FURTHER STUDY ON TWO SPECIES OF LOACH FISHES (Cypriniformes: Nemacheilidae: *Nemacheilus*) BASED ON MORPHOLOGY AND MOLECULAR DATA

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ABSTRACT

The identities of two local loaches, *Nemacheilus chrysolaimos* (Valenciennes, 1846) and *N. fasciatus* (Valenciennes, 1846) from six rivers, were obtained through a comprehensive examination of their morphology and molecular characteristics in Biltar Regency, East Java, Indonesia. Therefore, this study identified *Nemacheilus* spp. from Blitar based on morphology and partial sequence of COI. The meristic data obtained for *N. chrysolaimos* included DII. 7–8 (dorsal fin), AI. 3–5 (anal fin), PI. 9 (pectoral fin), VI. 6–7 (ventral fin), and C. 17 (caudal fin). On the other hand, *N. fasciatus* exhibited the following meristic data, namely D II 7–8 (dorsal fin), AI. 6 (anal fin), PI. 9–10 (pectoral fin), VI. 6–7 (ventral fin), and C. 17 (caudal fin). A significant difference was observed in the morphometric characteristics of *N. fasciatus* across various sampling sites, as determined by the Kruskal-Wallis Test. Furthermore, the nucleotide base composition sequences of *Nemacheilus spp.* consisted of Thiamine (T), Cytosine (C), Adenine (A), and Guanine (G) with a mean of 29.565%, 32.023%, 23.88%, and 16.244%. Maximum Likelihood (ML) and Minimum Evolution (ME) phylogenetic analysis was also conducted using the Kimura 2 Parameter model to establish two major clades on *Nemacheilus* spp. and one out-group significantly different from the *Nemacheilus* spp. The results showed that these major clades exhibited a close relationship at 100% bootstrap support and were grouped under the genus *Nemacheilus*. The study on *Nemacheilus* spp. from the Blitar locality differentiated COI sequences between *N. fasciatus* and *N. chrysolaimos*. Additionally, *N. chrysolaimos*, as inferred from reference sequences, was identified as the ancestral species to *N. chrysolaimos* MZB 26540 and MZB 26539. ABGD analyses, employing a prior maximal distance of 0.025, also indicated the separation of these species into distinct partitions. The integration of morphology and genetic data for *Nemacheilus* spp. should provide valuable insights for future genetic population studies and conservation initiatives.

Keywords: DNA barcoding, morphology, *Nemacheilus,* phylogenetic, taxonomy

INTRODUCTION

The family Nemacheilidae comprises one of the most diverse groups of freshwater fish worldwide, encompassing 43 genera and 714 species (Froese & Pauly 2022a). One of the members of this family is the genus *Nemacheilus*, which has been described and validated with a total of 55 species. According to (Kottelat 1984; Kottelat *et al.* 1993; Kottelat 2013a; Froese & Pauly 2022b; Kottela, 2022), the highest diversity of *Nemacheilus* species has been recorded in the Asiatic region. Among the total of 55 species, only two species, namely *Nemacheilus chrysolaimos* and *N. fasciatus*, have been reported to be highly abundant in Java Island (Kottelat 1984; Kottelat

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et al. 1993; Hadiaty and Yamahira 2014; Hubert *et al.* 2019).

The taxonomic history of the two *Nemacheilus* fishes was initially documented by Cuvier and Valenciennes in 1846. They classified these species under the genus Cobitis, naming them *Cobitis chrysolaimos* and *C. fasciata* (Cuvier & Valenciennes, 1846). Subsequently, the genus was revised by Bleecker in 1853, and the name *Nemacheilus* became a valid designation. These *Nemacheilus* spp. have gained significant attention due to the exclusive distribution in Indonesian waters, as well as their strikingly similar morphology but distinct body colorations. Kottelat (1984) elucidated the differences between these two species and in subsequent publications (Kottelat *et al.* 1993), additional information was provided. Moreover, Hadiaty and Yamahira (2014) presented an updated identification key specifically for the species of *Nemacheilus* spp. found in Asian waters within Indonesia.

In addition to the morphological analysis, the molecular examination of the two *Nemacheilus* fishes showed their dissimilarities. DNA barcoding identified the Most Recent Common Ancestor (MRCA) to have existed approximately 1.5 million years and 0.5 million years ago in *N. fasciatus* and *N. Chrysolaimos,* respectively (Hubert *et al.* 2019). Šlechtová *et al.* (2021) also supported that *N. chrysolaimos* was different from *N. masyae* through genomic DNA isolation. According to Kusuma *et al.* (2021), *Nemacheilus chrysolaimos* from Temanggung and Yogyakarta using the partial sequence of COI gene indicated a haplotype and nucleotide diversity (Hd) of 0.679 and 0.00117, respectively. The differentiation between these two species needs to be reinforced through DNA barcoding in contrast to the study conducted by Ath-thar *et al.* (2018) using PCR-RAPD analysis on *Neimacheilus fasciatus*, which revealed a high level of genetic diversity. This study also augments the understanding of *Nemacheilus* spp. by employing morphological and molecular approaches. A noteworthy addition is the inclusion of the fins formula, which has not been previously described. The outcomes are expected to offer valuable insights and resolve the taxonomy of these two fishes yet to be disclosed.

MATERIALS AND METHODS

Field Sampling

The specimens of *Nemacheilus* spp. from Blitar Regency were collected from three main locations, namely Garum, Ponggok, and Wlingi at six rivers. Specifically, Garum consisted of three rivers (Slorok, Sumber Ronje and Glawah), Ponggok comprised two rivers (Loadeng and Tunjung), and Wlingi included one river (Lekso), as shown in Figs. 1 & 2. To capture the fish samples, gill nets were employed, and the collected fishes were preserved in sample bottles containing a 10% formaldehyde solution. Furthermore, the samples were transported to the laboratory at the State University of Surabaya (Universitas Negeri Surabaya) for subsequent identification, measurement, and analysis.

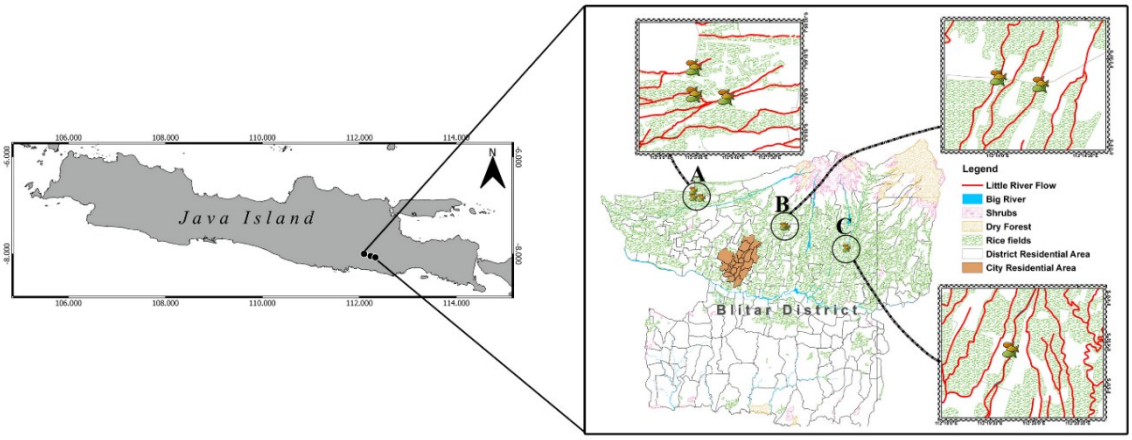

Figure 1 The map showing sampling localities of two Nemacheilus fishes at three rivers of Blitar, East Java, Indonesia. A. Garum (7°59.967'S, 112°5.775'E), B. Ponggok (8°2.604'S, 112°14.004'E) and C. Wlingi (8°4.215'S, 112°19.894'E)

Figure 2 The habitat of two *Nemacheilus* fishes in Blitar, East Java, Indonesia. A. Slorok river, B. Sumber Ronje river, C. Glawah river, D. Loadeng river, E. Tunjung river and F. Lekso river

Morphology Work

In the laboratory, all of the fish were sorted, washed, and cleaned for morphological observation and identification. Measurement of the morphological characteristics was made on 14 characters, as shown in Fig. 3, using modifications of Kottelat (1984) and Kottelat and Freyhof (2007) with a digital caliper and 0.01 mm accuracy. Furthermore, the identification was performed in line with the study of Kottelat (1984) and Kottelat *et al.* (1993). The specimens were then stored in 70% alcohol and deposited in the Museum Zoologicum Bogoriense (MZB), Cibinong, Indonesia. Before molecular work, the specimens were frozen at -20°C for DNA extraction.

DNA Extraction and Sequencing

The isolation of total DNA (whole genome) from stomach tissue samples was carried out using the DNA Isolation Kit (Roche), with several modifications. A DNA fragment of approximately 526 base pairs (bp), corresponding to the COI gene region of the mitochondrial DNA (mtDNA), was successfully amplified using gradient PCR. The universal primers LCO1490 (5' GGT CAA CAA ATC ATA AAG ATA TTG G 3') and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3') were used for this purpose (Folmer *et al.* 1994). The hotstart PCR method was employed, using a Kapa master and two Taq master mixes. The PCR process consisted of 35 cycles, each encompassing the following steps, initial double-strand attachment (pre-denaturation) at 95°C for 3 minutes, denaturation at 94°C for 45 seconds, annealing at 45°C for 45 seconds, and extension at 72°C for 2 minutes. A final elongation step was conducted at 72°C for 10 minutes. To visualize the PCR products, gel electrophoresis was performed on a 1% agarose gel prepared with 0.5 g of agarose and 50 mL of TAE buffer. Additionally, 4 µL of Ethidium Bromide (EtBr) was added as a dye to the gel, and the subsequent step was to mix 3 µL of the PCR samples with 1 µL of loading dye before putting the mixture in an agarose well. The electrophoresis was performed using a machine with a voltage of 220 V and a current of 400 mA, for 25 minutes. PCR products were also purified using a Qiagen purification kit according to the manufacturer's instructions and subsequently sequenced at First Base, Malaysia.

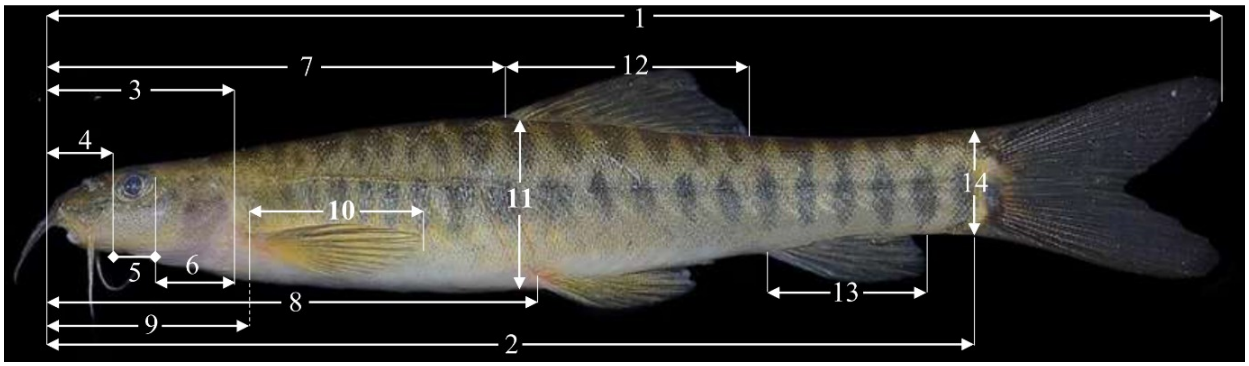

Figure 3 Morphometric of the *Nemacheilus* fish from Blitar, East Java. Abbreviation: **1.** Total length (TL); **2.** Standard length (SL); **3.** Head length; **4.** Snout length; **5.** Eye diameter; **6.** Postorbital length; **7.** Predorsal length; **8.** Prepelvic length; **9.** Prepectoral length; **10.** Pectoral fin length; **11.** Height of the body; **12.** Dorsal fin length; **13.** Anal fin length; **14.** Height of caudal peduncle. The fish image was adopted from Hubert *et al.* (2019); measurement was modified from Kottelat (1984) and also Kottelat & Freyhof (2007)

Data Analysis

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*, from sampling localities. This was because only this species occurred at all six rivers, while *N. chrysolaimos* had a limited number and was only found in one river. The molecular data obtained from the study were subjected to the following analytical procedures:

Sequence Composition and Genetic Diversity

A partial sequence of the COI gene along 503 bp was obtained from 11 *Nemacheilus* spp. from Blitar Regency, East Java, as the final dataset. Each sequence was initially translated into an amino acid to check and remove pseudogene (Song *et al.* 2008; Buhay 2009). Nucleotide sequencing was then continued by carrying out the chromatogram analysis, using Finch TV software and translating into amino acid sequence through the ExPASy website (Duvaud *et al.* 2021). Subsequently, all sequences were checked through the BLAST (*Basic Local Alignment Search Tool*) (Boratyn *et al.* 2013) and the BOLD System (Ratnasingham & Hebert 2007) to be compared with close relatives of *Nemacheilus* spp. A total of 5 accessions from GenBank (NCBI) were selected as in-group and out-group for the phylogenetic tree reconstruction. Multiple sequence alignment was performed by using the Clustal X (Larkin *et al.* 2007) and then checked manually through the BioEdit software (Hall 1999). Furthermore, partial sequences of the COI gene from *Nemacheilus* spp. were submitted to GenBank with referred accession numbers, as shown in Table 2. All the data including taxonomic characteristics and GenBank accession numbers were tagged with the voucher specimens preserved at Zoologicum Bogoriense (MZB) at Juanda Street, Bogor, West Java, Indonesia.

The calculation of similarity values was performed as follows: similarity percentage = (1-Genetic Distance) × 100%. The substitution of transitions and transversion of nucleotide bases was calculated by the K2P (Kimura 2-Parameter) model. Information on the genetic diversity of all sequences used for phylogenetic reconstruction was analyzed through items, such as the value of nucleotide diversity (Pi), the number of polymorphic sites (S), the haplotype analysis (haplotype diversity (Hd), and the number of haplotypes (nHap) (Nei 1972). In addition, the software version of MEGA X was 10.2.6 (Kumar *et al.* 2018) used to calculate nucleotide frequencies, transition/conversion ratio (k), transition/conversion, rate ratio bias (R), and probabilities.

Phylogenetic Reconstruction

Reconstruction of the phylogenetic tree based on the partial sequence of the COI gene was conducted on a total of 17 sequences using MEGA X version 10.2.6. The purpose is to determine the grouping of different species and

the applied methods were Minimum Evolution (ME) and Maximum-Likelihood (ML). The settings used for ME phylogenetic tree reconstruction begins with the setting of a bootstrap consensus tree inferred from 1000 replicates retrieved to represent the evolutionary history of the taxa (Felsenstein 1985). Branching corresponding to partitions reproduced in less than 50% of bootstrap replicates was eliminated. Furthermore, evolutionary distances were calculated using the Kimura 2-parameter (K2P) method and in units of the number of base substitutions per site (Kimura, 1980). The rate variation among sites was modeled with a gamma distribution (shape parameter = 63). The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at search level 2 (Nei & Kumar 2000) and the neighbor-joining algorithm was used to generate the initial tree (Saitou & Nei 1987).

The settings used for ML phylogenetic tree reconstructions were calculated by using the K2P substitution model (Saitou & Nei 1987), and the rate variation among sites was modeled with a Gamma distribution and bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985). In addition, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. The barcode gap analysis generated by Automatic Barcode Gap Discovery (ABGD) was used to strengthen the identification of this species, and grouping analysis (Puillandre *et al.* 2012) was conducted through a web interface to check the distribution and size of a potential barcoding gap for the partial sequence of COI gene dataset with the following settings: Pmin: 0.001, Pmax: 0.9, Step: 10, X (relative gap width):1.5, Kimura (K80), number of bins: 20.

RESULTS AND DISCUSSION

Class Actinopteri Cope, 1871
Ordo Cypriniformes Bleeker, 1859
Family Nemacheilidae Regan, 1911
Genus *Nemacheilus* Bleeker, 1863
***Nemacheilus chrysolaimos* (Valenciennes, 1846)** (Fig. 4)

Noemacheilus fasciatus Kuhl & van Hasselt in van Hasselt, 1823: 133 (Buitenzorg) (partim; nomen nudum).
Cobitis chrysolaimos Valenciennes in Cuvier & Valenciennes, 1846: 27, fig. 521.
Noemacheilus chrysolaimos Kottelat, 1984: 241, figs. 14a, 15.
Nemacheilus chrysolaimos Kottelat, 1993: 75, pl. 25.—Roberts, 1993: 25, fig. 29.—Hardiaty & Yamahira, 2014: 84 (list), 87 (list), 90 (list), 92 (key).

Material examined. Slorok river, Garum (MZB 26539, SL. 52.5 mm; MZB 26540, SL. 51.3 mm; 08°02'36.25"S, 112°14'00.23"E), Blitar, East Java, 26 June 2022, Coll. D.A. Rahayu & E.D. Nugroho.

Description. Morphometric data are presented in Table 1. The head is rounded with a pair of eyes, a pair of nares, a short blunt snout, a small subterminal mouth, and a circular lip around the mouth. Furthermore, the eyes are elliptical and nares are located between the snout and the eyes. The mouth contains three pairs of rostral barbels, two pairs on the upper jaw, and one pair on the upper snout maxillary barbels may reach half of the postorbital length of the head. The inner rostral spines are present and reach about half of the eye.

The body is elongated, fusiform, weakly compressed, and laterally flattened at the base of the tail without sharp scales. TL is about 1.18–1.28 (1.24 ± 0.01) times as long as SL and head length is about 2.21–3.09 (2.65 ± 0.09) times as the snout. Meanwhile, the eye diameter is about 0.38–0.57 (0.46 ± 0.02), 0.36–0.50 (0.41 ± 0.01), and 0.02–0.04 (0.03 ± 0.002) times as long as the snout, postorbital length, and SL, respectively. Predorsal length, Prepectoral length, and height of the body are about 0.99–1.10 (1.02 ± 0.01), 1.07 (0.94 ± 0.02), and 1.00–1.50 (1.19 ± 0.05) times as the prepelvic length, length of the head, and height of the caudal peduncle, respectively.

Pectoral fins almost reach more than half of pelvic fin bases. An axillary lobe presents at the pelvic fins bases under the first to third of branched dorsal rays. The anal fin does not reach the caudal fin bases. The dorsal fin is opposite the ventral fin or just behind the vertical fin. The anal fin is short, far behind the ventral fin. The ventral fin does not reach the anal fin; pectoral fins are shorter than the head. The caudal fin is very

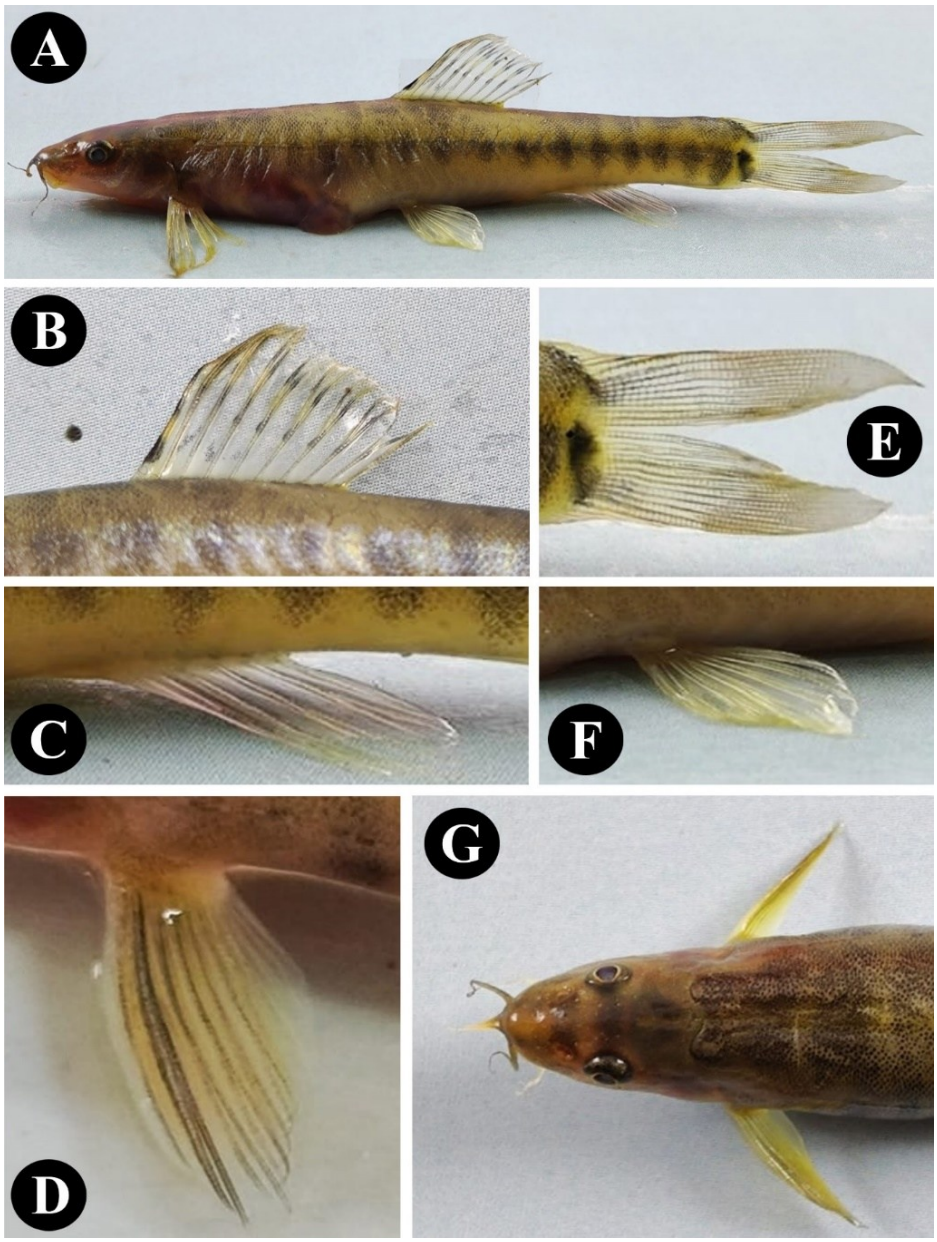

Figure 4 *Nemacheilus chrysolaimos* (Valenciennes, 1846) (Standar Length: 52.5 mm; MZB 26539) from Blitar, East Java, Indonesia. A. Habitus, lateral view; B. Dorsal fin, lateral view; C. Anal fin, lateral view; D. Pectoral fin, lateral view; E. Caudal fin, lateral view; F. Ventral fin, lateral view; G. Anterior part, dorsal view

emarginate, the lobes are acute, caudal fin is longer than the head with the rays all forked. The initial dorsal fin is in front of the vertical line of the anal fin base, closer to the tip of the snout than the base of the caudal fin; the dorsal fin is medium in size, the base of the posterior tip is the opposite the base of the ventral fin and the tip is not reaching anal fin. The caudal fin is crescent in shape, the pectoral fin is rounded, and the length is almost equal to the head, ending less than the length in front of the ventral fin; Anal fin is shorter than the pectoral fin and the shape is tapered or slightly pointed and bearing hard and soft rays, not or barely lower than the body, higher than the length of the base; the caudal fin is emarginate or crescent-shaped-emarginate. The first anterior dorsal rays are the longest. Dorsal fins DII. 7–8, anal fins AI. 3–5, pectoral fin PI. 9, ventral fins VI. 6–7 and caudal fin C. 17.

Coloration. The body is black-yellowish in color with 12–18 dark bars irregular shape on the lateral part. The two pairs of rostral barbels are black and one pair with yellowish coloration. The base of the caudal fin is red in the anterior part of the caudal peduncle and the initial base of the dorsal fin rays has a black spot. The head is brown with a dark color in the center; a darker pattern is also present on the snout and opercula.

Furthermore, the base and first rays of the pectoral fins are dark in color and the last dorsal fin rays are dark with black spots.

Body length. Standard length (SL) and total length (TL) ranged from 32.1–52.4 mm and 40.60–66.40 mm, with a mean of 43.47 ± 2.05 mm and 55.32 ± 2.93 mm (n = 9).

Distribution. According to Kottelat (1984) and Kottelat *et al.* (1993), the species *N. chrysolaimos* was distributed in Java Island, particularly in West Java. Meanwhile, Hubert *et al.* (2019) reported that species can be found in almost at all provinces in Java Island, from West Java, Central Java to East Java.

Remarks. There is a contradiction information on the distribution of this species between Kottelat's (1984) finding with Hubert *et al.* (2019) information. We believe that so far, the Kottelat's collected materials maybe restricted to the West Java only and have not ever been expanded to another location, meanwhile, Hubert *et al.* (2019) revisited and collected this species at all 3 provinces in Java Island, therefore, the information is totally different.

Nemacheilus fasciatus **(Valenciennes, 1846)**
(Fig. 5)

Noemacheilus fasciatus Kuhl & van Hasselt in van Hasselt, 1823: 133; 1824: 376; Kottelat, 1984: 247, fig. 18a.
Cobitis fasciata Valenciennes in Cuvier & Valenciennes, 1846: 25.—Bleeker, 1854: 96; 1860: 78.
Cobitis suborbltalis 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 & Yamahira, 2014: 84 (list), 87 (list), 90 (list), 92 (key).

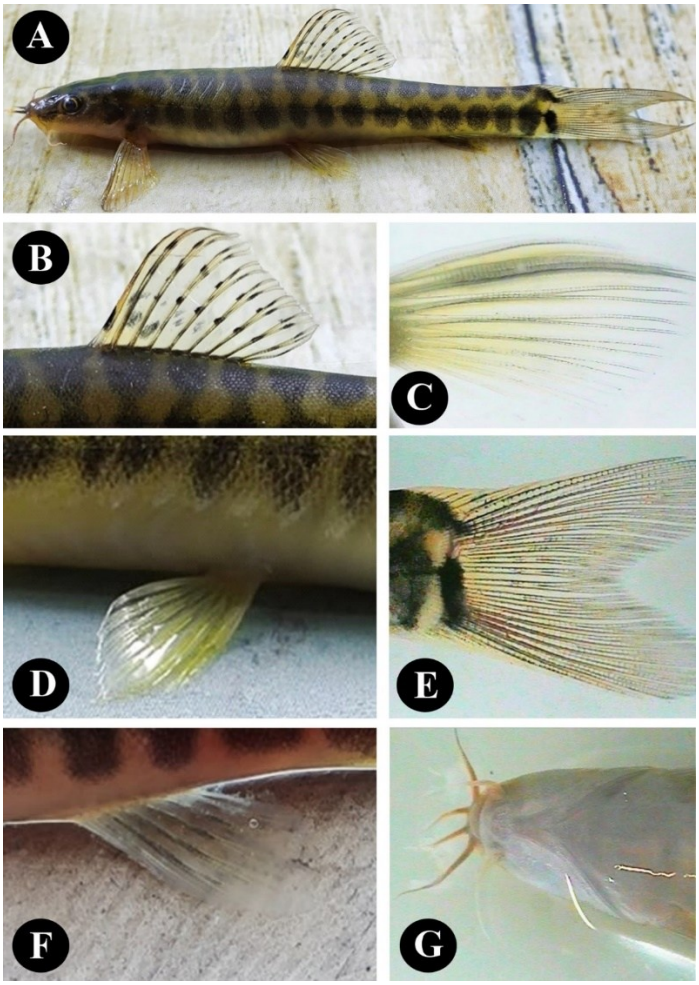

Figure 5 *Nemacheilus fasciatus* (Valenciennes, 1846) (Standar Length: 52.9 mm; MZB 26539) from Blitar, East Java, Indonesia. A. Habitus, lateral view; B. Dorsal fin, lateral view; C. Pectoral fin, lateral view; D. Ventral fin, lateral view; E. Caudal fin, lateral view; F. Anal fin, lateral view; G. Anterior part, ventral view

Material examined. Glawah River (MZB 26552, SL. 45.7 mm; 07°59'58.02"S, 112°05'46.52"E), Loadeng River (MZB 26545, SL. 52.9 mm; 08°00'01.61"S 112°06'30.43"E), Blitar, East Java, 26 June 2022, Coll. D.A. Rahayu & E.D. Nugroho.

Description. Morphometric and statistical data are presented in Table 1. The head is rounded with a pair of eyes, a pair of nares, a short blunt snout, a small subterminal mouth and circular lips around the mouth. The eyes are elliptical and nares are located between the snout and the eyes. Three pairs of rostral barbels are present at the mouth, two pairs on the upper jaw and one pair on the upper snout. The length of the snout is medium, slightly pointed and slightly shorter than the postorbital part of the head. The mouth is arched and the anterior lip is slightly furrowed anteriorly (see Fig. 5). The posterior lip has 4–5 deep grooves on each side of the different median incisions. The posterior part of the lips is smooth. The maxillary and outer rostral barbels reach the mid-length of the postorbital area of the head.

The body is elongated, fusiform, weakly compressed and without sharp scales at the base of the tail. Total length (TL) is about 1.14–1.50 (1.26 ± 0.06) times as long as SL. The head length is about 1.69–3.65 (2.56 ± 0.04) times as long as the snout. Eye diameter is about 0.33–0.69 (0.46 ± 0.005) times as long as the snout; 0.25–0.58 (0.39 ± 0.008) times as long as postorbital length; 0.02–0.06 (0.04 ± 0.0008) times as long as SL. Predorsal length is about 0.72–1.52 (1.01 ± 0.007) times as long as prepelvic length. Prepectoral length is about 0.70–1.22 (0.96 ± 0.008) times as long as the length of the head. The body height is about 1.02–3.22 (1.62 ± 0.04) times as long as the height of the caudal peduncle.

The position of the dorsal fin base is located in front of the vertical line of the pelvic fin. It is closer to the tip of the snout than the base of the caudal fin; the size of the dorsal fin is medium; the base of the posterior tip is opposite to the ventral fin and the tip does not reach the anal fin. The anal fin is short, far behind the ventral fin. The ventral fin does not reach the anal fin; the pectoral fins are shorter than the head. The caudal fin is emarginated, the lobes are pointed and caudal fin is longer than the head. The base of the dorsal fin is almost at the middle of the tip of the snout and the base of the caudal fin. There is no black spot at the base of the anterior dorsal fin. The pectoral fin is rounded and the length is almost equal to the head, ending less than its length in front of the ventral fin, shorter than the pectoral fin, ending less than its length in front of the anal fin; the anal fin is rounded or slightly pointed, not or barely branched lower than body size, higher than base length; the caudal fin is emarginated or crescent-emarginate. The pectoral fin does not reach the base of the ventral fin. A small axillary lobe presents at the base of the ventral fin which is inserted under the dorsal forked finger, the anal fin does not reach the base of the caudal fin and the last fin is branched with a subequal lobe. Dorsal fin D II 7–8; anal fin A I. 6; pectoral fin P I. 9–10; ventral fin V I. 6–7, Caudal fin C. 17.

Coloration. The body is yellowish with 16–18 vertical elongated black spots. The anterior spots are thinner than the posterior ones. There are about 5-6 black saddles on the back in front of the dorsal fin. There is a black spot at the base of the caudal fin. The head is dark. There are about 7 colors of the saddle with positions below and behind the dorsal fin. There is a black spot on the proximal third of the dorsal fin rays. Half of the dorsal fin rays are dark. There are two longitudinal rows of spots on the dorsal rays: in the middle and above all four fin rays. The other fin is hyaline.

Body length. Standard length (SL) ranged from 24.20 to 71.70 mm, with a mean of 42.59 ± 0.87 mm and total length (TL) ranged from 31.30 to 93.50 mm, with a mean of 53.56 ± 1.31 mm (n = 147). The smallest was found from Loadeng river and the largest was found from Lekso river.

Distribution. According to Kottelat (1984) and Kottelat *et al.* (1993), this species was distributed up to southern Sumatra and also Java Island (West Java, Central Java and East Java). On the other hand, Hubert *et al.* (2019) noted that this species was distributed only in West Java. In this study, *N. fasciatus* was found in all six rivers from Blitar, East Java.

Remarks. There is a contradiction information on the distribution of this species between Kottelat (1984), Kottelat *et al.* (1993) with Hubert *et al.* (2019). We believe that Kottelat's information is correct compared to Hubert *et al.* (2019), because Hubert's claimed that this species only distributed in West Java after revisiting the Java Island. Meanwhile, we

found *N. fasciatus* in East Java, different from Hubert's information and similar to Kottelat's information.

Statistical analysis. The Kruskal-Wallis test showed that all of the 14 measured characters were significantly different ($P < 0.001$) (Table 1).

The morphological characteristics of the two *Nemacheilus* fishes can be distinguished from their body colorations (*N. chrysolaimos* with black-yellowish, while *N. fasciatus* with yellowish), pattern (irregular shape/bars or dots), ray ornamentation on the dorsal fin (black dots in *N. chrysolaimos*, while reddish spot in *N. fasciatus*), anterior naris (*N. chrysolaimos* with valve pierced tube-like, while in *N. fasciatus*, anterior margin with a winged flap) and also the fins meristic. The four preceding characters were almost similar and also found from Kottelat's (1984) observations but Kottelat (1984) and Kottelat *et al.* (1993) did not mention the detail on the meristic fins of *N. chrysolaimos* or *N. Fasciatus*. The fins provide valuable insights into the morphological characteristics of the two fish species. *N. chrysolaimos* has shorter proportions of eye diameter, head length, and lateral length of the head concerning SL, as opposed to *N. longipectoralis*. This information contributes to a more comprehensive understanding of the morphological distinctions between the two species. Similarly, it becomes apparent that *N. fasciatus* bears a resemblance to *N. masyae*, with a few notable differences. The upper caudal lobe and the eye diameter of *N. fasciatus* are larger in comparison to *N. Masyae*, and this distinction in size provides an updated perspective on the morphological variations between the two closely related species.

The significant difference in the morphometric of *N. fasciatus* may be related to the condition of the rivers or the habitats. For example, the differences in the river flow have caused a morphological variation in western rainbowfish (*Melanotaenia australis*) (Kelley *et al.* 2017). Flow regime differences in the streams result in morphological variation in *Cyprinella venusta* (Haas *et al.* 2010). The availability and prey type also lead to differences in morphological features (Hendry *et al.* 2002) but there was no evidence to support the differences among *N. fasciatus*. Therefore, further study should be conducted to elucidate the morphological variation among *N. fasciatus* from the six rivers in Blitar, East Java, Indonesia.

Sequence Composition and genetic diversity

A total of 11 partial sequences of the COI gene with a length of 503 base pairs (bp) for *N. fasciatus* and *N. chrysolaimos* were successfully amplified and analyzed to determine genetic variations within the related species based on the database in Table 2. A universal primer for the partial sequence of the COI gene carried out through accurate calculations, was successfully

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

Characters	***N. chrysolaimos*** **(n = 9)**						***N. fasciatus*** **(n = 147)**					
	Min	Max	Mean	Std. Error	H	*p*	Min	Max	Mean	Std. Error	H	*p*
Total length (TL)	40.6	66.4	55.3	2.9	N/A	N/A	31.3	93.5	53.6	1.1	92.6	< 0.001
Standard length (SL)	32.1	52.4	44.4	2.4	N/A	N/A	24.2	71.7	42.6	0.9	87.3	< 0.001
Head length	6.1	10.6	8.7	0.5	N/A	N/A	5.2	13.9	8.6	0.1	69.5	< 0.001
Snout length	2.1	4.8	3.4	0.3	N/A	N/A	1.8	6.4	3.4	0.1	16.4	< 0.001
Eye diameter	0.8	2.2	1.5	0.1	N/A	N/A	1	3.6	1.7	0	24.7	< 0.001
Postorbital length	2	5.4	3.8	0.4	N/A	N/A	2	7.9	4.2	0.1	41.2	< 0.001
Predorsal length	14.5	25.9	21	1.3	N/A	N/A	10.5	34.4	21.3	0.4	70.4	< 0.001
Pre-pelvic length	14.2	25.7	20.6	1.4	N/A	N/A	10.8	34.8	21.2	0.4	66.1	< 0.001
Pre-pectoral distance	5.7	10.4	8.2	0.5	N/A	N/A	4.8	80	8.8	0.5	40.7	< 0.001
Pectoral fin length	4.9	8.6	7.1	0.4	N/A	N/A	0.9	12.6	6.1	0.2	85	< 0.001
Body height	2.6	8.1	4.9	0.5	N/A	N/A	3.3	11.3	6.1	0.1	41.8	< 0.001
Dorsal fin length	5.4	10.2	7.9	0.4	N/A	N/A	3.2	17.3	7.2	0.2	77.9	< 0.001
Anal fin length	3.7	6.6	5.5	0.3	N/A	N/A	1.3	9.5	4.9	0.2	84	< 0.001
Height of caudal peduncle	2.6	5.4	4	0.3	N/A	N/A	1.9	6.6	3.9	0.1	36.3	< 0.001

Note: N/A is not available.

Table 2 GenBank accession numbers for partial sequence of COI gene of *Nemachaeilus* spp. with references

No	Species	MZB Code	Locality	GenBank Accession
1	*Nemacheilus chrysolaimos*	MZB 26539	Slorok River, Garum	OP379585
2	*Nemacheilus chrysolaimos*	MZB 26540	Slorok River, Garum	OP381212
3	*Nemacheilus fasciatus*	MZB 26550	Slorok River, Garum	OP412496
4	*Nemacheilus fasciatus*	MZB 26545	Loadeng river, Ponggok	OP412495
5	*Nemacheilus fasciatus*	MZB 26543	Sumber Ronje River, Ponggok	OP379588
6	*Nemacheilus fasciatus*	MZB 26544	Sumber Ronje River, Ponggok	OP412493
7	*Nemacheilus fasciatus*	MZB 26552	Glawah River, Ponggok	OP412492
8	*Nemacheilus fasciatus*	MZB 26551	Glawah River, Ponggok	OP412789
9	*Nemacheilus fasciatus*	MZB 26547	Kali Tanjung River, Garum	OP412790
10	*Nemacheilus fasciatus*	MZB 26541	Lekso River, Wlingi	OP380384
11	*Nemacheilus fasciatus*	MZB 26549	Slorok River, Garum	OP412790
12	*Nemacheilus fasciatus*	BIF3609	Dauwan River, Mojokerto	KU692665.1
13	*Nemacheilus fasciatus*	BIF0832	Pelus River, Purwokerto	KT960792.1
14	*Nemacheilus fasciatus*	BIF3609	Dauwan River, Mojokerto	KU692665.1
15	*Nemacheilus chrysolaimos*	BIF0170	Ci Seupan, Sukabumi	KU692664.1
16	*Rasbora argyrotaenia*	BIF0975	Teluk Peny, Cilacap, West Java	KT960805.1

applied to *Nemacheilus* spp. From Blitar Regency, East Java, Indonesia. The details of the sequence characteristics based on a length of 503 bp are summarized in Table 3. The percentage of base adenine (A), cytosine (C), guanine (G), and thymine (T) in all *Nemacheilus* spp. were 16.244%, 32.023%, 23.88%, and 29.565%, as shown in Table 3. Furthermore, the percentage of G+C content in the partial sequence of the COI gene was 48.26%.

The absence of stop codons in these sequences indicated a successful amplification of functional mitochondrial COI sequences. Therefore, nuclear DNA sequences derived from the mitochondrial DNA (NUMTs) were not sequenced since vertebrates NUMT was less than 600 bp (Wong *et al.* 2009). The characteristics of the partial sequence of the COI gene were analyzed which included haplotype diversity (Hd) 0.978 with nucleotide (π) 0.10532, Frequency of parsimony informative sites 25.646%, Polymorphic sites 165, ts/tv ratio (k) Purines= 7.006, Pyrimidines= 0.042; ts/tv ratio (R) 1.322; and mean of evolutionary rate 0.00, 0.02, 0.05, 0.09, 0.15, 0.22, 0.31, 0.42, 0.56, 0.72, 0.93, 1.19, 1.53, 2.01, 2.79, and 4.99 substitutions per site. The characteristics indicated that the partial sequence of the COI gene was suitable for determining the species of *Nemacheilus* spp. Genetic distance referred to the ratio of genetic differences between species or populations. Based on the genetic distance matrix of 2 species of *Nemacheilus* spp., the highest distance was found between *N. fasciatus* and *N. Chrysolaimos* with a value of 0.22 (Table 4). Therefore, a smaller genetic distance value generated a more similar appearance partial sequence of COI genes compared to related species.

DNA barcoding distinguished freshwater fish species with barcodes in Australia, Canada, India, Thailand, Germany, and Indonesia (Ward *et al.* 2005, Hubert *et al.* 2008, Knebelsberger *et al.* 2014; Lakra *et al.* 2015, Pampromin *et al.* 2019, Rahayu *et al.* 2019). The partial sequences of the COI gene profile for *N. chrysolaimos* and *N. fasciatus*, which were local freshwater fish species in different locations were compiled. Sequence validation was also performed using the online facility provided by BLAST (NCBI) and the BOLD system. The results indicated that the sequence samples matched the available accessions in the database, with query coverage ranging from 98% to 99.8%, and this confirmed the effectiveness of using DNA barcodes for species identification. After analyzing the nucleotide sequences, no insertions, deletions, or codon stops were observed. This supported the notion that all the amplified sequences represented functional mitochondrial COI sequences. Additionally, the average length of the amplified sequences exceeded 503 bp, which was typically the limit observed for nuclear DNA sequences originating from mtDNA (NUMT). These findings strengthened the reliability of the results and underscored the suitability of the COI gene as a marker for distinguishing between *N. chrysolaimos* and *N. fasciatus* in the local freshwater fish populations of Blitar Regency, Indonesia (Buhay, 2009; Gunbin *et al.* 2017).

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			
	1st	2nd	3rd	Total
Thyrosine frequency	26.757%	41.737%	20.203%	503 bp
Cytosine frequency	30.382%	31.723%	33.964%	503 bp
Adenine frequency	22,875%	12.5%	31.127%	503 bp
Guanine frequency	19.986%	14.041%	14.706%	503 bp
Frequency of invariable sites	67.196%			
Frequency of parsimony informative sites	25.646%			
Nucleotide diversity (Pi)	0,10532			
Haplotype diversity	0.882			
Number of haplotypes	8			
Total number of mutations	199			
Polymorphic sites	165			
ts/tv ratio (k)	Purines= 7.006, Pyrimidines= 0.042			
ts/tv ratio (R)	1.322			
Gamma discrete distribution	0.5428			
Mean of evolutionary rate	0.00, 0.02, 0.05, 0.09, 0.15, 0.22, 0.31, 0.42, 0.56, 0.72, 0.93, 1.19, 1.53, 2.01, 2.79, and 4.99 substitutions per site			

Note: The COI gene sequence characteristics were based on the 503 bp sequence length.

The partial sequence of the COI gene of *Nemacheilus* spp. showed that the values of the nucleotide base composition of G+C and A+T were between 48.26% and 51.74%, as shown in Table 3. The value of the nucleotide base composition and content of the A+T result was higher than G+C, consistent with the characteristics of the mitochondrial base composition. The analysis of the partial sequence of the COI gene showed that AT content (54.44%) was higher than GC content (48.26%), These data were observed in Australia (Ward *et al.* 2005), Canadian (Steinke *et al.* 2009); Cuban (Lara *et al.* 2010), and Bangladesh (Ahmed *et al.* 2020) fish species. Clusters 1 and 2 were resolved as sister taxa with 99% bootstrap and the genetic diversity was very low or less than 2%. A genetic distance value of more than 2% indicated that there were species different from other group members. Meanwhile, a genetic distance value of less than 3% indicated that the group or cluster was obtained from the same species (Hebert *et al.* 2003; Hebert *et al.* 2004). Based on the standards from Nei (1972), the genetic distance of *Nemacheilus* spp. obtained in Blitar Regency waters was categorized into low (0.01–0.045) and medium (0.17–0.18) similar to *Nemacheilus* spp. genetic distance calculations reported by Hubert *et al.* (2019).

Phylogenetic reconstruction

Phylogenetic relationships were shown in the ME tree (Fig. 6) and ML tree (Fig. 7). Each species was associated with a specific DNA barcode cluster and the relationship among these species was obtained. Closer species in terms of genetic divergence, were clustered at the same nodes to determine the distance between the terminal branches of the ME & ML trees, consisting of two divergent clusters.

Table 4 Pairwise genetic distance of *Nemacheilus fasciatus* dan *N. crysolaimos* compared to all congeners and outgroups

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
NF MZB 26551																
NF MZB 26552	0.000															
NF MZB 26549	0.000	0.000														
NF MZB 26550	0.000	0.000	0.000													
NF MZB 26545	0.004	0.004	0.004	0.004												
NF MZB 26547	0.045	0.045	0.045	0.045	0.045											
NF MZB 26543	0.045	0.045	0.045	0.045	0.045	0.000										
NF MZB 26541	0.045	0.045	0.045	0.045	0.045	0.000	0.000									
KU692665.1_NF	0.045	0.045	0.045	0.045	0.045	0.000	0.000	0.000								
KU692666.1_NF	0.060	0.060	0.060	0.060	0.060	0.014	0.014	0.014	0.014							
NF MZB 26544	0.047	0.047	0.047	0.047	0.047	0.002	0.002	0.002	0.002	0.012						
KT960792.1_NF	0.047	0.047	0.047	0.047	0.047	0.002	0.002	0.002	0.002	0.012	0.000					
NC MZB 26540	0.229	0.229	0.229	0.229	0.229	0.181	0.181	0.181	0.181	0.170	0.179	0.179				
KU692664.1_NC	0.229	0.229	0.229	0.229	0.229	0.181	0.181	0.181	0.181	0.170	0.179	0.179	0.000			
NC MZB 26539	0.229	0.229	0.229	0.229	0.229	0.181	0.181	0.181	0.181	0.170	0.179	0.179	0.000	0.000		
KT960805.1	0.278	0.278	0.278	0.278	0.278	0.229	0.229	0.229	0.229	0.223	0.226	0.226	0.243	0.243	0.243	
LC130692	0.288	0.288	0.288	0.288	0.288	0.241	0.241	0.241	0.241	0.241	0.238	0.238	0.244	0.244	0.244	0.144

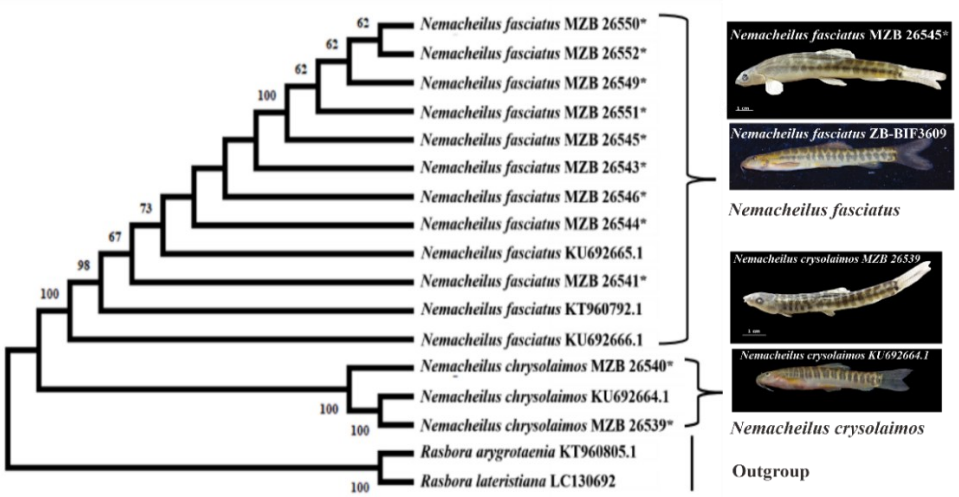

Figure 6 Minimum Evolution (ME) phylogenetic tree of *Nemacheilus* spp. based on partial sequence of COI gene. The asterisk (*) denotes the sequence of *Nemacheilus* spp. obtained from Blitar Regency, East Java, Indonesia and *Rasbora* spp. as the outgroup

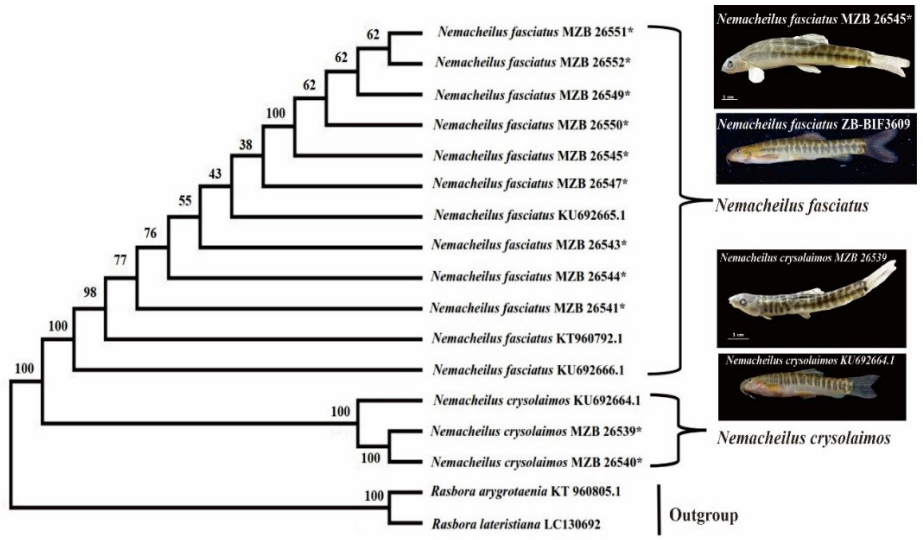

Figure 7 Maximum-Likelihood (ML) phylogenetic tree of *Nemacheilus* spp. based on partial sequence of COI gene. The asterisk (*) denotes the sequence of *Nemacheilus* spp. obtained from Blitar Regency, East Java, Indonesia and *Rasbora* spp. as the outgroup

The phylogenetic analysis of *Nemacheilus* spp. using both ME and ML methods resulted in unambiguous branching patterns, as illustrated in Figs. 6 and 7. The phylogenetic trees showed that *N. fasciatus* and *N. chrysolaimos* species formed distinct monophyletic branches. However, their proximity at the same node indicated genetic relatedness and the positioning of these branches corresponded with a calculated genetic distance of 0.22, signifying the greatest divergence between these two species. The ME, ML, and genetic distance data collectively provided strong evidence that *N. fasciatus* and *N. chrysolaimos* were genetically distant from each other.

In addition, the ABGD method identified 3 groups for *Nemacheilus* spp. specimens in with the initial approach and the barcode gap threshold calculated by the COI dataset as shown in Figs. 8A & 8B). The value of the barcode gap distance was 0.025 in line with the results of the ABGD grouping which divided the species into 3 groups, as shown in Fig 8C. Group [1] (*Nemacheilus fasciatus* MZB 2655, *Nemacheilus fasciatus* MZB 26549, *Nemacheilus fasciatus* MZB 26550, *Nemacheilus fasciatus* MZB 26552, *Nemacheilus fasciatus* MZB 26545, Group [2] (*Nemacheilus fasciatus* MZB 26543, *Nemacheilus fasciatus* MZB 26546, *Nemacheilus fasciatus* MZB 26544, *Nemacheilus fasciatus* MZB 26541), and Group [3] (*Nemacheilus crysolaimos* MZB 26540, and *Nemacheilus crysolaimos* MZB 26539).

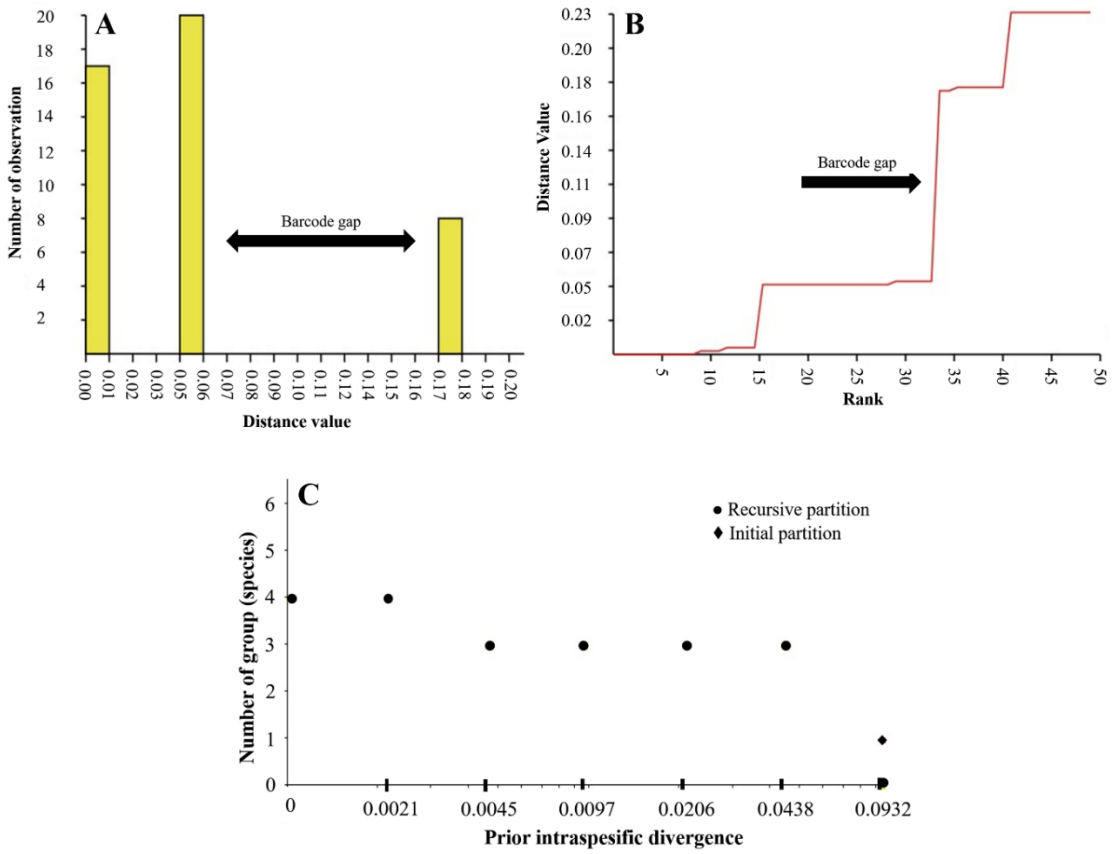

Figure 8 Barcode Gap Analysis of COI sequences performed by ABGD (Puillandre *et al.* 2012). Histograms show the distribution of pairwise genetic distances (uncorrected p-distances) between each pair of specimens. The arrow indicates the gap that allows to distinguish intraspecific (left) and interspecific (right) distances for the COI region. (A) Histogram of distance, (B) Ranked distance, and (C) Number of Primary Species Hypotheses (PSHs) obtained, for each prior intraspecific divergence

The application of ABGD analyses, with a prior maximal distance set at 0.025, further reinforced the separation of *N. chrysolaimos* and *N. fasciatus* into distinct partitions. These additional analyses align with differentiation of these species. Consequently, the combination of genetic distance, phylogenetic analysis, and ABGD analyses collectively confirmed the successful identification of *Nemacheilus spp.* from Blitar Regency. Based on the comprehensive evidence derived from DNA barcoding, with morphological characteristics, it can be concluded that the targeted utilization of these tools offered an efficient and reliable means of identifying *Nemacheilus* spp. at the species level. Therefore, this study was the first to report on the morphology in accordance with Kottelat (1984); Kottelat *et al* (1993); Hardiaty *et al.* (2014)] genetic identification and phylogenetic reconstruction of *Nemacheilus* spp. using the partial sequence of the COI gene. Conservation management of *N. chrysolaimos* and *N. fasciatus* in grouping animal units should be conducted according to species and genetic entity, as well as the potential of developing cryopreservation for sustainability. A molecular approach using the partial sequence supported the identification results based on a morphological approach in *Nemacheilus* spp. and obtained an accession number from GenBank (NCBI) database. This study indicated that improved morphology and molecular characteristics of local loaches (*Nemachelius* spp.) were obtained from Blitar Regency. Therefore, a reliable DNA barcode reference library for East Java, Indonesia, freshwater fish was established to assign fish species by screening sequences. This initiative aimed to enhance the achievement of better monitoring, conservation, and management of fisheries in this overexploited region.

CONCLUSION

In conclusion, this study has successfully identified analysis for loach fishes such as *N. chrysolaimos* and *N. fasciatus* from six rivers at Biltar, East Java, Indonesia, based on morphology and molecular data. The main characteristic used to distinguish these species is the color pattern on their lateral bodies, such as dark bars or spots, along with the morphological variations of anal fins. Moreover, through the utilization of genetic approaches, including phylogenetic reconstruction, sequence composition, genetic diversity analysis, and ABGD analysis, it was determined that *N. chrysolaimos* and *N. fasciatus* are distinct species.

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