specific species in food. Additionally, research by Ghouri et al. (2020) successfully created QR codes from DNA barcoding of fish that are safe for consumption in Pakistan.

CONCLUSION

This study first identifies the species in Sumber Umbulan Ngenep, Malang Regency, East Java, Indonesia. Morphological, genetic, and phylogenetic tree reconstruction analyses based on the COI gene revealed the presence of three identified species at

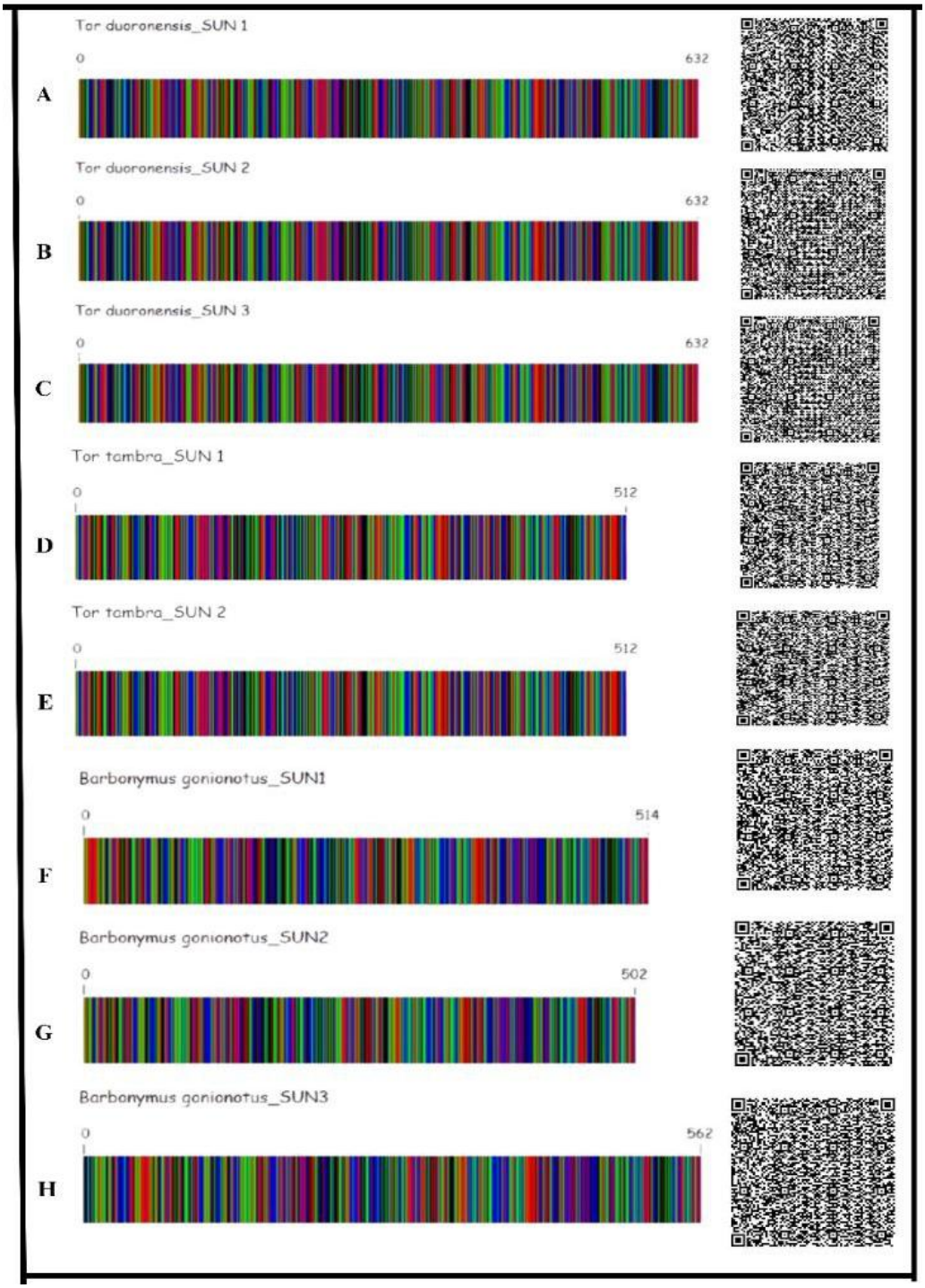

Figure 10. DNA QR code (DQR) based on the COI gene. A = PV576247; B = PV576262; C = PV576263; D = PV576316; E = PV576512; F = PV570356; G = PV576115; H = PV576116

Sumber Umbulan Ngenep: *Tor douronensis*, *Tor tambra*, and *Barbonymus gonionotus*. These findings provide new information about the presence of these species and can serve as a basis for conservation efforts and future management strategies. This publication is an output of academic mobility with international research collaboration.

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