

# Morphology and COI barcodes reveal local loaches (*Nemacheilus* spp.) from Pasuruan River, East Java, Indonesia

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**Abstract.** Sulung CAVT, Chair NE, Rahayu DA, Nugroho ED, Rusdianto. 2024. Morphology and COI barcodes reveal local loaches (*Nemacheilus* spp.) from Pasuruan River, East Java, Indonesia. *Biodiversitas* 25: 2806-2819. The family Nemacheilidae is one of the most diverse and widespread family of freshwater fishes. The nemacheilid loach in the Pasuruan river is relatively abundant; however, there is limited information available about this species. Therefore, further research is necessary to identify loaches from the Pasuruan area. This research aimed to identify the local loach species from Pasuruan District. The pectoral fins were stored in pure-grade ethanol and subjected to DNA extraction, polymerase chain reaction (PCR), and sequence analysis using bioinformatics tool, BOLD systems, and Automatic Barcode Gap Discovery (ABGD) web. The morphological identification revealed two local loaches, *Nemacheilus chrysolaimos* Valenciennes 1846 and *Nemacheilus fasciatus* Valenciennes 1846. The high haplotype diversity (Hd) of 0.9 and nucleotide diversity ( $\pi$ ) of 0.1 indicate a wide range of genetic variations. The frequency of parsimony informative sites at 25.6% and 16 polymorphic sites and the overall ts/tv ratio of 2.3. The phylogenetic trees demonstrated a clear and unambiguous branching pattern for *N. fasciatus* and *N. chrysolaimos* forming their own monophyletic branches. Kimura's 2-parameter genetic distance of the COI gene sequences between these two species was 0.11. This finding was strengthened by morphological and molecular identification results, which indicated that there are two local loach species in Pasuruan District.

**Keywords:** Diagnostic characters, DNA barcodes, loach fish, genetic composition, morphological

## INTRODUCTION

Indonesia, as an archipelago, with a rich diversity of biological wealth, including abundant freshwater fish species. Among these species is the local loach from the Nemacheilidae family. The distribution of loaches in Indonesia is divided into several islands such as Sumatra, Kalimantan, and Java (Hubert et al. 2019; Kusuma et al. 2021). In Central Java, loaches, including *Nemacheilus chrysolaimos* Valenciennes 1846 and *Nemacheilus fasciatus* Valenciennes 1846 have been identified in rivers such as the Pelus, Mengaji, Banjarn River, and Kranji. Additionally, *N. fasciatus* has been observed in Jepara and Purwokerto (Baumgartner and Wibowo 2018; Chapsos and Hamilton 2018; Haryani 2022). In East Java, Indonesia particularly in Blitar, *N. chrysolaimos* and *N. fasciatus* have been documented, with a higher abundance of *N. fasciatus* and *N. chrysolaimos* with diagnostic characters (Rahayu et al. 2023).

The distribution and abundance of loaches are influenced by habitat profiles through water temperature, water movement, and water solutes (Siswarini et al. 2022; Prakoso et al. 2016). The loaches inhabit rivers with clear water, fast-flowing water, and rocky riverbeds (Jubaedah and Sayida 2022; Kusuma et al. 2021). Pasuruan District is one of the areas that fulfill the characteristics of loach

habitat because it has a range of mountains and hills in the southern region and is directly adjacent to the Madura Strait in the northern region, so that streams flow from highland to lowland areas (Iksan et al. 2021; Tjahjanti and Luliafan 2021).

In the East Java region, *Nemacheilus* spp. that belong to the Nemacheilidae family are a valuable commodity that can be traded and consumed, according to a number of publications (Hadiaty and Kottelat 2009; Hadiaty and Yamahira 2014; Wibisono 2014; Nurhidayat et al. 2017; Prakoso et al. 2016; Ath-thar et al. 2018; Suriadi et al. 2019). Rahayu et al. (2023) reported an loach family member, locally known as Uceng (*Nemacheilus* spp.) in Blitar. This loach is in the Pasuruan river relatively abundant, however there is limited information available about this species. Therefore, further research is necessary to identify *Nemacheilus* spp. from the Pasuruan area. Molecular characterization can be used to strengthen morphological data for species identification. DNA barcoding with one molecular marker can be used to strengthen identification based on morphological data (Hubert and Hanner 2016; Trivedi et al. 2016; Abdullah et al. 2019; Alcudia et al. 2020; Ambarwati et al. 2021; Basith and Kusri 2021; Juniar et al. 2021; Radulovici et al. 2021; Sari et al. 2021; Vieira et al. 2021; Nugroho et al. 2023; Rahayu et al. 2023;).

Several studies have highlighted the effectiveness of DNA barcoding in species identification within fish populations in Indonesia. For example, Arisusanti et al. (2019) reveal fish species diversity in Lake Lebo Taliwang using Cytochrome c oxidase I (COI) gene. Xing et al. (2018) demonstrate the high efficiency of species identification using DNA barcoding, particularly through COI sequencing. Tadmor-Levi et al. (2022) established a genetic database for fish species identification using DNA barcoding, showcasing its utility in resolving species identification. Additionally, Roesma et al. (2022) identify and determine freshwater fish in Siberut Island. Furthermore, Shokralla et al. (2015) and Venera-Ponton et al. (2020) emphasize the rapid and effective DNA barcoding for accurate identification in species level. Joesidawati et al. (2023) show that DNA barcoding can be used to reveal morphologically similar fish species for *Encrasicholina* and *Stolephorus* in Tuban District. Wang et al. (2018) demonstrate the useful of DNA barcoding for identifying various fish components, which are challenging for identification based on morphology alone.

DNA barcoding facilitates rapid and accurate species differentiation, often proving superior to traditional morphological identification methods (Khedkar et al. 2016; Li et al. 2022). Moreover, the COI gene within mitochondrial DNA is esteemed for its stability, characterized by minimal deletions and insertions in its sequences. This useful guarantees the consistency and reliability of genetic data derived from COI sequences for species identification purposes. This stability enhances the utility of COI sequences in molecular identification studies, allowing for quick and reliable differentiation between species, which is particularly useful in large-scale

biodiversity assessments and conservation efforts. Based on this background, this study aims to identify *Nemacheilus* sp. from Pasuruan river based on morphological and molecular characterization using DNA Barcoding of COI gene.

## MATERIALS AND METHODS

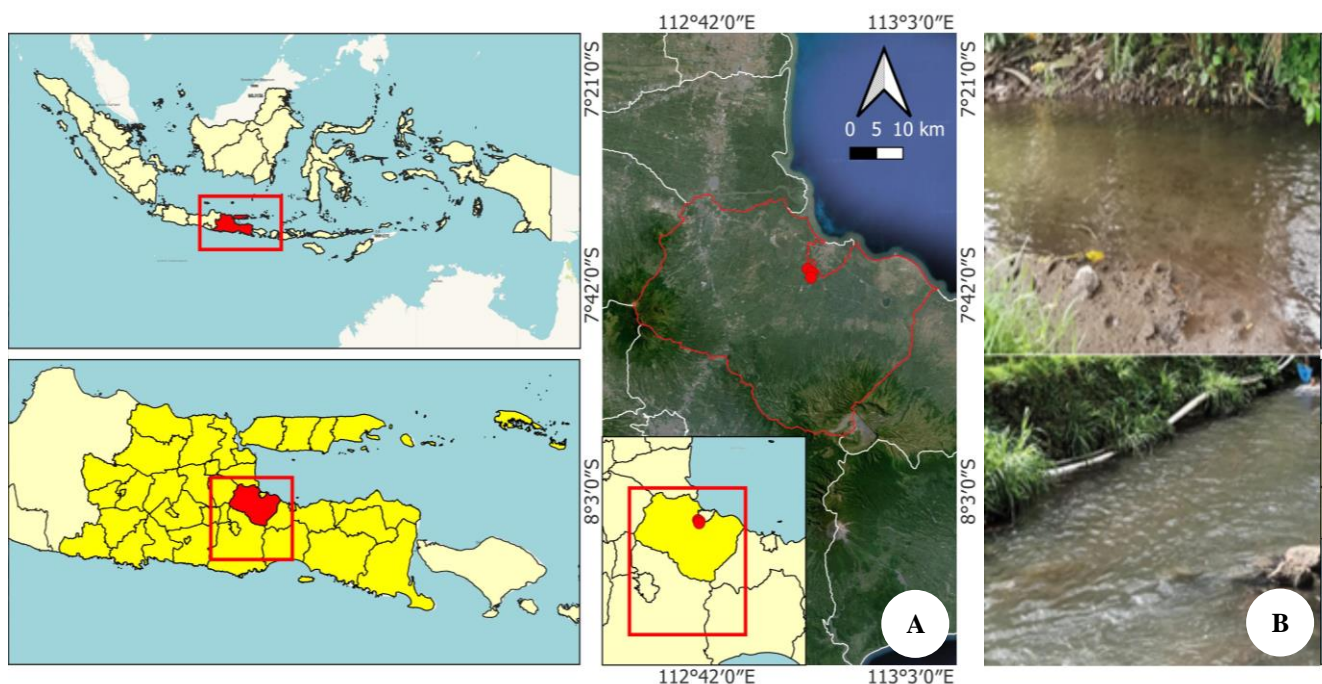
### Study area

Specimens of *Nemacheilus* sp. were collected from Warung Dowo Rivers, Pasuruan District, East Java, Indonesia (Figure 1) at each loach sampling station. Purposive sampling was used, loaches were captured using fishing nets in 1 meter. All samples were placed into sample bottles filled with 96% absolute ethanol.

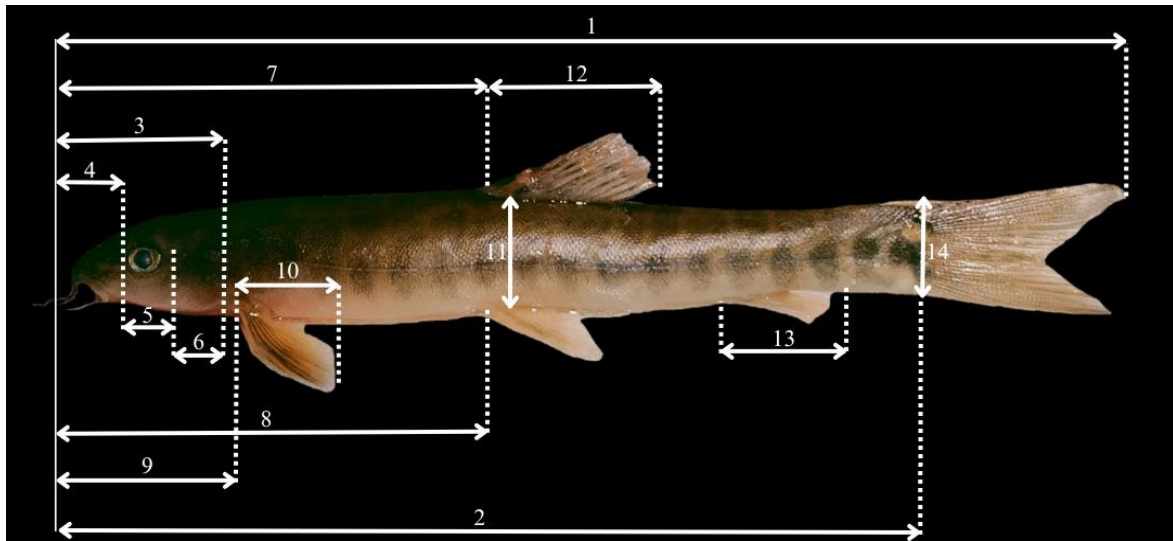
### Procedures

#### Morphological work

In the laboratory, all fish specimens were sorted and washed, to prepare them for morphological observation and identification. Measurements of morphological characteristics were conducted on 14 characters, as followed in Figure 2, using a digital caliper with 0.01 mm precision, following a modified protocol based on Kottelat (1984) and Kottelat and Freyhof (2007) and Rahayu et al. (2023). Identification procedures which provided by Kottelat (1984) and Kottelat et al. (1993). Subsequently, all specimens were preserved in 70% alcohol and deposited at the Zoological Taxonomy, Universitas Negeri Surabaya, Indonesia. Prior to molecular analysis, the specimens were frozen at -20°C for DNA extraction.



**Figure 1.** The map of A. Warung Dowo River, Pasuruan District, East Java, Indonesia and B. Their habitat indicating the sampling sites of loaches



**Figure 2.** The characteristics of morphometrics loach fish in Warung Dowo River, East Java, Indonesia. Notes: 1. Total Length (TL); 2. Standart Length (SL); 3. Head length; 4. Snout length; 5. Eye diameter; 6. Head length behind the eye; 7. Body length before dorsal fin; 8. Body length before pelvic fin; 9. Body length before pectoral fin; 10. Pectoral fin length; 11. Body height; 12. Dorsal fin length; 13. Anal fin length; 14. Caudal fin height (modified from Kottelat (1984) and also Kottelat and Freyhof (2007) and Rahayu et al. 2023

#### DNA extraction

The isolation of total DNA (whole genome) from tissue fin samples was carried out using the DNA Isolation Kit (Roche), with several modifications. The Buffer GT1 200  $\mu$ L pipette was added into a 1.5 mL tube and vortexed. Subsequently, 200  $\mu$ L of GT2 buffer and 20  $\mu$ L of Proteinase K were added to the mixture and thoroughly mixed using a vortex. The mixture underwent an incubation period of 10 minutes at 56°C, with the incubation tube being briefly inverted every 5 minutes. Following this, 200  $\mu$ L of absolute ethanol was added to the mixture and briefly vortexed. The sample was then transferred to a Spin Column and centrifuged for 1 minute at 13,000 rpm. The resulting flow-through was discarded, and 500  $\mu$ L of buffer W1 was added to the Spin Column, followed by another round of centrifugation for 1 minute at 13,000 rpm. After discarding the flow-through, 700  $\mu$ L of W2 buffer (containing ethanol) was added to the Spin Column and centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded again, and the Spin Column was centrifuged for an additional 2 minutes at 13,000 rpm. The DNA retained in the Spin Column was then transferred to a new 1.5 mL tube. Subsequently, 50-100  $\mu$ L of Elution Buffer was added and incubated at room temperature for 1 minute before being centrifuged for 1 minute at 13,000 rpm.

#### DNA extraction and sequencing

A fragment of approximately 619 bp (base pairs) that amplify to the COI gene region of the mtDNA was amplified by PCR using universal primer LCO1490: (5' GGT CAA CAA ATC ATA AAG ATA TTG G 3') dan HCO2198: (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3') (Folmer et al. 1994). The hotstart PCR method uses a master mix Kapa and two Taq master mixes. The PCR process was carried out in 35 cycles. Each cycle

consisted of a double thread attachment process (pre-denaturation) at 95°C for 3 minutes, a denaturation at 94°C for 45 seconds, an annealing at 45°C for 45 seconds and an extension at 72°C for 2 minutes. Then, it proceeded further, with final elongation at 72°C for 10 minutes. PCR products were checked on 1% agarose gel (0.5 g agarose and 50 mL TAE Buffer) mixed with 4  $\mu$ L Ethidium Bromide (EtBr) as a dye. The next step was to mix 3  $\mu$ L of the PCR samples with 1  $\mu$ L of loading dye, the mixture was then put into an agarose well. The electrophoresis was performed using an electrophoresis machine with a voltage of 220 V and a current of 400 mA, for 25 minutes. PCR products were purified using Qiagen purification kit according to manufacturer's instructions and subsequently sequenced at First Base, Malaysia.

#### Data analysis

##### Morphology

The description of the two *Nemacheilus* species was provided based on morphological observations using morphometrics, meristics and diagnostic characters. A description of the two *Nemacheilus* spp. was presented based on morphological observation. Since the data from morphological measurements were not normally distributed, a non-parametric test (Kruskal-Wallis test) was employed to investigate any significant difference in the morphometric of *N. fasciatus* and *N. chrysolaimos* from sampling localities. This species was selected for analysis as it was present in this warung dowo river.

##### DNA barcode

The study obtained partial sequences of the COI gene based on 619 base pairs from 12 *Nemacheilus* spp. collected in Warung Dowo river, Pasuruan, East Java, Indonesia, which constituted the final dataset. Initially, each sequence was translated into an amino acid sequence



to eliminate any pseudogenes, following protocols outlined by Song et al. (2008), Šlechtová et al. (2021) and Muhala et al. (2024). Subsequently, chromatogram analysis was performed using Finch TV software. The translated amino acid sequences were then verified via the ExPASy website (<https://web.expasy.org/translate/>) (Duvaud et al. 2021). All sequences were compared with close relatives of *Nemacheilus* spp. through BLAST (Basic Local Alignment Search Tool) (Boratyn et al. 2013) and the BOLD System (Bejarano et al. 2023). Six accessions from GenBank (NCBI) were chosen as in-group and out-group for phylogenetic tree construction. Multiple sequence alignment was carried out using Clustal X (Ferrari and Patrizio 2021) followed by manual verification with BioEdit software. The partial COI gene sequences from *Nemacheilus* spp. in this research were submitted to GenBank with corresponding accession numbers (refer to Table 1).

#### Phylogenetic reconstruction

Phylogenetic reconstruction based on partial sequences of the COI gene involved a total of 21 sequences, including both in-group and out-group accessions obtained from GenBank. The analysis aimed to construct the grouping of different species alongside related species. Phylogenetic tree reconstruction was conducted using MEGA X version 10.2.6, employing the Neighbor-Joining (NJ) and Maximum-Likelihood (ML) methods. For both NJ and ML tree reconstructions, the Kimura 2-Parameter (K2P) substitution model was utilized (Nishimaki and Sato 2019). Additionally, the rate variation among sites was modeled with a Gamma distribution, and a bootstrap consensus tree was inferred from 1000 replicates (Russo and Selvatti 2018).

#### Species delimitation using ABGD

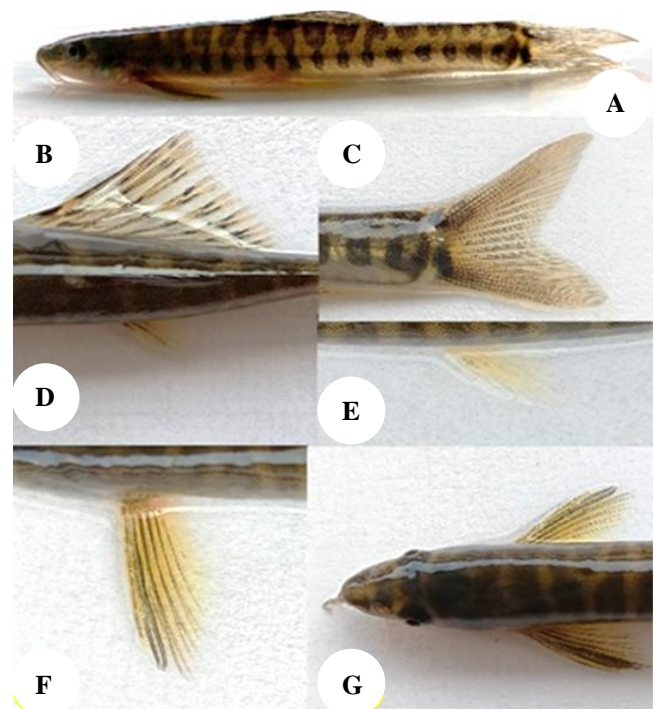
Species delineation in fish using COI gene was conducted using the Automatic Barcode Gap Discovery (ABGD) method, as described by Mu et al. 2023. This algorithm identifies genetic clusters or putative species by detecting the inflection point in the frequency distribution of ranked pairwise genetic distances between aligned homologous sequences. The ABGD method iteratively partitions the dataset into candidate species, aiming to obtain the finest partition. To begin, a matrix of pairwise uncorrected *p*-distances was calculated in MEGA, excluding all ambiguous positions, for each pair of sequences. The ABGD analysis was performed using the web-interface available at <http://www.abi.snv.jussieu.fr/public/abgd/>. The analysis incorporated three implemented models: Jukes-Cantor and Kimura 2-Parameter (K2P).

Class	: Actinopteri Cope 1871
Ordo	: Cypriniformes Bleeker 1859
Family	: Nemacheilidae Regan 1911
Genus	: <i>Nemacheilus</i> Bleeker 1863

*Nemacheilus chrysolaimos* (Valenciennes 1846) variant 1 (Figure 3)

**Material examined.** Warung Dowo River in Pasuruan District, East Java, Indonesia.

**Description.** The head is rounded and triangular with a pair of eyes, a pair of nostrils, a blunt and short snout, a small lower mouth, and lips that form a semicircle at the mouth. In addition, it has an elliptical eye shape and a pair of holes located between the snout and eyes. It has 3 pairs of rostral rattles consisting of 2 pairs on the upper jaw and 1 pair on the snout of the upper jaw. The body shape is elongated fusiform, slightly flattened to the sides and sharpened towards the base of the tail. The pectoral fins are almost as long as the pelvic fins. The anal fin does not reach the base of the caudal fin. The dorsal fin is located opposite the pelvic fin in a vertical direction. The caudal fin is shaped like a V with both ends pointed, having hard and soft fingers. Dorsal fin DII.7-8, anal fin AI.6-7, pectoral fin PI.9-10, ventral or pelvic fin VI.4-7, caudal fin C.18-19.



**Figure 3.** *Nemacheilus chrysolaimos* variant 1 (Standard Length: 52.5 mm) from Warung Dowo River, Pasuruan, East Java, Indonesia. A. Habitus, lateral view; B. Dorsal fin, lateral view; C. Caudal fin, lateral view, D. Pectoral fin, lateral view, E. Anal fin, lateral view; F. Ventral fin, lateral view; G. Anterior part, dorsal view

## RESULTS AND DISCUSSION

### Morphological characters

The research results revealed the description of *Nemacheilus* spp. that were found, namely *Nemacheilus fasciatus* and *Nemacheilus chrysolaimos* with each species having two morphological variants.

**Coloration.** The body has a yellowish-black or golden-yellow color with 14-18 irregularly shaped vertical dark lines on the lateral parts of the body with 5 interrupted lines near the base of the caudal fin. It has two pairs of grey colored scutes.

**Body length.** Standard length (SL) and total length (TL) ranged from 40.0-56.0 mm and 50.0-68.0 mm, respectively, with a mean of  $40.1 \pm 5.3$  mm and  $56.0 \pm 4.9$  mm ( $n=16$ ).

**Distribution.** Based on Hubert et al. (2019) states that the species *N. chrysolaimos* is almost found in all provinces on the island of Java such as Central Java and East Java, Indonesia. Kusuma et al. (2021) stated that this species was also found in Central Java, precisely in Banyumas, Temanggung, and Yogyakarta. Meanwhile, Kottelat (1984) and Hardiaty and Yamahira 2014 stated that *N. chrysolaimos* is distributed in Java, Sumatra, and Kalimantan.

**Remarks.** The existence of information on the distribution of loach that has been found by previous researchers does not ensure that all regions have known the same characteristics so this study will provide different information obtained from previous studies regarding the characteristics of *Nemacheilus* sp. in different regions.

*Nemacheilus chrysolaimos* (Valenciennes 1846) variant 2 (Figure 4)

**Material examined.** Warung Dowo River in Pasuruan District, East Java, Indonesia.

**Description.** The head is rounded and triangular with a pair of eyes, a pair of nostrils, a blunt and short snout, a small lower mouth, and lips that form a semicircle at the mouth. In addition, it has an elliptical eye shape and a pair of holes located between the snout and eyes. It has 3 pairs of rostral snout consisting of 2 pairs on the upper jaw and 1 pair on the snout of the upper jaw. The body shape is elongated fusiform, slightly flattened to the side and sharpened towards the base of the tail. The caudal fin has a pointed lobe with all fingers branched. The dorsal fin is located at the center of the body length. The caudal fin is shaped like a V with both ends slightly blunted, having hard and soft fingers. Dorsal fin DII.7-8, anal fin AI.6-7, pectoral fin PI.9-10, ventral or pelvic fin VI.4-7, caudal fin C.18-19.

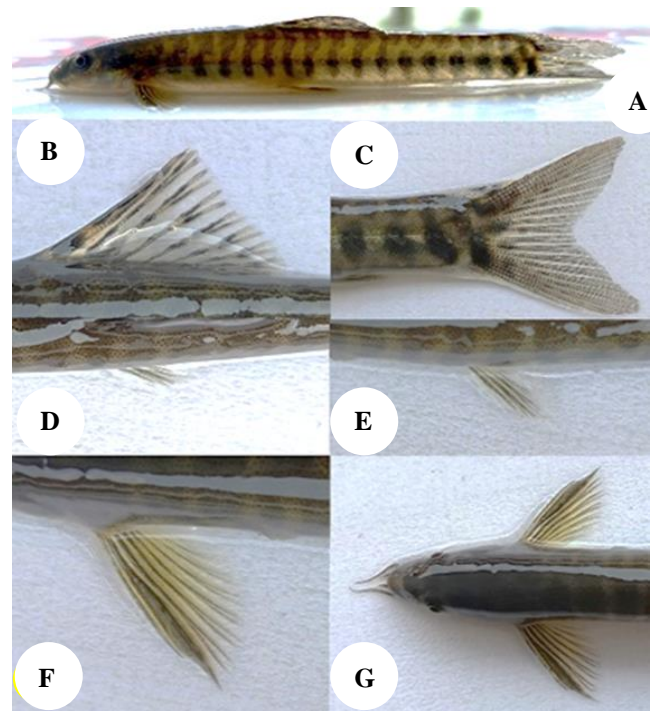
**Coloration.** The body is yellowish black or golden yellow with 14-18 irregularly shaped vertical dark stripes on the lateral parts of the body with 11 connected lateral stripes and 5 disconnected stripes near the base of the caudal fin. It has two pairs of black colored scutes.

**Body length.** Standard length (SL) and total length (TL) ranged from 40.0-56.0 mm and 50.0-68.0 mm, respectively, with a mean of  $40.1 \pm 5.3$  mm and  $56.0 \pm 4.9$  mm ( $n=16$ ).

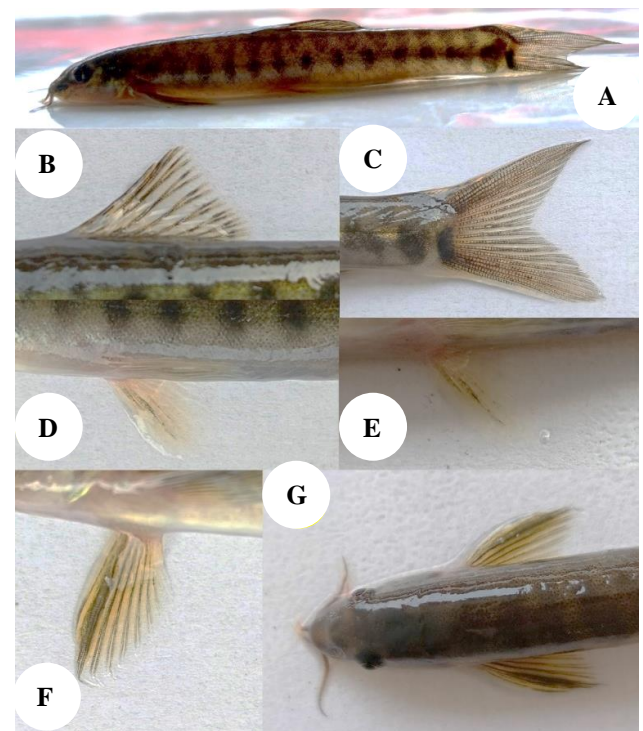
*Nemacheilus fasciatus* (Valenciennes 1846) (Figures 5 and 6)

*Noemacheilus fasciatus* Kuhl and van Hasselt in van Hasselt 1823: 133; 1824: 376; Kottelat 1984: 247.

*Cobitis fasciata* Valenciennes in Cuvier and Valenciennes, 1846: 25.-Bleeker 1854: 96; 1860: 78.



**Figure 4.** *Nemacheilus chrysolaimos* variant 2 (Standard Length: 48.5 mm) from Warung Dowo River, Pasuruan, East Java, Indonesia. A. Habitus, lateral view; B. Dorsal fin, lateral view; C. Caudal fin, lateral view; D. Pectoral fin, lateral view; E. Anal fin, lateral view; F. Ventral fin, lateral view; G. Anterior part, dorsal view



**Figure 5.** *Nemacheilus fasciatus* variant 1 (Standard Length: 58.2 mm) from Warung Dowo River, Pasuruan, East Java, Indonesia. A. Habitus, lateral view; B. Dorsal fin, lateral view; C. Caudal fin, lateral view; D. Pectoral fin, lateral view; E. Anal fin, lateral view; F. Ventral fin, lateral view; G. Anterior part, dorsal view



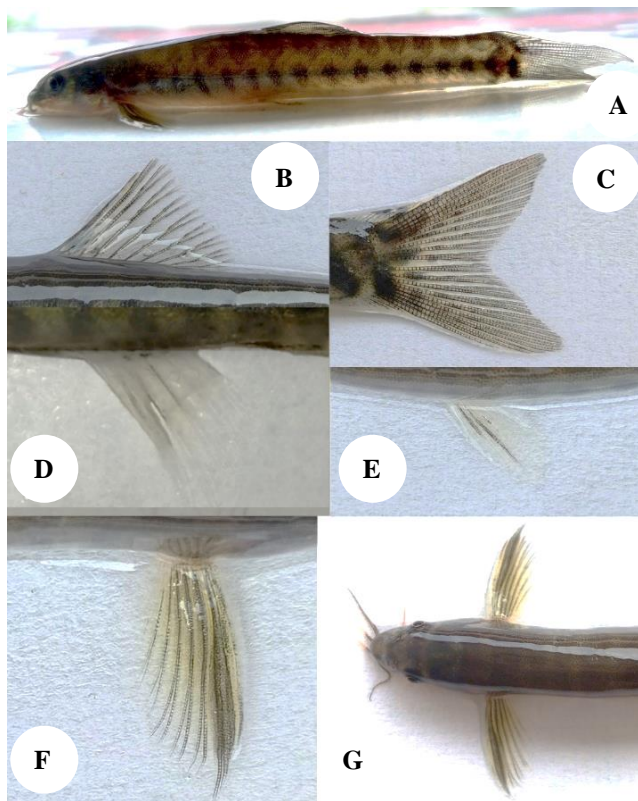
*Cobitis suborbitalis* Valenciennes in Cuvier and Valenciennes 1846: 26.

*Cobitis chrysolaimos* Valenciennes in Cuvier and Valenciennes 1846: 27.

*Nemacheilus fasciatus* Bleeker 1863a: 41, 366 (in part); 1863b: 7 (in part). Kottelat 1993: 25.-Roberts 1993: 26.-Hardiaty and Yamahira 2014: 84 (list), 87 (list), 90 (list), 92 (key).

**Material examined.** River in Pasuruan District, East Java, Indonesia.

**Description.** The head is triangular with a semicircular tip and has a pair of eyes, a pair of nostrils, a blunt and short snout, a small, downturned mouth, and lips that form a semicircle at the mouth. In addition, it has an elliptical eye shape and a pair of holes between the snout and eyes. It has 3 pairs of rostral snout consisting of 2 pairs on the upper jaw and 1 pair on the snout of the upper jaw. The body shape is elongated fusiform, slightly flattened to the side and sharpened towards the base of the tail. The pectoral fins are almost as long as the pelvic fins. The anal fin does not reach the base of the caudal fin. The dorsal fin is located at the center of the body length. The caudal fin is shaped like a V with both ends pointed and the upper end shorter than the lower end, having hard and soft fingers. Dorsal fin DII.7-8, anal fin AI.4-5, pectoral fin PI.7-9, ventral or pelvic fin VI.6-7, caudal fin C.17-19.



**Figure 6.** *Nemacheilus fasciatus* variant 2 (Standard Length=45.7 mm) from Warung Dowo River, Pasuruan, East Java, Indonesia. A. Habitus, lateral view; B. Dorsal fin, lateral view; C. Caudal fin, lateral view; D. Pectoral fin, lateral view; E. Anal fin, lateral view; F. Ventral fin, lateral view; G. Anterior part, dorsal view

**Coloration.** The body has a yellowish-black color with 12 to 18 body bladders that are located opposite each other on the dorsal and ventral sides. It has two pairs of brown colored scutes. The base of the caudal fin is black on the underside and yellow on the top.

**Body length.** Standard length (SL) ranged from 44.0-55.0 mm, with a mean of  $51.2 \pm 0.7$  and total length (TL) ranged from 60.0-68.0 mm, with a mean of  $59.6 \pm 2.7$  mm (n=21).

**Distribution.** Based on Kottelat (1984) and Hardiaty et al. (2014), the distribution of *Nemacheilus chrysolaimos* is in Java Island, especially in West Java. (Hubert et al. 2019) stated that the species has a distribution and is found in all provinces in Java such as Central Java and East Java, Indonesia. Meanwhile, Rahayu (2023) stated that the species is found in East Java, precisely in Blitar District.

**Remarks.** Rahayu (2023) stated that there is a distribution of *N. fasciatus* in East Java precisely in Blitar District which will be different from what we found in Pasuruan District.

**Material examined.** Warung dowo river, Pasuruan District, East Java, Indonesia.

**Description.** The head is triangular with a semicircular tip and has a pair of eyes, a pair of nostrils, a blunt and short snout, a small, downturned mouth, and lips that form a semicircle at the mouth. In addition, it has an elliptical eye shape and a pair of holes between the snout and eyes. It has 3 pairs of rostral snout consisting of 2 pairs on the upper jaw and 1 pair on the snout of the upper jaw. The body shape is elongated fusiform, slightly flattened to the side and sharpened towards the base of the tail. The pectoral fins are almost as long as the pelvic fins. The anal fin does not reach the base of the caudal fin. The dorsal fin is located opposite the pelvic fin in a vertical direction. The anal fin is shorter than the pelvic fin. The pectoral fins do not reach the length of the head, the pelvic fins do not reach the base of the anal fin. The caudal fin has a pointed lobe with all fingers branched. The dorsal fin is located at the center of the body length. The caudal fin is shaped like a V with both ends blunt, having hard and soft fingers. Dorsal fin DII.7-8, anal fin AI.4-5, pectoral fin PI.7-9, ventral or pelvic fin VI.6-7, caudal fin C.17-19.

**Coloration.** The body is yellowish-black in color with 12 to 18 body bladders that are set opposite each other on the dorsal and ventral sides. It has two pairs of black colored scutes. The base of the caudal fin is black.

**Body length.** Standard length (SL) ranged from 44.0-55.0 mm, with a mean of  $51.2 \pm 0.7$  and total length (TL) ranged from 60.0-68.0 mm, with a mean of  $59.6 \pm 2.7$  mm (n=21).

**Remarks.** Rahayu et al. (2023) stated that there is a distribution of *N. fasciatus* in East Java precisely in Blitar District which will be different from what we found in Pasuruan District.

Each variant found was different from another one by morphological characteristics based on the body pattern of bars or blotches. In *N. chrysolaimos* variant 1 has 10 irregular bars between the head and caudal peduncle, widening at their upper end near the dorsal side and narrowing towards the lower end near the ventral side, on

the caudal peduncle are 5 irregularly shaped dorsal saddles and the midlateral line on the caudal peduncle is overlaid by 5 blotches. This feature distinguishes variant 1 of *N. chrysolaimos* from variant 2 which has 11 more regular bars between the head and the caudal peduncle and then continued with 5 dorsal saddles and 5 blotches along the midlateral line that cross each other at the ventral side of the body. These characteristics are different from the characteristics found in *N. fasciatus*. Within *N. fasciatus* also 2 different morphological variants were found. *Nemacheilus fasciatus* variant 1 has the first 5-6 dorsal blotches between the head and the dorsal fin connected with the first 5 midlateral blotches and those together form 5 irregular bars. From under the origin of the dorsal fin it continues with 8 dorsal saddles and 8 midlateral blotches. This feature is different from that of *Nemacheilus fasciatus* variant 2, which has 11-12 dorsal saddles and 14-15 midlateral blotches.

The two fish species, namely *N. chrysolaimos* and *N. fasciatus* with each species having 2 morphological variants can be distinguished based on morphological characteristics of their body pattern, particularly the number of blotches and the number as well as regularity of bars. The four morphological variants of the two species are almost similar and are also found in the observations of Rahayu (2023) who mentions complete morphological characteristics based on body color, body style, and meristic counts of fin rays. *N. chrysolaimos* has a yellowish-black body color while *N. fasciatus* has a yellowish body color. In addition, there are also morphological characteristics in the fins where *N. chrysolaimos* has dorsal fins with black dots and *N. fasciatus* has dorsal fins with reddish spots. On the body pattern, *N. chrysolaimos* has vertical elongated black spots while *N. fasciatus* has irregularly shaped dark lines on the lateral parts. Thus, this information provides further understanding of the morphological differences between the species found and other species where there are differences in morphological variants in the species found

but are still close relatives of each other and in one species. Furthermore, differences can be observed in the structure of the anterior naris. In *N. chrysolaimos*, the anterior naris typically features a valve pierced tube-like structure, whereas in *N. fasciatus*, the anterior margin may have a winged flap.

The Kruskal-Wallis test showed that all of the 14 measured characters were significantly different ( $P < 0.001$ ) (Table 1). The differences in morphological characteristics can be caused by river conditions or habitat profiles of *Nemacheilus* spp. allowing for morphometric differences and variation within species (Hardiaty and Yamahira 2014). Morphological differences between individuals that cause different variants are closely related to habitat factors such as temperature, turbidity, depth, pH, water flow, fluctuating habitats, and the type and amount of food (Hu et al. 2019; Freudiger et al. 2021).

### Sequence composition and genetic diversity

The successful amplification of the COI gene target was confirmed by the presence of a distinct DNA band without any smearing, as visualized at approximately 619 base pairs (Table 2). The absence of stop codons in these sequences indicates that the amplification process effectively captured functional mitochondrial COI sequences. Consequently, nuclear DNA sequences originating from mitochondrial DNA (NUMTs) were excluded from sequencing, as vertebrate NUMTs typically exhibit lengths of less than 619 base pairs (Ricardo et al. 2020). The utilization of the COI gene for species identification is widely useful for its accuracy, primarily because pseudogenes are eliminated during the protein translation process. Studies have consistently demonstrated the effectiveness of COI sequences in distinguishing species across diverse taxa. For instance, recent research on biodiversity has led to the generation of new COI sequences for a significant percentage of species (Venera-Ponten et al. 2020).

**Table 1.** Morphometric data (mm) and Kruskal-Wallis's test (H) on two species of *Nemacheilus* From Pasuruan, East Java, Indonesia

Characters	<i>N. chrysolaimos</i> (n=16)						<i>N. fasciatus</i> (n=21)					
	Min	Max	Mean	Std.error	H	p	Min	Max	Mean	Std.error	H	p
Total length (TL)	50.0	68.0	56.0	4.9	28.8	<0.001	60.0	68.0	59.6	2.7	43.1	<0.001
Standard length (SL)	40.0	56.0	40.1	5.3	20.3	<0.001	44.0	55.0	51.2	0.7	37.5	<0.001
Head length	1.0	12.0	5.7	1.1	37.3	<0.001	1.0	12.0	7.7	0.7	94.5	<0.001
Snout length	3.0	5.0	3.3	0.1	20.4	<0.001	2.0	4.0	3.1	0.1	55.8	<0.001
Eye diameter	2.0	4.0	2.6	0.1	11.4	<0.001	2.0	3.0	2.3	0.1	70.7	<0.001
Postorbital length	2.0	8.0	4.0	0.3	26.8	<0.001	3.0	7.0	4.0	0.2	11.1	<0.001
Predorsal length	2.0	27.0	23.3	1.4	21.3	<0.001	21.0	28.0	24.0	0.4	28.8	<0.001
Pre-pelvic length	21.0	28.0	24.6	0.4	23.4	<0.001	23.0	29.0	25.2	0.3	30.8	<0.001
Pre-pectoral length	1.0	13.0	9.2	1.0	93.1	<0.001	1.0	13.0	7.6	0.9	12.6	<0.001
Pre-pectoral distance	1.0	23.0	8.0	1.6	54.7	<0.001	1.0	22.0	7.8	1.0	81.9	<0.001
Pectoral fin length	1.0	11.0	7.0	0.7	55.6	<0.001	1.0	22.0	7.0	1.0	73	<0.001
Body height	1.0	14.0	10.8	0.7	12.7	<0.001	1.0	15.0	11.9	0.6	18.3	<0.001
Dorsal fin length	1.0	13.0	6.9	0.9	53.7	<0.001	1.0	24.0	8.5	1.0	88.3	<0.001
Anal fin length	1.0	14.0	6.8	0.9	53.3	<0.001	1.0	25.5	9.6	1.0	87.3	<0.001
Height of caudal peduncle	1.0	16.0	8.5	1.4	69.2	<0.001	1.0	15.0	7.4	0.8	81.5	<0.001

DNA barcoding to be highly effective in distinguishing freshwater fish species across various regions, including India, Thailand, Germany, and Indonesia (Kneibelsberger et al. 2015; Lakra et al. 2015; Panpromin et al. 2019; Rahayu et al. 2019). In our study, we have compiled the partial sequence profiles of the COI gene for *N. chrysolaimos* and *N. fasciatus*, two local freshwater fish species found in Pasuruan District, Indonesia, across different locations. Our findings confirm the efficacy of DNA barcodes in accurately identifying these species. Importantly, no insertions/deletions or codon stops were observed upon translation of the nucleotide sequences. These results is reliability and utility of DNA barcoding as a powerful tool for species identification and biodiversity assessment in freshwater fish populations.

The high haplotype diversity (Hd) of 0.9 and nucleotide diversity ( $\pi$ ) of 0.1 (Table 3) indicate a wide range of genetic variations within the studied *Nemacheilus* spp. The frequency of parsimony informative sites at 25.6% suggests the presence of informative genetic variations that can aid in

species differentiation. The 16 polymorphic sites and the overall ts/tv ratio of 2.3 further highlight the genetic variability and evolutionary dynamics within the studied *Nemacheilus* spp. in Pasuruan District. The mean evolutionary rate ranging from 0.00 to 2.79 substitutions per site indicates the rate of genetic changes over time in the COI gene sequences. The assessment of genetic distance between species is crucial for understanding the relationships and similarities among different *Nemacheilus* spp. A greater genetic distance, such as the value of 0.11 or 11% between *N. fasciatus* and *N. chrysolaimos*, indicates a different genetic relationship in the partial COI gene sequences between these species. The highest distance observed between *N. fasciatus* and *N. Chrysolaimos* 12% while the smallest distance was between *N. fasciatus* and *N. chrysolaimos* at 0.112 or 11.2% (Table 3). The findings of this composition can provide insights into the evolutionary history and divergence patterns within the studied *Nemacheilus* spp. from Warung Dowo River, Pasuruan District.

**Table 2.** DNA Sequences from NCBI GenBank used as comparisons

Species	Code	Sample location	Acc. number GenBank
<i>Nemacheilus fasciatus</i>	MZB 26551	Slorok River, Garum	OP412496.1
<i>Nemacheilus fasciatus</i>	MZB 26545	Loadeng River, Ponggok	OP412495.1
<i>Nemacheilus fasciatus</i>	BIF3609	Dauwan River, Mojokerto	KU692665.1
<i>Nemacheilus fasciatus</i>	BIF0832	Pelus River, Purwokerto	KT960792.1
<i>Nemacheilus chrysolaimos</i>	MZB 26539	Slorok River, Garum	OP381212.1
<i>Nemacheilus chrysolaimos</i>	MZB 26540	Slorok River, Garum	OP379585.1
<i>Nemacheilus chrysolaimos</i>	BIF0170	Dauwan River, Mojokerto	KU692664.1
<i>Rasbora argyrotaenia</i>	BIF9657	Peny Bay, Cilacap	MN869838.1
<i>Rasbora argyrotaenia</i>	BIF0975	Peny Bay, Cilacap	KT960805.1
<i>Nemacheilus chrysolaimos</i>	PAS 01 Type 1	Warung Dowo, Pasuruan*	PP627145
<i>Nemacheilus chrysolaimos</i>	PAS 02 Type 1	Warung Dowo, Pasuruan*	PP627149
<i>Nemacheilus chrysolaimos</i>	PAS 03 Type 1	Warung Dowo, Pasuruan*	PP627150
<i>Nemacheilus chrysolaimos</i>	PAS 04 Type 2	Warung Dowo, Pasuruan*	PP627154
<i>Nemacheilus chrysolaimos</i>	PAS 05 Type 2	Warung Dowo, Pasuruan*	PP627503
<i>Nemacheilus chrysolaimos</i>	PAS 06 Type 2	Warung Dowo, Pasuruan*	PP627499
<i>Nemacheilus fasciatus</i>	PAS 11 Type 1	Warung Dowo, Pasuruan*	PP627185
<i>Nemacheilus fasciatus</i>	PAS 12 Type 1	Warung Dowo, Pasuruan*	PP627498
<i>Nemacheilus fasciatus</i>	PAS 13 Type 1	Warung Dowo, Pasuruan*	PP627500
<i>Nemacheilus fasciatus</i>	PAS 14 Type 2	Warung Dowo, Pasuruan*	PP627200
<i>Nemacheilus fasciatus</i>	PAS 15 Type 2	Warung Dowo, Pasuruan*	PP627501
<i>Nemacheilus fasciatus</i>	PAS 16 Type 2	Warung Dowo, Pasuruan*	PP627502

Note: \* this study

**Table 3.** Characteristics of partial sequence of COI gene used for phylogenetic trees reconstruction and genetic distance analysis include sequences from the study sample and the GenBank/BOLD system (in group and out group)

Parameters	Position at codon			Total
	1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	
Thyrosine frequency	26.74%	41%	22%	619 bp
Cytosine frequency	30.20%	29.1%	35.1%	619 bp
Adenine frequency	23.65%	15.5%	30.3%	619 bp
Guanine frequency	19.41%	14.0%	12.8%	619 bp
Frequency of invariable sites	60.352%			
Frequency of parsimony informative sites	25.646%			
Nucleotide diversity (Pi)	0.09762			
Haplotype diversity	0.882			
Number of haplotypes	12			
Polymorphic sites	166			
Variance of Haplotype diversity		0.00152		
ts/tv ratio (R)	2.267			
Gamma discrete distribution	0.4428			
Mean of evolutionary rate	0.00, 0.04, 0.05, 0.09, 0.22, 0.26, 0.31, 0.42, 0.56, 0.72, 0.93, 1.20, 1.53, 2.01, and 2.79			
	substitutions per site			

Note: The COI gene sequence characteristics were based on the 619 bp sequence length



The base composition analysis of the partial sequence of the COI gene, revealing a higher AT content (50.39%) compared to GC content (49.61%), is a significant finding that aligns with similar observations in various fish species across different regions. Studies by Modeel et al. (2024) in Beas River, and Bingpeng et al. (2018) in Taiwan straits have reported comparable trends in base composition analysis of the COI gene, indicating a prevalent pattern of AT richness in fish species. A lower GC composition and G were observed, characteristics of mitochondrial DNA in fishes. The GC content in the first codon position was notably higher than in the other two positions, with the base usage bias at the third codon position being the lowest.

### Identification using BOLD Systems

The highest identification accuracy achieved for loaches from Pasuruan District using the Barcode of Life Data Systems (BOLD) online platform, ranging from 99.52 to 99.9%, is an impressive testament to the effectiveness of DNA barcoding in species identification. This level of accuracy is particularly notable given the complexity and diversity of loach fish species, which often exhibit subtle morphological differences that can be challenging to discern using traditional taxonomy alone. This study supports the use of the BOLD system as a superior tool for fish identification compared to other databases, aligning well with the objective of achieving high identification accuracy, same findings with the study by Modeel et al. (2023). Our findings indicate that there were no differences in BOLD identification between the variants observed in *N. fasciatus* and *N. chrysolaimos* (Table 4).

### Phylogenetics reconstruction

Phylogenetic reconstruction analysis resulted in the construction of Neighbor-Joining (NJ) and Maximum-Likelihood (ML) trees, as depicted in Figures 7 and 8, respectively. Each species was linked to a distinct DNA barcode cluster, facilitating the clear delineation of phylogenetic relationships among them. Both NJ and ML trees exhibited two divergent clusters, with bootstrap values exceeding 99%, indicating robust support for the inferred relationships. The phylogenetic trees demonstrated

a clear and unambiguous branching pattern for *Nemacheilus* spp., with *N. fasciatus* and *N. chrysolaimos* forming their own monophyletic branches. However, the close proximity of these two species at the same node suggests a close evolutionary relationship, consistent with the calculated genetic distance of 0.11, which indicates the furthest genetic separation between them. Overall, the NJ, ML, and genetic distance analyses support the genetic distinctiveness of *N. fasciatus* and *N. chrysolaimos*, highlighting their divergence and evolutionary relationships within the *Nemacheilus* genus.

The findings of this research revealed that *N. fasciatus* and *N. chrysolaimos* are resolved sister taxa with a robust bootstrap support of 99%. The genetic diversity observed between these clusters was very low, with values less than 2%. According to established criteria, a genetic distance value exceeding 2% typically indicates species differentiation, while values below 3% suggest intra-specific or conspecific groupings (Chakraborty and Ghosh 2014; Bektas et al. 2018). Overall, the genetic distance analysis supports the distinction between species and provides insight into the genetic diversity within *Nemacheilus* spp. in Pasuruan District.

### Species delimitation using ABGD

In addition, ABGD method identified 3 groups for *Nemacheilus* spp. specimens in with the initial approach and the barcode gap threshold calculated by the ABGD analysis of the COI dataset (see Figures 9.A and 9.B). Initial Partition with prior maximal distance  $P=2.15e-02$ ; Barcode gap distance = 0.100. instance K80 Kimura = 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 3 to 7 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 3 groups (see Figure 9.C).

**Table 4.** The highest Bold Identification of Local loach From Pasuruan District, Indonesia

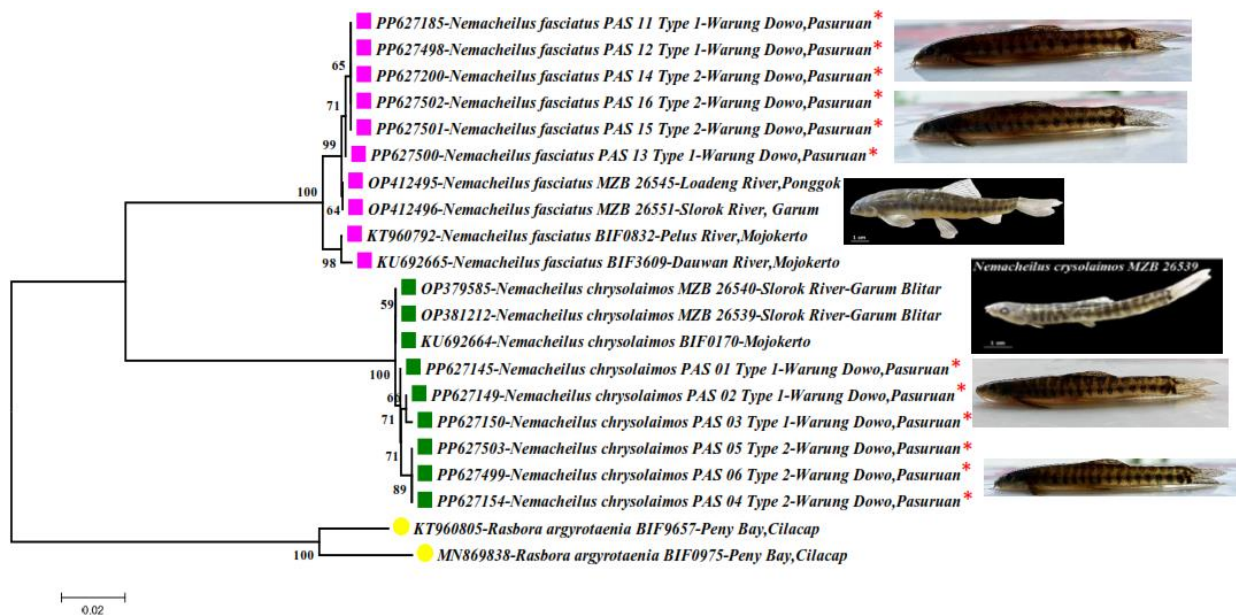
Species	Highest BOLD Identification	Similarities (%)	Status
PP627503- <i>Nemacheilus chrysolaimos</i> PAS 05 Type 2	<i>Nemacheilus chrysolaimos</i>	99.84	Published
PP627499- <i>Nemacheilus chrysolaimos</i> PAS 06 Type 2	<i>Nemacheilus chrysolaimos</i>	99.68	Published
PP627154- <i>Nemacheilus chrysolaimos</i> PAS 04 Type 2	<i>Nemacheilus chrysolaimos</i>	99.52	Published
PP627145- <i>Nemacheilus chrysolaimos</i> PAS 01 Type 1	<i>Nemacheilus chrysolaimos</i>	99.58	Published
PP627149- <i>Nemacheilus chrysolaimos</i> PAS 02 Type 1	<i>Nemacheilus chrysolaimos</i>	99.72	Published
PP627150- <i>Nemacheilus chrysolaimos</i> PAS 03 Type 1	<i>Nemacheilus chrysolaimos</i>	99.8	Published
PP627501- <i>Nemacheilus fasciatus</i> PAS 15 Type 2	<i>Nemacheilus fasciatus</i>	99.9	Published
PP627502- <i>Nemacheilus fasciatus</i> PAS 16 Type 2	<i>Nemacheilus fasciatus</i>	99.78	Published
PP627200- <i>Nemacheilus fasciatus</i> PAS 14 Type 2	<i>Nemacheilus fasciatus</i>	99.68	Published
PP627185- <i>Nemacheilus fasciatus</i> PAS 11 Type 1	<i>Nemacheilus fasciatus</i>	99.68	Published
PP627498- <i>Nemacheilus fasciatus</i> PAS 12 Type 1	<i>Nemacheilus fasciatus</i>	99.52	Published
PP627500- <i>Nemacheilus fasciatus</i> PAS 13 Type 1	<i>Nemacheilus fasciatus</i>	99.54	Published

The automatic barcode gap discovery (ABGD) analysis conducted at a prior maximal distance has successfully delineated *N. chrysolaimos* and *N. fasciatus* into separate partitions, supporting the phylogenetic tree's findings. This method has proven effective in identifying distinct genetic clusters within these loach species, delineation of their evolutionary relationships.

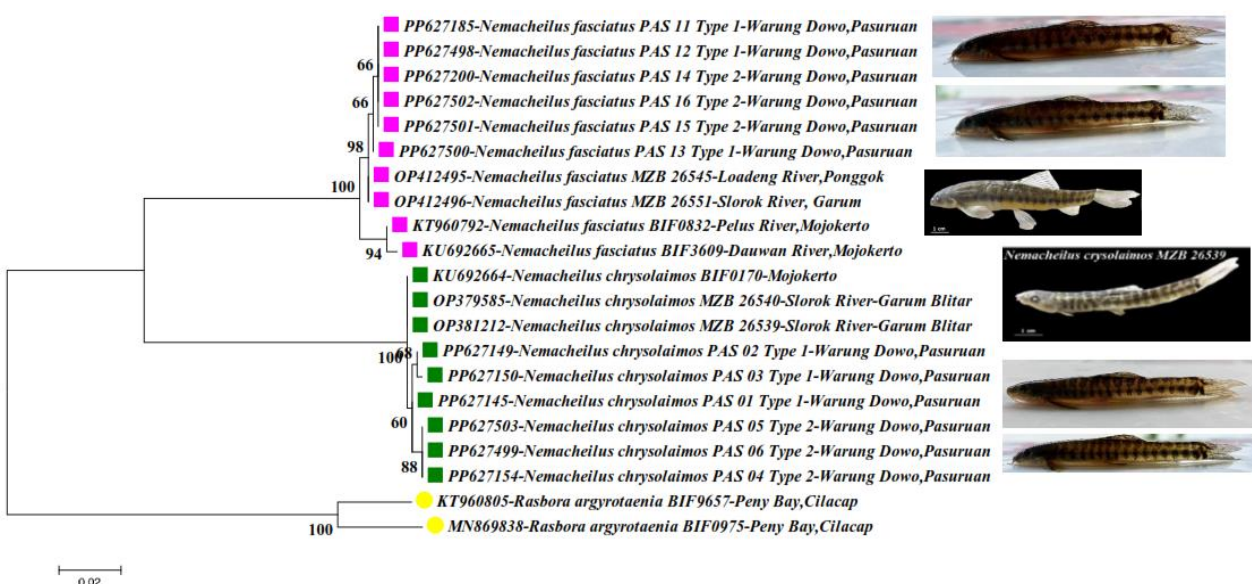
### Haplotype network *Nemacheilus* spp.

The Median Joining network analysis has provided a comprehensive description of the genetic variation within

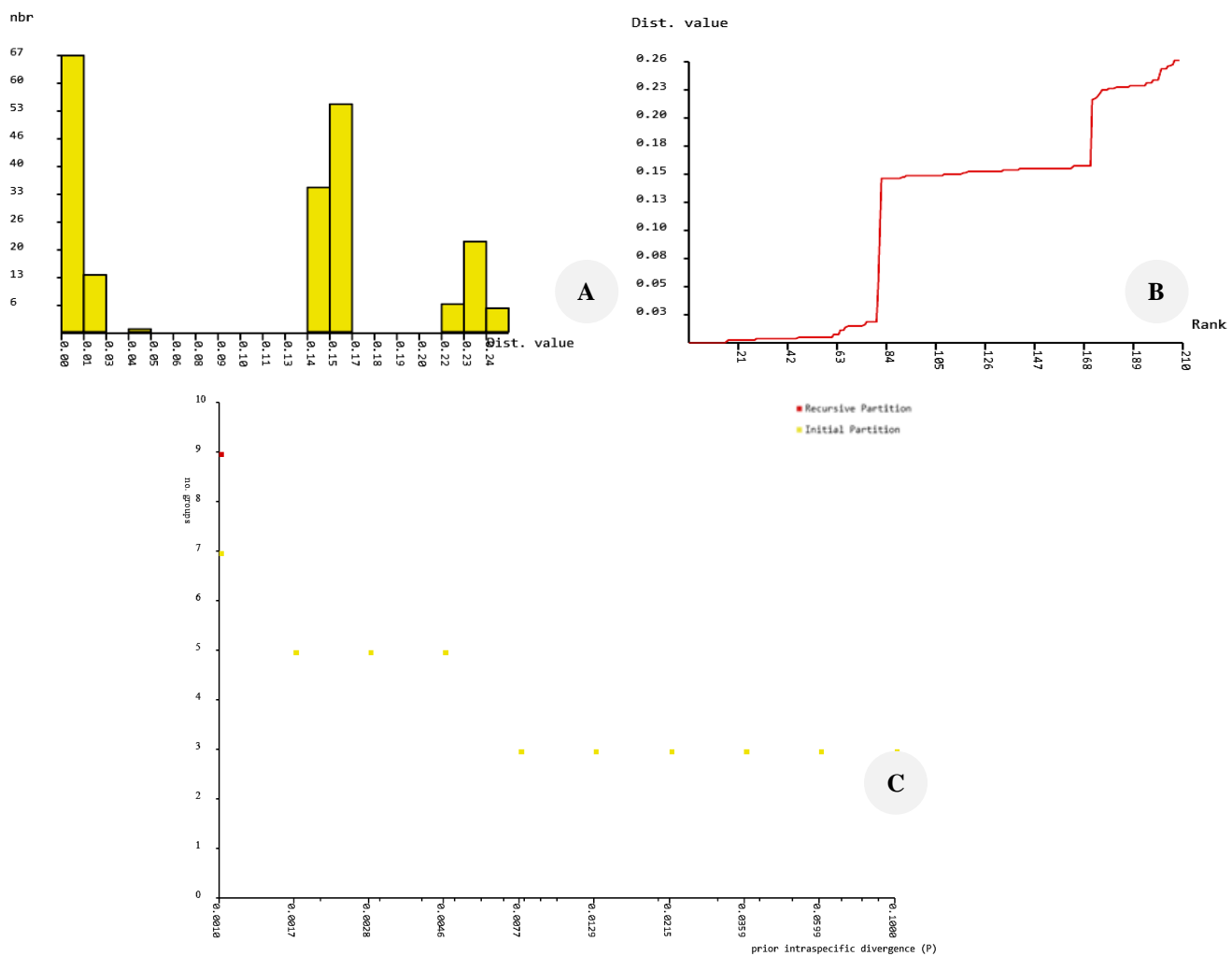
*Nemacheilus* spp., highlighting 12 haplotypes grouped into 3 distinct haplogroups. The network illustrates that *Nemacheilus* species form an ingroup based on their unique haplotypes, with each fish possessing a distinct, non-homologous haplotype. Specifically, *N. chrysolaimos* exhibits its own homologous haplotype (haplotype 1-variant 1), along with haplotypes 2, 3, and 4 (variant 2), while *Nemacheilus* ingroup is represented by Haplotype 5. This genetic clustering suggests a close relationship of *N. chrysolaimos* with the origin locations of Blitar and Mojokerto, as depicted in Figure 10.



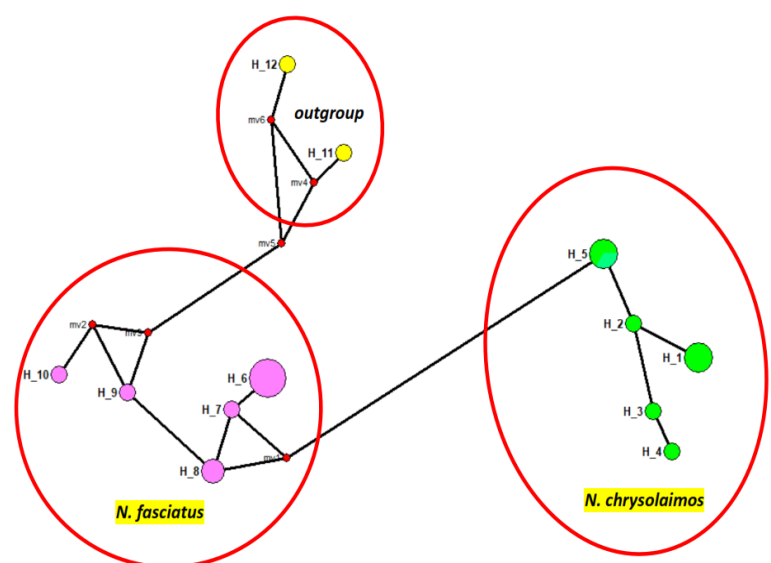
**Figure 7.** Neighbour Joining (NJ) phylogenetic tree of *Nemacheilus* spp based on partial sequence of COI gene. The asterisk (\*) denotes the sequence of *Nemacheilus* spp. obtained from Warung Dowo river, Pasuruan District, East Java, Indonesia and *Rasbora* spp. as the outgroup



**Figure 8.** Maximum Likelihood (ML) phylogenetic tree of *Nemacheilus* spp based on partial sequence of COI gene. The asterisk (\*) denotes the sequence of *Nemacheilus* spp. obtained from Warung Dowo river, Pasuruan District, East Java, Indonesia and *Rasbora* spp. as the outgroup



**Figure 9.** Analysis of Gap Barcodes of *Nemacheilus* sp. species generated by Automatic Barcode Discovery Gap Discovery (Puillandre et al. 2012). Distribution of K2P distances between each pair of specimens for the COI gene; A=Distance histogram; B=Rank distance, and; C=Number of PSH obtained for each previous intraspecific divergence



**Figure 10.** Haplotype network of *Nemacheilus* spp. Haplotype network of 12 haplotypes based on the COI gene sequence. Haplotypes are shown in different circle shapes and patterns, the number of individuals is shown in different circle shapes (large=3 individuals; small=1 individual). Branching between haplotypes is shown by substitution based on the alignment position of the COI gene sequence



Similarly, the grouping of *N. fasciatus* fish also clusters according to their types, but they are not homologous due to geographical differences. These findings provide valuable insights into the genetic diversity and relationships within *Nemacheilus* species in East Java, emphasizing the importance of further research and conservation efforts for these unique fish populations.

In conclusion, this study confirms the efficacy and reliability of targeted DNA barcoding and morphological evidence for the identification of *Nemacheilus* spp. at the species level. It represents the first report on the morphology (consistent with Kottelat 1984; Kottelat et al. 1993; Hardiaty and Yamahira 2014; Rahayu et al. 2023), genetic identification, and phylogenetic reconstruction of *Nemacheilus* spp. from Pasuruan District using partial sequences of the COI gene. Moreover, the conservation management of *N. chrysolaimos* and *N. fasciatus* is made feasible through the grouping of fish units according to species and genetic composition, as well as the potential development of cryopreservation for sustainability and domestication efforts. The results of this study demonstrate that a molecular approach utilizing partial COI gene sequences supports the identification results based on morphological characteristics in *Nemacheilus* spp., with accession numbers obtained from the GenBank (NCBI) database. This research provides approved and improved identification of the morphology and molecular characteristics of local loaches (*Nemacheilus* spp.) from Pasuruan District. By establishing a reliable DNA barcode reference library for East Java, Indonesia freshwater fish, this study first effort for enhanced monitoring, conservation, and management of fisheries in these overexploited regions.

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