BIODIVERSITAS
Volume 26, Number 6, June 2025
Pages: 2896-2907

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d260632

Morphological and genetic variation of local loach (*Nemacheilus* spp.) from Lumajang, East Java, Indonesia

DWI ANGGOROWATI RAHAYU[1,♥], WIDOWATI BUDIJASTUTI[1], SUNU KUNTJORO[1], WINARSIH[1], ENDIK DENI NUGROHO[2], RENI AMBARWATI[1], ULFI FAIZAH[1], CAHYA AJENG VALENTA TRESNA SULUNG[1], RUSDIANTO RUSDIANTO[3], NOORHIDAYAH BINTI MAMAT[4]

[1]Laboratory of Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya. Jl. Ketintang, Surabaya 60213, East Java, Indonesia. Tel./fax.: +62-31-8296427, ♥email: dwirahayu@unesa.ac.id

[2]Department of Biology Education, Faculty of Science Education, Universitas Nahdlatul Ulama Pasuruan. Jl. Raya Warung Dowo, Pasuruan 67171, East Java, Indonesia

[3]Research Center for Biosystematics and Evolution, National Research and Innovation Agency. Jl. Raya Jakarta- Bogor KM.46 Cibinong, Bogor 16911, West Java, Indonesia

[4]Institute of Biological Sciences, Faculty of Science, University of Malaya. 50603 Kuala Lumpur, Malaysia

Manuscript received: 27 January 2025. Revision accepted: 5 June 2025.

Abstract. *Rahayu DA, Budijastuti W, Kuntjoro S, Winarsih, Nugroho ED, Ambarwati R, Faizah U, Sulung CAVT, Rusdianto R, Mamat NB. 2025. Morphological and genetic variation of local loach (*Nemacheilus spp.*) from Lumajang, East Java, Indonesia. Biodiversitas 26: 2896-2907.* The genus *Nemacheilus* is a part of the family Nemacheilidae within the order Cypriniformes, comprising several morphologically similar fish species found in East Java, Indonesia. However, species identification in Lumajang remains unavailable. This study aimed to analyze morphological and genetic diversity of local loach species from Lumajang, East Java, contributing to the existing knowledge with new records. The experiment was carried out through diagnostic identification, meristic, and morphometric analyses, using Past.403 software for correlation assessments. Pectoral fin samples were preserved in high-purity ethanol for DNA extraction, followed by Polymerase Chain Reaction (PCR) targeting mitochondrial genomes, Recombinase-Activating Gene proteins 1 (RAG1), and mitochondrial Cytochrome C Oxidase I (COI) to explore phylogenetic relationships among Nemacheilidae species through maximum likelihood methods. Morphological identification showed two local loaches, *Nemacheilus chrysolaimos* (Valenciennes, 1846) and *Nemacheilus fasciatus* (Valenciennes, 1846) with two variants (dominant black color and yellow color). Several morphometric parameters showed high correlations, such as TL with SL, HL with Snout Length (SnL), and Dorsal Fin Length (DFL) with Anal Fin Length (AFL). The COI locus showed high genetic variation with 126 variable sites across 489 base pairs. Meanwhile, the RAG1 gene had a lower variation with 33 variable sites across 681 base pairs, with phylogenetic analysis showing two distinct clades. This comprehensive study enhanced the understanding of morphological and genetic variation in *Nemacheilus* species, related to conservation efforts and sustainable aquaculture.

Keywords: DNA barcodes, diagnostic characters, phylogenetic, similarity

INTRODUCTION

The genus *Nemacheilus* is a part of the family Nemacheilidae in the order Cypriniformes, with significant morphological variation and genetic diversity across its species (Hubert et al. 2019). This variation is influenced by multiple factors, including geographical distribution, ecological adaptations, and genetic mechanisms such as mutation and recombination. The geographical distribution of loaches throughout Indonesia is segmented across various islands, including Sumatra, Kalimantan, and Java (Hubert et al. 2019; Kusuma et al. 2021; Rahayu et al. 2023). In the Central Java region, several species of loaches have been recorded, specifically *Nemacheilus chrysolaimos* (Valenciennes, 1846) and *Nemacheilus fasciatus* (Valenciennes, 1846). These species have been found in several river systems such as the Pelus, Mengaji, Banjaran, and Kranji Rivers, particularly *N. fasciatus* in the areas of Jepara and Purwokerto (Baumgartner and Wibowo 2018; Chapsos and Hamilton 2018; Haryani 2022). In East Java, particularly within the Blitar region, both *N. chrysolaimos* and *N. fasciatus* have been observed (Rahayu et al. 2023) and Pasuruan (Sulung et al. 2024). This shows the need for continuous investigations and conservation efforts to protect the aquatic species and their environments, particularly from Lumajang District, Indonesia.

Morphological characteristics of *Nemacheilus* species are essential for species identification and classification within the genus. These species show variations in body shape, fin structure, and coloration patterns (Rahayu et al. 2023), which are essential for taxonomic differentiation (Kusuma et al. 2021). Page et al. (2020) reported that variations in dorsal fin ray counts and body shapes were essential for distinguishing between species within the genus. Furthermore, Sgouros et al. (2019) reported differences in the arrangement of spots or stripes along the bodies of various populations, suggesting the possibility of cryptic speciation within *Nemacheilus*. This morphological diversity shows the complex evolutionary history of *Nemacheilus*, which is not often in line with molecular data (Hubert et al. 2019; Rahayu et al. 2023).

Tornow and Skelton (2011) reported that morphological data could lack congruence with molecular hypotheses, showing the need to integrate both types of data for a more

comprehensive understanding of evolutionary relationships. Although loach species found in the Lumajang River are relatively abundant, there is limited information regarding their diversity and description. Therefore, further studies are required to accurately identify *Nemacheilus* spp. in the Lumajang region.

The use of DNA barcoding with a single molecular marker can further reinforce the identification process based on morphological characteristics (Hubert and Hanner 2016; Trivedi et al. 2016; Abdullah et al. 2019; Alcudia-Catalma et al. 2020; Basith et al. 2021; Juniar et al. 2021; Radulovici et al. 2021; Sari et al. 2021, 2023; Vieira et al. 2021; Nugroho et al. 2023; Rahayu et al. 2023).

The use of mitochondrial Cytochrome C Oxidase I (COI) and Recombination Activating Gene 1 (RAG1) has proven to be highly effective for species identification (Šlechtová et al. 2021), particularly in the context of *Nemacheilus* species. The COI gene is widely recognized as a standard DNA barcode due to the high level of conservation within species and moderate variability among different species (Bingpeng et al. 2018; Riyadini et al. 2020; Naz et al. 2023). The use of COI in species identification has been documented across various studies, showing effectiveness in distinguishing closely related species and providing a reliable method of taxonomic classification (Falade et al. 2016; Riyadini et al. 2020). In addition to COI, RAG1 serves as a valuable nuclear marker that can complement mitochondrial barcoding methods (Panijpan et al. 2015). The inclusion of RAG1 enhances the resolution of phylogenetic relationships, particularly in cases where only mitochondrial data are insufficient due to hybridization events (Reid et al. 2011; Sember et al. 2015; Fujimoto et al. 2017; Page et al. 2020).

The combination of COI and RAG1 allows for a more comprehensive method of species identification (Panijpan et al. 2015; De la Ossa-Guerra et al. 2020). This method integrates both mitochondrial and nuclear genetic information, thereby addressing potential limitations associated with relying solely on one type of genetic marker (Reid et al. 2011). Morphological variation and genetic diversity of *Nemacheilus* are correlation for both taxonomic classification and ecological studies. Recent investigations using advanced molecular methods alongside traditional morphological assessments will continue to refine the understanding of this diverse group of freshwater fishes.

MATERIALS AND METHODS

Study area

Specimens of *Nemacheilus* spp. were collected from Lumajang Rivers, Lumajang District, East Java, Indonesia (Figure 1) at each loach sampling station. This geographical coordinate 113.1149556 (longitude) and -8.154858333 (latitude). Purposive sampling was applied, where loaches were captured using fishing net measuring 1 meter in [length/width] with a mesh size of 5 mm. All samples were placed into bottles filled with 96% absolute ethanol.

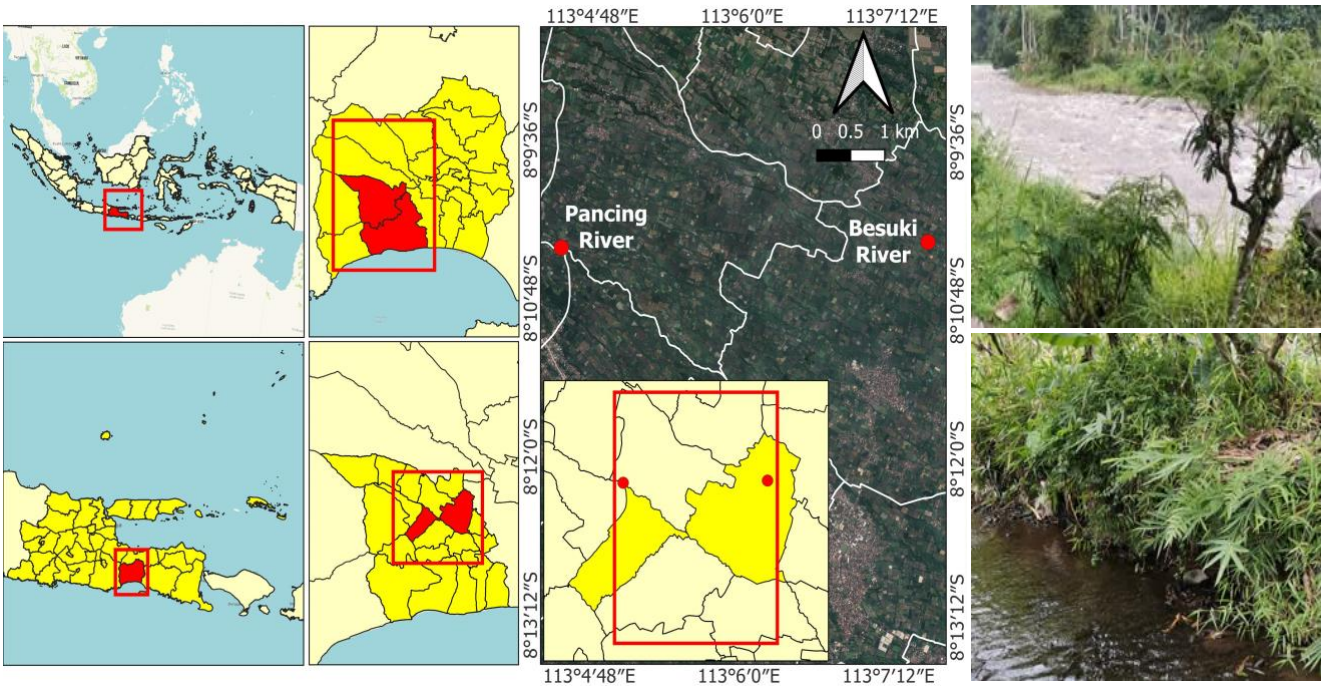

Figure 1. The map of Lumajang Rivers, Lumajang District, East Java, Indonesia and their habitat indicating the sampling sites of loaches fish procedures

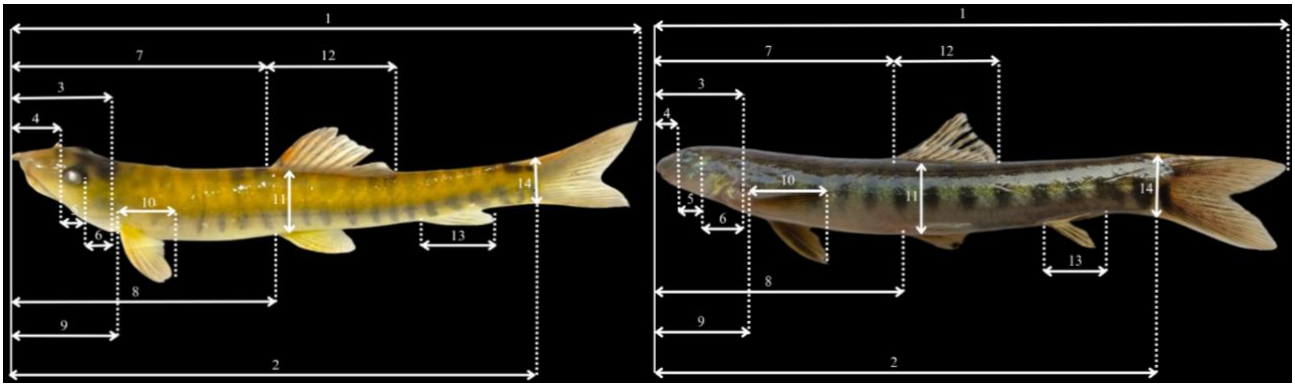

Figure 2. The morphometric of two *Nemacheilus* sp. from Lumajang. 1. Total Length (TL), 2. Body Length (SL), 3. Head length, 4. Snout length, 5. Eye diameter, 6. Head length behind the eye, 7. Body length before dorsal fin, 8. Body length before pelvic fin, 9. Body length before pectoral fin, 10. Pectoral fin length, 11. Body height, 12. Dorsal fin length, 13. Anal fin length, 14. Caudal fin height (modified from Kottelat (1984); Kottelat and Freyhof (2007); and Rahayu et al. 2023)

Morphological work

In the laboratory, all fish specimens were carefully sorted and cleaned in preparation for morphological examination and identification. Measurements of 14 morphological characteristics were taken, as illustrated in Figure 2, using a digital caliper with a precision of 0.1 mm, adhering to a modified protocol based on the works of Kottelat (1984), Kottelat and Freyhof (2007), and Rahayu et al. (2023). Each sample was carefully excised using sterile scissors and immediately preserved in 96-100% ethanol to prevent DNA degradation. The identification procedures were based on guidelines provided by Kottelat (1984) and Kottelat et al. (1993). Afterward, all specimens were preserved in 96-100% ethanol and stored at the Zoological Taxonomy Biology Program of Universitas Negeri Surabaya, Indonesia. Prior to conducting molecular analyses, the specimens were frozen at -20°C for subsequent DNA extraction. Furthermore, samples were isolated and extracted using a silica column through four stages of lysis, binding, washing, and elution, following the DNeasy Blood and Tissue Kit Qiagen commercial kit procedure.

PCR and sequencing

A fragment of approximately 489 base pairs (bp) corresponding to the COI gene region of mitochondrial DNA (mtDNA) was amplified through PCR using universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') as described by Folmer et al. (1994), RAG1-F 5′-AGCTGTAGTCAGTAYCACAARATG-3′ (Quenouille et al. 2004), and RAG-RV1 5′-TCCTGRAAGATYTTG-TAGAA-3′ (Šlechtová et al. 2007). The hot start PCR method used a Kapa master mix along with two Taq master mixes and the PCR was conducted over 35 cycles. Each cycle included a pre-denaturation step at 95°C for 3 minutes, followed by denaturation at 94°C for 45 seconds, annealing at 45°C for 45 seconds, and extension at 72°C for 2 minutes. The process concluded with a final elongation step at 72°C for 10 minutes. The PCR products were analyzed on a 1% agarose gel (composed of 0.5 g agarose and 50 mL TAE Buffer) mixed with 4 µL of Ethidium Bromide (EtBr) as a dye. Subsequently, 3 µL of the PCR samples were combined with 1 µL of loading dye, and the mixture was loaded into an agarose well (Rahayu et al. 2023). Electrophoresis was performed using an electrophoresis machine set to 220 V and 400 mA for 25 minutes. The PCR products were purified using the Qiagen purification kit according to the manufacturer's instructions and were sequenced at First Base, Malaysia.

Data analyses

Morphological analysis of two Nemacheilus *species*

The characterization of the two species within the genus *Nemacheilus* was conducted through a comprehensive examination of their morphological characteristics using morphometric, meristic, and diagnostic features. Morphological assessment of the two *Nemacheilus* species was systematically carried out. All measurements were recorded with precision to the nearest 0.1 mm, using digital calipers, and were taken predominantly from the left side of the specimens whenever feasible. Morphological data were analyzed using Principal Component Analysis (PCA), following procedures adapted from Bookstein et al. (1985). This analysis was executed using the R programming environment. The second and third principal components were specifically plotted to the variations in morphology while controlling for size-related effects.

Phylogenetic analysis

Phylogenetic relationships of the COI and RAG1 gene sequences were examined to facilitate species identification. The translated amino acid sequences were then verified through the ExPASy website (https://web.expasy.org/translate/) (Duvaud et al. 2021). Successful species assignments were achieved when reference sequences clustered into groups of conspecific sequences, forming a monophyletic clade. The DNA sequencing chromatograms were scrutinized, edited, and compiled into contiguous sequences using Finch TV and BioEdit version 7.0.5.3 (Hall 1999).

Table 1. DNA Sequences specimens published and submitted to NCBI GenBank

Species	Location	COI
Nemacheilus fasciatus LMJ 1	Lumajang, East Java	PQ596216
Nemacheilus fasciatus LMJ 2	Lumajang, East Java	PQ596221
Nemacheilus fasciatus LMJ 3	Lumajang, East Java	PQ596223
Nemacheilus fasciatus LMJ 4	Lumajang, East Java	PQ596224
Nemacheilus fasciatus LMJ 5	Lumajang, East Java	PQ596292
Nemacheilus fasciatus LMJ 6	Lumajang, East Java	PQ596300
Nemacheilus fasciatus LMJ 7	Lumajang, East Java	PQ625856
Nemacheilus fasciatus LMJ 8	Lumajang, East Java	PQ625857
Nemacheilus fasciatus LMJ 9	Lumajang, East Java	PQ625888
Nemacheilus fasciatus LMJ 10	Lumajang, East Java	PQ625893
Nemacheilus chrysolaimos LMJ1	Lumajang, East Java	PQ625895
Nemacheilus chrysolaimos LMJ2	Lumajang, East Java	PQ625897
Nemacheilus chrysolaimos LMJ3	Lumajang, East Java	PQ625900
Nemacheilus chrysolaimos LMJ4	Lumajang, East Java	PQ625903
Nemacheilus chrysolaimos LMJ5	Lumajang, East Java	PQ625910
Nemacheilus fasciatus voucher MZB 26551	Blitar, East Java	OP412496
Nemacheilus fasciatus voucher MZB 26551	Blitar, East Java	OP412495
Nemacheilus fasciatus voucher BIF0832	Blitar, East Java	KT960792
Nemacheilus fasciatus voucher BIF3609	Blitar, East Java	KU692665

For the reconstruction of phylogenetic trees based on COI and RAG1 sequences, a total of 9 COI and 9 RAG1 reference sequences of *Nemacheilus* species were sourced from the National Center for Biotechnology Information (NCBI) database, as shown in Table 1. Multiple sequence alignment was carried out using Clustal X (Ferrari and Patrizio 2021) followed by manual verification with BioEdit software. The assembled consensus sequences from the specimens combined with the GenBank sequences (Larkin et al. 2007) to create datasets for both COI and RAG1.

The COI and RAG1 sequences were concatenated to form a combined dataset, allowing for an assessment of whether the performance of the concatenated markers exceeded individual markers. The datasets for COI, RAG1, and the concatenated COI + RAG1 were subjected to Maximum Likelihood (ML) tree reconstruction using Mega X, incorporating 1000 bootstrap replicates. For both Neighbor-Joining (NJ) and ML tree reconstructions, the Kimura 2-Parameter (K2P) substitution model was used (Nishimaki and Sato 2019). Additionally, the variability in substitution rates among sites was modeled using a Gamma distribution, and a bootstrap consensus tree was derived from 1000 replicates (de Moraes Russo and Selvatti 2018).

RESULTS AND DISCUSSION

Morphological variation

Synapomorphy shows that the species head is triangular, with a semicircular tip. This includes a pair of eyes, a pair of nostrils, a short and blunt snout, and a small downturned mouth, with semicircular lips. The species possesses three pairs of rostral barbels, with two located on the upper jaw and one situated on the snout of upper jaw. The body shows an elongated fusiform shape, slightly flattened laterally, and tapering towards the tail base.

The diagnostic characteristics of *N. chrysolaimos* include an anal fin with 6-7 rays (AI.6-7) (Figure 3), a pectoral fin with 9-10 rays (PI.9-10), a ventral or pelvic fin with 4-7 rays (VI.4-7), and a caudal fin with 18-19 rays (C.18-19). The body coloration is either black or golden-yellow, adorned with 14-18 irregularly shaped vertical dark stripes along the lateral sides, and features five interrupted lines near the base of the caudal fin. In comparison, the diagnostic features of *N. fasciatus* (yellow variant) show a yellowish-black body coloration. This is characterized by 12 to 18 body bladders arranged oppositely on the dorsal and ventral sides, with a predominance of yellow on the body. Based on fin count, the anal fin has 4-5 rays (AI.4-5), pectoral 7-9 rays (PI.7-9), ventral or pelvic 6-7 rays (VI.6-7), and caudal 17-19 rays (C.17-19). *Nemacheilus fasciatus* (black variant) is characterized by a black body coloration, featuring 12 to 18 body bladders that are arranged oppositely on the dorsal and ventral sides.

Cluster analysis dimensional reduction (PCA) of loach fish

Table 2 shows the correlation values among the morphometric parameters of loach fish. High correlation values (>0.5) show a strong correlation between the two parameters, while low values (<0.5) suggest a weak or no correlation at all. The parameters observed included Total Length (TL), Standard Length (SL), Head Length (HL), and Body Height (BH). The correlation variance explained by Principal Component 1 (PC1) showed that these size-related parameters had a high correlation with PC1. The strong influence of these variables could be inferred from their potential contribution to the overall size of the fish, which was the primary source of variance. Several morphometric parameters showed high correlations, such as TL with SL, HL with Snout Length (SnL), and Dorsal Fin Length (DFL) with Anal Fin Length (AFL). This suggested that the parameters tended to correspond with one another. However, other parameters showed low correlations, such as Eye Diameter (ED) with Height of Caudal Peduncle (HCP), suggesting that these parameters do not have a strong correlation.

The correlation values show differences among morphometric parameters for each group of loach fish, comprising *N. fasciatus* (black and yellow) with *N. chrysolaimos,* as presented in Table 1. This suggested that the correlation among morphometric parameters could vary across different groups of loach fish. For instance, the correlation between TL and SL was higher in the *N. fasciatus* variant black and yellow groups compared to *N. chrysolaimos*. In the context of discriminant analysis, the plot visually represents discriminant functions that optimize separation among three predefined groups, namely *N. fasciatus* variant black and yellow, with *N. chrysolaimos*. The plot's axes corresponded to these functions, which were derived from linear combinations of variables differentiating the groups. Based on confidence levels for each group, the black ellipse (*N. fasciatus* variant

black) showed greater variability, while the yellow ellipse (*N. fasciatus* variant yellow) showed moderate variability. The light blue ellipse (*N. chrysolaimos*) showed lower variability and tighter clustering. The *N. fasciatus* variant black group was distinctly separated from the other two along the first axis, showing morphological differences. Although the *N. fasciatus* variant yellow and *N. chrysolaimos* groups overlap, there was a strong correlation with *N. chrysolaimos* being slightly more separated.

The results of the discriminant analysis showed that TL and SL were high differentiators for *N. fasciatus* variant black group, effectively distinguishing from both the yellow variant and *N. chrysolaimos*. In comparison, ED, Pre-Pectoral Length (PPvL), and Post-Dorsal Length (PDL) were found to be more influential in differentiating between the yellow variant of *N. fasciatus* and *N. chrysolaimos*. The black variant showed greater morphological diversity, suggesting a broader range of adaptations or evolutionary pressures compared to the more homogeneous yellow variant and *N. chrysolaimos*.

Based on discriminant analysis, the results showed that distinct morphological characteristics could effectively differentiate between the three groups of fish, namely *N. fasciatus* variant black and yellow, with *N. chrysolaimos*. The discriminant functions derived from linear combinations of various morphological variables showed high differences, particularly along the first axis where the *N. fasciatus* variant black was separated from others. This observation suggested that morphological diversity in fish could be influenced by ecological factors, showing the influence of competition and predation on the observed morphology (Kristjánsson et al. 2011). Additionally, the black variant showed greater variability, suggesting a broader range of adaptations or evolutionary pressures compared to the more homogeneous yellow variant and *N. chrysolaimos*. Variations in morphology among individuals are influenced by environmental factors, including temperature, turbidity, depth, pH levels, water flow, habitat fluctuations, and the nature and availability of food sources (Hu et al. 2019; Freudiger et al. 2021).

Table 2. Eigenvalues and variance explained

Principal component	Eigenvalue	% Variance explained
PC1	7.28997	56.077
PC2	1.9751	15.193
PC3	1.16298	8.946
PC4	0.710142	5.4626
PC5	0.599727	4.6133
PC6	0.411397	3.1646
PC7	0.288792	2.2215
PC8	0.180996	1.3923
PC9	0.135036	1.0387
PC10	0.0924897	0.71146
PC11	0.0657818	0.50601
PC12	0.0582453	0.44804
PC13	0.029343	0.22572

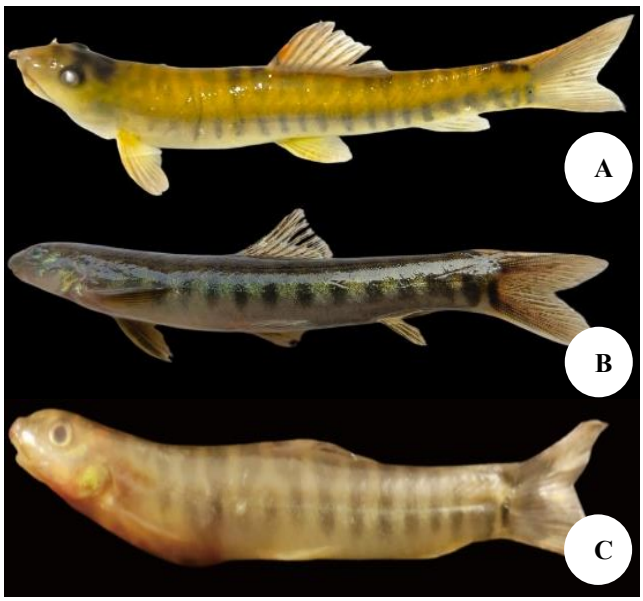

Figure 3. *Nemacheilus* Diagnostic characters. A. *Nemacheilus fasciatus* (variant yellow), B. *Nemacheilus fasciatus* (variant black), C. *Nemacheilus chrysolaimos*

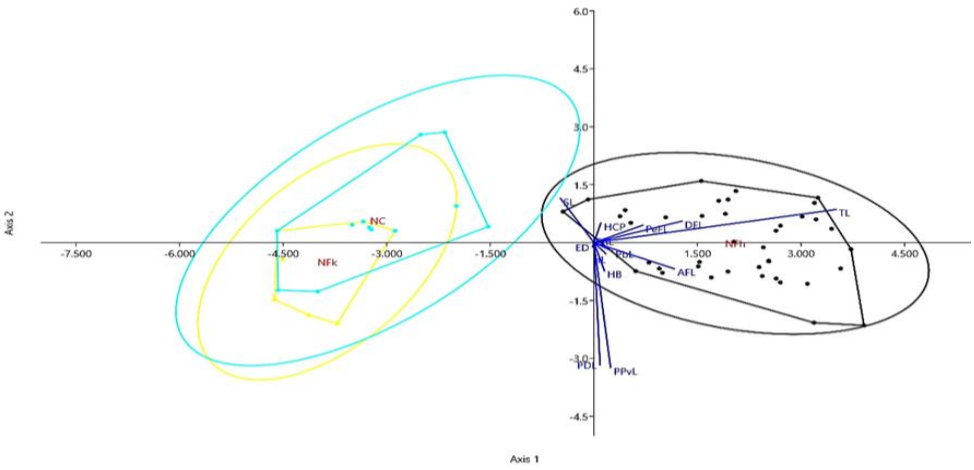

Figure 4. Discriminant analysis of *Nemacheilus* spp. from Lumajang District, East Java, Indonesia

The data representing confidence regions for each group in the plot provided a visual representation of the variability. The black ellipse (Figure 4), representing *N. fasciatus* variant black, showed a wider spread and a higher level of morphological diversity. In comparison, the yellow variant's ellipse was more compact, showing moderate variability. The light blue ellipse for *N. chrysolaimos* showed the least variability and tighter clustering. Yedier et al. (2022) reported that morphological diversity among freshwater fish varied significantly across different ecological realms, with some species showing extreme characteristics. Similarly, Yedier and Bostanci (2021) reported challenges in identifying species based on visual morphological features, emphasizing the importance of quantitative methods like discriminant analysis for accurate species identification. For instance, Aminan et al. (2020) demonstrated a 100% classification accuracy in morphometric analyses of *Rasbora* species, underscoring the reliability of morphological traits in distinguishing closely related species.

The discriminant analysis results showed TL and SL were high differentiators for the *N. fasciatus* variant black, effectively distinguishing from both the yellow variant and *N. chrysolaimos*. This was supported by the role of morphological traits in ecological niche partitioning among fish species (Ayebare et al. 2023). However, the yellow variant of *N. fasciatus* and *N. chrysolaimos* were differentiated primarily by ED, PPvL, and PDL. This showed that the characteristics were more influential in distinguishing between the two groups. Brlík et al. (2024) stated that specific morphological characteristics could serve as key indicators for species differentiation within closely related taxa. In summary, the analysis of morphological diversity and the application of discriminant analysis reveal correlation insights into the ecological and morphological differentiation among the studied fish species. The findings underscore the relevance of specific morphological traits in understanding species coexistence and niche partitioning, which is crucial for in the use of growth models and mortality models and conservation efforts using domestication models (Indarjo et al. 2022).

Sequence composition and genetic diversity

The successful amplification of the target genes, specifically the COI gene and RAG 1, was validated by the observation of distinct DNA bands free from smearing. These bands were observed at approximately 489 base pairs (bp) and 681 bp, respectively, as shown in Table 3. The lack of stop codons suggested that the amplification process successfully captured functional sequences of mitochondrial COI and nuclear DNA. Therefore, sequences of nuclear DNA derived from mitochondrial DNA (NUMTs) were excluded from the sequencing analysis. This was because vertebrate NUMTs typically had lengths shorter than 619 base pairs. The COI gene was widely recognized for its use in species identification due to the high accuracy caused by the elimination of pseudogenes during the protein translation process (Hubert and Hanner 2016; Li et al. 2022; Muhala et al. 2024). Several studies have consistently shown the efficacy of COI sequences in differentiating species across various taxonomic groups.

The COI locus (489 bp) had a high number of variable sites (126), showing significant genetic diversity, while the RAG1 locus (681 bp) had fewer variable sites (33) (Table 3). This suggested that COI was more suitable for population genetic and phylogenetic studies due to greater variability. Generally, parsimony informative sites are essential for resolving evolutionary relationships. In this study, only COI and the combined dataset (COI + RAG1) had 6 parsimony informative sites, showing the greater contribution of COI to phylogenetic resolution. The combined dataset's parsimony informative sites suggested that integrating loci could enhance phylogenetic analysis. Singleton sites are unique variations occurring only once in the dataset. The COI locus does not specify the number of singleton sites while RAG1 has none. The combined dataset contains 6 singleton sites, showing potential unique mutations relevant to specific evolutionary events or population dynamics. This finding underscores the greater contribution of the COI locus to phylogenetic resolution, as it offers more informative data compared to RAG1. The integration of loci, as demonstrated by the combined dataset, enhances the robustness of phylogenetic analyses, allowing for a more comprehensive understanding of evolutionary relationships (Medina et al. 2023).

The analysis of genetic diversity using the COI and RAG1 gene provided significant insights into the evolutionary relationships among *Nemacheilus* species. The COI gene, with 489 base pairs and 126 variable sites, showed a high level of genetic diversity. Therefore, COI gene was considered a more suitable marker for population genetic and phylogenetic studies compared to the RAG1 gene, which had only 33 variable sites across the 681 base pairs. This result was consistent with the established understanding that mitochondrial DNA, such as COI showed higher variability than nuclear DNA due to faster mutation rates (Pappalardo et al. 2015; Chen et al. 2019). The presence of parsimony informative sites was crucial for resolving evolutionary relationships. Furthermore, COI contributed significantly to phylogenetic resolution, as shown by the six parsimony informative sites found in both COI and the combined dataset (Edwards et al. 2016; Luo et al. 2024).

The analysis of genetic distances in *Nemacheilus* species showed important insights into genetic structure and evolutionary relationships. Intraspecific distances showed low genetic variability within each species, while interspecific distances had significant genetic divergence. This suggested that *N. fasciatus* and *N. chrysolaimos* represented distinct evolutionary lineages, as shown in Table 4. The use of both mitochondrial and nuclear genetic markers provided a comprehensive understanding of genetic relationships, with COI showing greater variability compared to RAG1. These results contributed to the broader understanding of biodiversity and evolutionary processes within the *Nemacheilus* genus.

Nucleotide diversity (Pi) values showed a moderate level of genetic variation, with COI indicating the highest diversity (0.09762) compared to RAG1 (0.089) and the combined dataset (0.09331). This pattern was consistent with the results in other studies where mitochondrial DNA

showed higher nucleotide diversity than nuclear DNA due to faster mutation rates. Haplotype diversity was also high across all loci, with COI (0.957) showing the greatest diversity, followed by the combined dataset (0.9135), and RAG1 (0.87). The presence of eight haplotypes in each locus suggested a rich genetic reservoir within populations. Furthermore, the findings underscore the importance of utilizing both mitochondrial and nuclear markers in genetic studies. While mitochondrial DNA provides insights into population structure and evolutionary history due to its maternal inheritance and rapid mutation rates, nuclear markers like RAG1 contribute to a more comprehensive understanding of genetic relationships and evolutionary processes (Chen et al. 2019).

The number of polymorphic sites was significantly higher in COI (126) compared to RAG1 (33), showing that the mitochondrial genes were more informative for assessing genetic diversity. The variance of haplotype diversity was low across all loci, showing a stable genetic structure within populations. The mean evolutionary rates for both loci showed a range of substitutions per site, where COI had a broader range compared to RAG1. The ts/tv ratio (R) was higher for COI (2.32) than RAG1 (2.1), suggesting that transitions were more frequent compared to transversions, a common trend in mitochondrial DNA evolution. The gamma discrete distribution values showed a higher rate of nucleotide substitution in COI (0.62) compared to RAG1 (0.34) (Table 5), which was in line with the expectations of faster evolution in mitochondrial genes. Modeel et al. (2024) and Bingpeng et al. (2018) reported a consistent trend of higher AT content compared to GC content in the COI gene of fish species. Specifically, Modeel et al. (2024) observed an AT richness of approximately 60% across various populations in the Beas River, while Bingpeng et al. (2018) documented similar findings in the Taiwan Straits, with AT content exceeding 65%. This AT bias is particularly pronounced in mitochondrial DNA, which is characterized by a lower GC composition.

Table 3. Composition of characters in the COI, RAG1 and COI + RAG1 aligned datasets

Loci	Sequence length (bp)	Variable site	
		Parsimony informative	Singleton
COI	489	126	6
RAG1	681	33	0
COI+RAG1	1170	169	6

Table 4. Genetic distance of *Nemacheilus* specimens using COI, RAG1 and COI + RAG1 aligned datasets. Pairwise intra- and interspecific genetic distances were evaluated based on the K2P evolutionary model

Taxa	COI			RAG 1			COI+RAG1		
	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max
Intraspecific									
Nemacheilus fasciatus	0.0	2.2	4.7	0.0	0.3	1.1	0.0	1.5	3.0
Nemacheilus chrysolaimos	0.0	0.2	2.3	0.0	0.4	0.8	0.0	0.1	1.0
Interspecific	11.2	11.1	11.9	3.2	3.0	3.6	2.3	2.6	2.8

Table 5. Characteristics of partial sequence of COI, RAG1, and COI + RAG1 gene used for phylogenetic trees reconstruction and genetic distance analysis include sequences from the study sample and the GenBank/Barcode of Life Data System (BOLD) system (in group and out group)

Parameters	Loci		
	COI	RAG1	COI+RAG1
Tyrosine frequency	40%	26.74%	33%
Cytosine frequency	30.10%	29.20%	30%
Adenine frequency	14.50%	22.65%	18%
Guanine frequency	15.00%	20.41%	19%
Frequency of invariable sites	60.10%	59.35%	59.73%
Frequency of parsimony informative sites	26.48%	23.24%	24.86%
Nucleotide diversity (Pi)	0.09762	0.089	0.09331
Haplotype diversity	0.957	0.87	0.9135
Number of haplotypes	8	8	8
Polymorphic sites	126	33	159
Variance of haplotype diversity	0.00097	0.0087	0.00809
ts/tv ratio (R)	2.32	2.1	2.21
Gamma discrete distribution	0.62	0.34	0.48
Standard deviation of haplotype diversity	0.031	0.024	0.0275
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, 2.01, and 2.69 substitutions per site	0.00, 0.05, 0.06, 0.09, 0.21, 0.24, 0.31, 0.43, 0.57, 1.20, 2.03, and 2.64 substitutions per site	

The analysis of nucleotide diversity (Pi) showed that COI had the highest diversity value (0.09762), followed by the combined dataset (0.09331), and RAG1 (0.089). This pattern was in line with previous studies, where mitochondrial genes showed greater nucleotide diversity than nuclear loci (Panprommin et al. 2019; Venera-Pontón et al. 2020; Yan et al. 2021; Lee et al. 2023). Haplotype diversity was also significantly high across all loci, with COI having the greatest diversity (0.957), showing a rich genetic reservoir within the populations studied. The presence of eight haplotypes in each locus suggested a substantial level of genetic variation, which is essential for understanding population dynamics and evolutionary history (Lee et al. 2023). Mitochondrial DNA (mtDNA), particularly the COI gene, is often utilized in phylogenetic and population genetic studies due to its higher mutation rates and variability. This characteristic allows for a more nuanced understanding of evolutionary relationships and genetic diversity within and among species. In contrast, nuclear genes, such as RAG1, typically exhibit lower nucleotide diversity, which can limit their effectiveness in resolving fine-scale evolutionary relationships among closely related taxa.

Moreover, Alal et al. (2021) emphasized the importance of genetic diversity in the conservation of fish species, noting that populations with higher haplotype diversity are better positioned to adapt changing of environments and resist diseases. The implications of high haplotype diversity extend beyond more genetic variation; they also provide insights into the evolutionary history of populations. For example, Alghozali et al. (2019) conducted a study on scalloped hammerhead sharks and found that genetic diversity metrics, including haplotype diversity, could reveal historical population expansions and contractions, thereby informing conservation strategies. This aligns with Lee et al. (2023), where the substantial genetic variation observed is indicative of complex demographic histories within the studied fish populations.

Phylogenetic analysis

The analysis conducted for phylogenetic reconstruction using Maximum-Likelihood (ML) trees used the COI gene, the RAG1 gene, and a concatenated dataset, as shown in Figures 5, 6, and 7. Each species was associated with a unique gene cluster, which enabled a clear representation of phylogenetic relationships. The ML trees constructed from the COI gene showed two distinct clusters, providing strong support for the proposed phylogenetic relationships. Phylogenetic trees had a definitive and clear branching structure for the *Nemacheilus* species, with *N. fasciatus* and *N. chrysolaimos* forming separate monophyletic branches. The close positioning of these two species at the same node suggested a close evolutionary connection, which was corroborated by the calculated genetic distance of 11.2, showing the maximum genetic separation.

The results showed that *N. fasciatus* and *N. chrysolaimos* were identified as sister taxa, supported by a bootstrap value of 99%. Genetic variability observed between these clusters was minimal, with values falling below 2%. According to established criteria, genetic distance exceeding 2% was generally indicative of species differentiation, while distances below 3% suggested intra-specific variation or conspecific groupings (Chakraborty and Ghosh 2014; Bektas et al. 2019). Therefore, genetic distance analysis supported the distinction between the species, offering valuable insights into the diversity of *Nemacheilus* species in the Lumajang District.

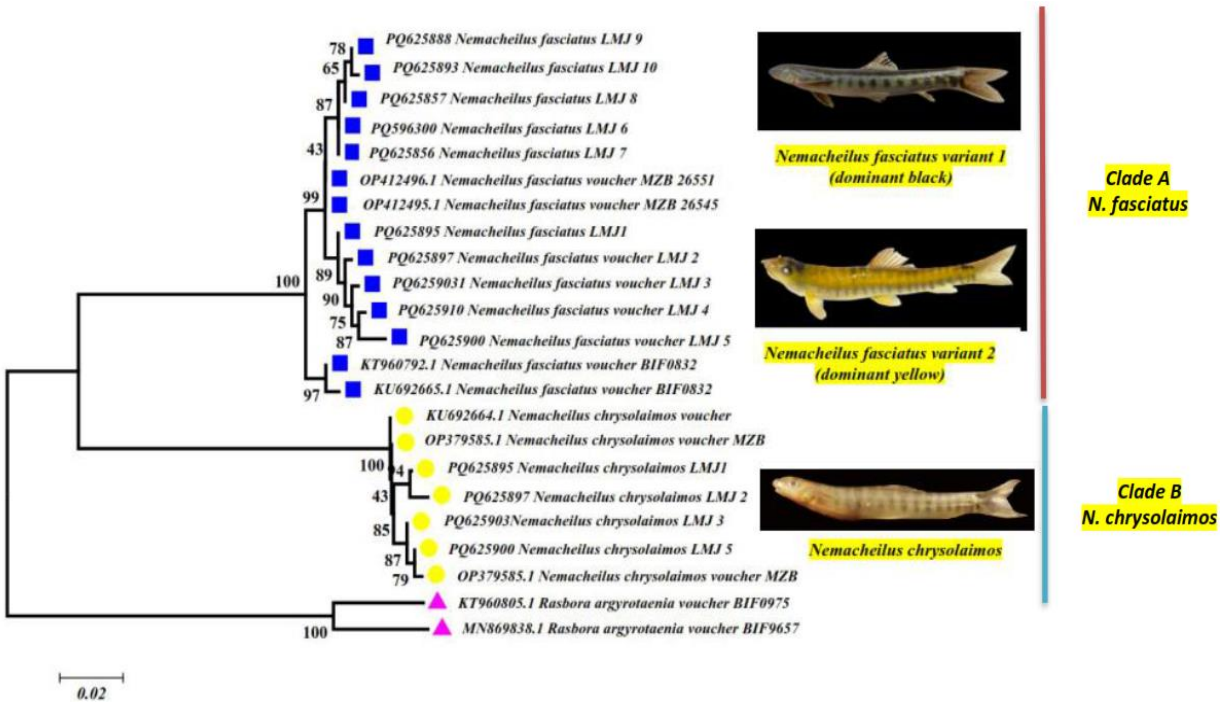

Figure 5. Maximum Likelihood (ML) phylogenetic tree of *Nemacheilus* spp. based on partial sequence of COI gene. *Rasbora* spp. as the outgroup. LMJ code is sample in this study

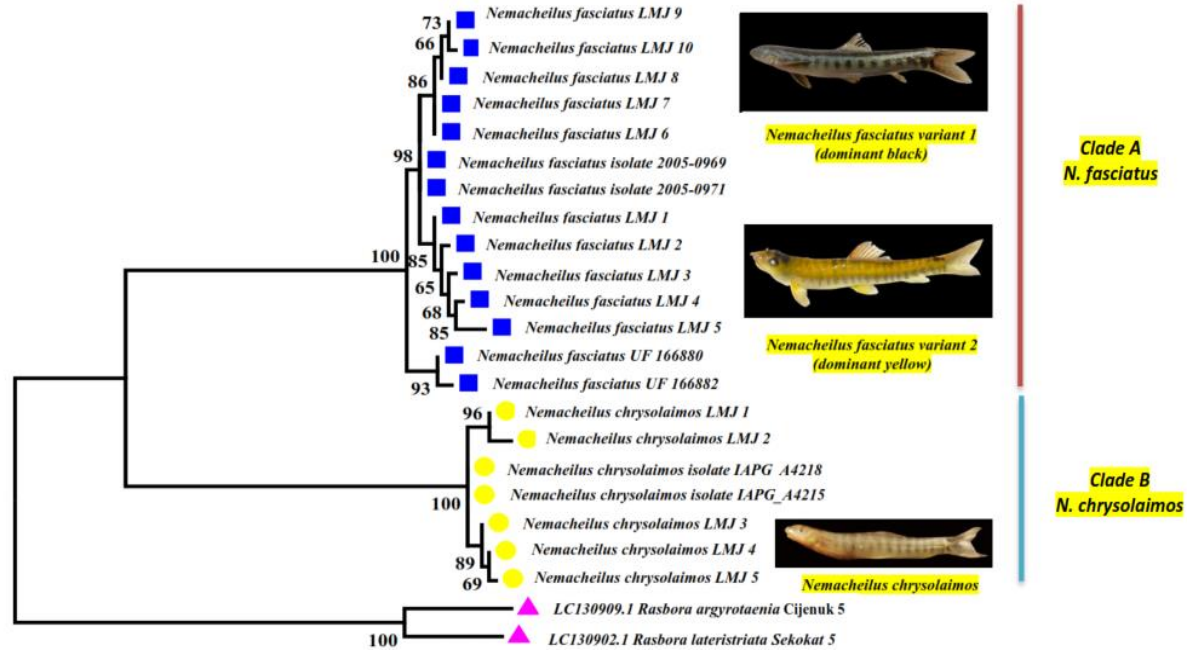

Figure 6. Maximum Likelihood (ML) phylogenetic tree of *Nemacheilus* spp. based on partial sequence of RAG1 gene. *Rasbora* spp. as the outgroup

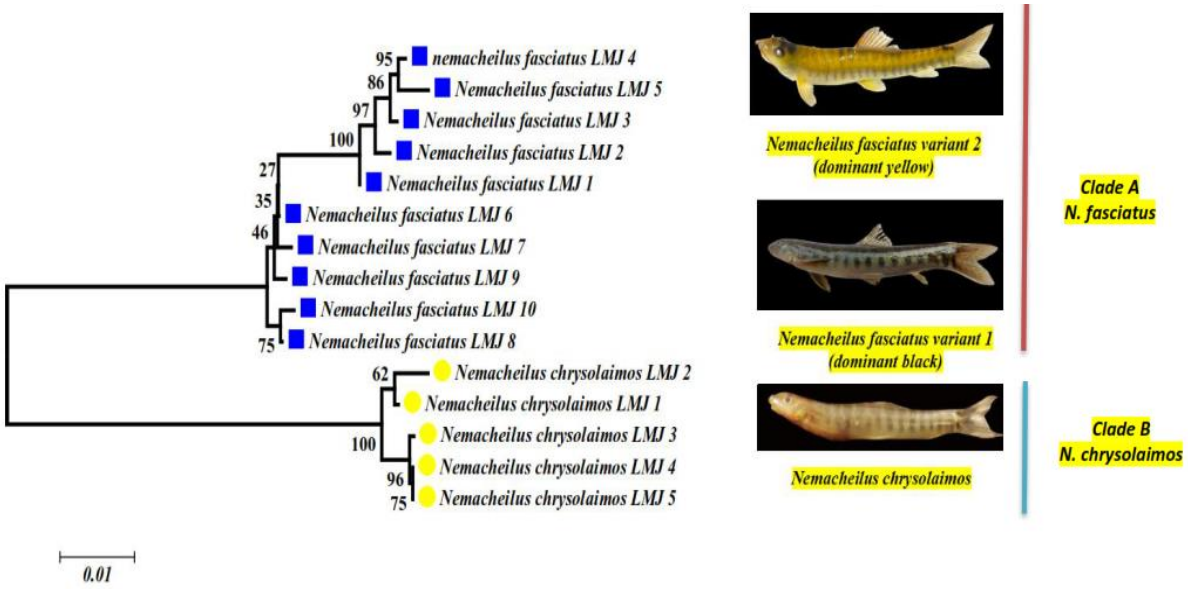

Figure 7. Maximum Likelihood (ML) phylogenetic tree of *Nemacheilus* spp. based on partial sequence of concatenated COI + RAG1 gene

When phylogenetic analyses of both the COI and RAG1 genes were compared, all specimens were classified according to their morphological characteristics. Phylogenetic analysis showed that *N. fasciatus* and *N. chrysolaimos* were monophyletic groups from a shared ancestor. The specimens examined in this study were organized into Clade A and Clade B, with each containing 21 specimens. By using the single-gene phylogenies of COI and RAG1 in Figure 7 as references for species identification, the specimens in Clade A were identified as *N. fasciatus*, while Clade B contained *N. chrysolaimos*. Furthermore, when the species identities derived from phylogenetic analysis of each specimen were compared, consistencies were observed between the COI and the combined RAG1 + COI phylogenetic trees, as shown in Figures 6 and 7.

Phylogenetic reconstruction using Maximum-Likelihood (ML) methods further showed the relationships among the species. The ML trees constructed from the COI gene had two distinct clusters, supporting the proposed phylogenetic relationships. Both *N. fasciatus* and *N. chrysolaimos* formed

separate monophyletic branches, showing their close evolutionary connection, which was supported by genetic distance of 11.2. This distance suggested minimal genetic variability between clusters, reinforcing the identification of *N. fasciatus* and *N. chrysolaimos* as sister taxa, supported by a robust bootstrap value of 99% (Page et al. 2020). According to established criteria, genetic distance exceeding 2% shows species differentiation, while distances below 3% suggest intra-specific variation or conspecific groupings (Lakra et al. 2015; Edwards et al. 2016; Wolf and Ellegren 2016). These results provided valuable insights into genetic diversity and evolutionary relationships within the *Nemacheilus* species in the Lumajang District, showing the importance of using multiple genetic loci for comprehensive phylogenetic analysis.

In conclusion, this study validated the effectiveness and dependability of targeted COI and RAG1 alongside morphological data for accurately identifying *Nemacheilus* species at the species level. The results provided fundamental data for the inaugural documentation of morphological characteristics, genetic identification, and phylogenetic analysis of *N. chrysolaimos* and *N. fasciatus* from the Lumajang District as new records. Furthermore, the conservation management of *N. chrysolaimos* and *N. fasciatus* could be effectively implemented through the classification of fish units based on species and genetic relationship, alongside the potential for developing to support sustainability and domestication initiatives.

ACKNOWLEDGEMENTS

The authors are grateful to the people around the river of Lumajang Regency, East Java, Indonesia, for their assistance during sampling session. Mr. Didik for his great help at the molecular laboratory. This research has received funding from LPPM Universitas Negeri Surabaya schema Faculty with grant number 309/UN38/HK/PP/2024.

REFERENCES

Abdullah A, Nurilmala M, Jacoeb AM, Sitaresmi KP. 2019. Mini DNA-barcode as molecular marker for heavily processed Hairtail fish products authentication. IOP Conf Ser: Earth Environ Sci 278: 012001. DOI: 10.1088/1755-1315/278/1/012001.

Alal GW, Barasa JE, Chemoiwa EJ, Kaunda-Arara B, Akoll P, Masembe C. 2021. Genetic diversity and population structure of selected lacustrine and riverine populations of African catfish, *clarias gariepinus* (burchell, 1822), in Kenya. J Appl Ichthyol 37 (3): 427-438. DOI: 10.1111/jai.14167.

Alcudia-Catalma MN, Diaz MGQ, Garcia RN, Ocampo PP, Laurena AC, Tecson-Mendoza EM. 2020. DNA barcoding and diversity analysis of 19 economically important Philippine sea cucumbers (Holothuroidea). Philipp J Sci 149 (2): 335-346. DOI: 10.56899/149.02.09.

Alghozali FA, Wijayanti DP, Sabdono A. 2019. Short Communication: Genetic diversity of scalloped Hammerhead sharks (*Sphyrna lewini*) landed in Muncar Fishing Port, Banyuwangi. Biodiversitas 20 (4): 1154-1159. DOI: 10.13057/biodiv/d200430.

Aminan AW, Kit LLW, Hui CH, Sulaiman B. 2020. Morphometric analysis and genetic relationship of *Rasbora* spp. in Srawak, Malaysia. Trop Life Sci Res 31 (2): 33-49. DOI: 10.21315/tlsr2020.31.2.3.

Ayebare S, Doser JW, Plumptre AJ, Owiunji I, Mugabe H, Zipkin EF. 2023. An environmental habitat gradient and within-habitat segregation enable co-existence of ecologically similar bird species. Proc Biol Sci 290 (2005): 20230467. DOI: 10.1098/rspb.2023.0467.

Basith A, Abinawanto, Kusrini E, Yasman. 2021. Genetic diversity analysis and phylogenetic reconstruction of groupers *Epinephelus* spp. from Madura Island Indonesia based on partial sequence of CO1 gene. Biodiversitas 22 (10): 4282-4290. DOI: 10.13057/biodiv/d221020.

Baumgartner LJ, Wibowo A. 2018. Addressing fish-passage issues at hydropower and irrigation infrastructure projects in indonesia. Mar Freshw Res 69: 1805-1813. DOI: 10.1071/mf18088.

Bektas Y, Aksu I, Kaya C, Turan D. 2019. DNA barcoding of the genus *Capoeta* (Actinopterygii: Cyprinidae) from Anatolia. Turk J Fish Aquat Sci 19 (9): 739-752. DOI: 10.4194/1303-2712-v19_9_03.

Bingpeng X, Heshan L, Zhilan Z, Chunguang W, Yanguo W, Jianjun W. 2018. DNA barcoding for identification of fish species in the Taiwan Strait. PLoS One 13: e0198109. DOI: 10.1371/journal.pone.0198109.

Bookstein FL, Chernoff B, Elder RL, Humphries Jr JM, Smith GR, Strauss RF. 1985. Morphometrics in Evolutionary Biology: The Geometry of Size and Shape Change, With Examples from Fishes (The Academy of Natural Sciences of Philadelphia, Special Publication No. 15). Academy of Natural Sciences of Philadelphia, Pennsylvania.

Brlík V, Procházka P, Yohannes E, Sedláček O, Albrecht T, Hořák D. 2024. Isotopes complement morphology: Niche partitioning among greenbuls in the afrotropical lowland forest. Afr J Ecol 62 (3): e13315. DOI: 10.1111/aje.13315.

Chakraborty M, Ghosh SK. 2014. An assessment of the DNA barcodes of Indian freshwater fishes. Gene 537 (1): 20-28. DOI: 10.1016/j.gene.2013.12.047.

Chapsos I, Hamilton S. 2018. Illegal fishing and fisheries crime as a transnational organized crime in Indonesia. Trends Organ Crime 22: 255-273. DOI: 10.1007/s12117-018-9329-8.

Chen W, Yang J, Li Y, Li X. 2019. Exploring taxonomic diversity and biogeography of the family nemacheilinae (cypriniformes). Ecol Evol 9 (18): 10343-10353. DOI: 10.1002/ece3.5553.

De la Ossa-Guerra LE, Santos MH, Artoni RF. 2020. Genetic diversity of the cichlid *Andinoacara latifrons* (Steindachner, 1878) as a conservation strategy in different Colombian basins. Front Genet 11: 815. DOI: 10.3389/fgene.2020.00815.

de Moraes Russo CA, Selvatti AP. 2018. Bootstrap and rogue identification tests for phylogenetic analyses. Mol Biol Evol 35 (9): 2327-2333. DOI: 10.1093/molbev/msy118.

Duvaud S, Gabella C, Lisacek F, Stockinger H, Ioannidis V, Durinx C. 2021. Expasy, the Swiss bioinformatics resource portal, as designed by its users. Nucleic Acids Res 49 (W1): W216-W227. DOI: 10.1093/nar/gkab225.

Edwards SV, Potter S, Schmitt JJ, Bragg JG, Moritz C. 2016. Reticulation, divergence, and the phylogeography-phylogenetics continuum. Proc Natl Acad Sci 113 (29): 8025-8032. DOI: 10.1073/pnas.1601066113.

Falade MO, Opene AJ, Benson O. 2016. DNA barcoding of *Clarias gariepinus*, *Coptodon zillii* and *Sarotherodon melanotheron* from southwestern nigeria. F1000Res 5: 1268. DOI: 10.12688/f1000research.7895.1.

Ferrari IV, Patrizio P. 2021. Study of Basic Local Alignment Search Tool (BLAST) and multiple sequence alignment (Clustal-X) of monoclonal mice/human antibodies. bioRxiv 2021: 07. DOI: 10.1101/2021.07.09.451785.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial Cytochrome C Oxidase Subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3 (5): 294-299.

Freudiger A, Josi D, Thünken T, Herder F, Flury JM, Marques DA, Taborsky M, Frommen JG. 2021. Ecological variation drives morphological differentiation in a highly social vertebrate. Funct Ecol 35 (10): 2266-2281. DOI: 10.1111/1365-2435.13857.

Fujimoto T, Yamada A, Kodo Y, Nakaya K, Okubo-Murata M, Saito T, Ninomiya K, Inaba M, Kuroda M, Arai K, Murakami M. 2017. Development of nuclear DNA markers to characterize genetically diverse groups of *Misgurnus anguillicaudatus* and its closely related species. Fish Sci 83: 743-756. DOI: 10.1007/s12562-017-1108-y.

Hall TA. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symp Ser 41: 95-98.

Haryani GS. 2022. Migratory freshwater fish in Indonesia: Threats and conservation efforts. IOP Conf Ser: Earth Environ Sci 1062: 012001. DOI: 10.1088/1755-1315/1062/1/012001.

Hu C-C, Wu Y-Q, Ma L, Chen Y-J, Ji X. 2019. Genetic and morphological divergence among three closely related *Phrynocephalus* species

(Agamidae). BMC Evol Biol 19 (1): 114. DOI: 10.1186/s12862-019-1443-y.

Hubert N, Hanner R. 2016. DNA barcoding, species delineation and taxonomy: A historical perspective. DNA Barcodes 3: 44-58. DOI: 10.1515/dna-2015-0006.

Hubert N, Lumbantobing D, Sholihah A, Dahruddin H, Delrieu-Trottin E, Busson F, Sauri S, Hadiaty R, Keith P. 2019. Revisiting species boundaries and distribution ranges of *Nemacheilus* spp. (Cypriniformes: Nemacheilidae) and *Rasbora* spp. (Cypriniformes: Cyprinidae) in Java, Bali and Lombok through DNA barcodes: Implications for conservation in a biodiversity hotspot. Conserv Genet 20: 517-529. DOI: 10.1007/s10592-019-01152-w.

Indarjo A, Salim G, Nugraeni CD, Anggoro S, Maryanto TI, Prakoso LY. 2022. Preliminary study on the domestication of giant freshwater prawn, Macrobrachium rosenbergii (De Man, 1879) from North Kalimantan, Indonesia. AACL Bioflux 15 (6): 3121-3130.

Juniar AE, Ambarwati R, Rahayu DA. 2021. Genetic identification of *Clithon oualaniense* (Gastropoda: Neritidae) from Madura, Indonesia. AACL Bioflux 14 (2): 1046-1056.

Kottelat M, Freyhof J. 2007. Handbook of European Freshwater Fishes. Cornol, CH : Publications Kottelat, Berlin.

Kottelat M, Whitten AJ, Kartikasari SN, Wirjoatmodjo S. 1993. Freshwater Fishes of Western Indonesia and Sulawesi. Periplus, Hong Kong.

Kottelat M. 1984. Revision of the Indonesian and Malaysian loaches of the subfamily Nemacheilinae. Jpn J Ichthyol 31: 225-260.

Kottelat M. 2013a. The fishes of the inland waters of Southeast Asia: A catalog and core bibliography of the fishes known to occur in freshwaters, mangroves and estuaries. Raffles Bull Zool 27: 1-663.

Kottelat M. 2013b. Dates of publication of Bleeker's Atlas ichthyologique and Poissons de Madagascar. Zootaxa 3681: 281-285. DOI: 10.11646/zootaxa.3681.3.7.

Kristjánsson BK, Malmquist HJ, Ingimarsson F, Antonsson T, Snorrason SS, Skúlason S. 2011. Relationships between lake ecology and morphological characters in icelandic arctic charr, *Salvelinus alpinus*. Biol J Linn Soc 103 (4): 761-771. DOI: 10.1111/j.1095-8312.2011.01670.x.

Kusuma RO, Dadiono MS, Kusuma B, Syakuri H. 2021. Genetic diversity of Stone loaches (*Nemacheilus*) in river of Banyumas area based on Cytochrome Oxidase Subunit I (COI). J Perikanan Universitas Gadjah Mada 23 (2): 89-94. DOI: 10.22146/jfs.61167. [Indonesian]

Lakra WS, Singh M, Goswami M, Gopalakrishnan A, Lal KK, Mohindra V, Sarkar UK, Punia PP, Singh KV, Bhatt JP, Ayyappan S. 2015. DNA barcoding Indian freshwater fishes. Mitochondrial DNA A DNA Mapp Seq Anal 27 (6): 4510-4517. DOI: 10.3109/19401736.2015.1101540.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948. DOI: 10.1093/bioinformatics/btm404.

Lee J, Kwon S, Ubagan MD, Lee T, Shin S. 2023. Genetic diversity and population dynamics of invasive *Ascidiella aspersa*: Insights from Cytochrome Oxidase Subunit I and 18S rDNA analyses in Korean and global populations. Water 15: 3886. DOI: 10.3390/w15223886.

Li H, Fang Y, Wang X, Li Y, Zhang N, Zhang R, Liu C, Zhang H, Lin L, Song P. 2022. Demersal fish diversity and molecular taxonomy in the Bering Sea and Chukchi Sea. Conserv Genet Resour 14: 85-97. DOI: 10.1007/s12686-021-01241-4.

Luo X, Hines HN, Lu X, Song W, Zhang, S. 2024. Multi-gene-based investigation on the molecular phylogeny of the hypotrichous family Strongylidiidae with notes on the ontogeny of a new genus and new species. Mar Life Sci Technol 6 (3): 1-15. DOI: 10.1007/s42995-024-00243-z.

Medina N, Tank DC, Espíndola A. 2023. Calceolariaceae809: A bait set for targeted sequencing of nuclear loci. Appl Plant Sci 11 (6): e11557. DOI: 10.1002/aps3.11557.

Modeel S, Negi RK, Sharma M, Dolkar P, Yadav S, Siwach S, Yadav P, Negi T. 2024. A comprehensive DNA barcoding of Indian freshwater fishes of the Indus River system, Beas. Sci Rep 14: 2763. DOI: 10.1038/s41598-024-52519-0.

Muhala V, Guimarães-Costa A, Macate IE, Rabelo LP, Bessa-Silva AR, Watanabe L, Dos Santos GD, Sambora L, Vallinoto M, Sampaio I. 2024. DNA barcoding for the assessment of marine and coastal fish diversity from the coast of Mozambique. PLoS One 19 (2): e0293345. DOI: 10.1371/journal.pone.0293345.

Naz S, Chatha AMM, Nazir U, Iqbal SS, Danabas D. 2023. Morphological and genetic identification of Head Taunsa Barrage fish species. Curr Trends Omics 3 (1): 39-59. DOI: 10.32350/cto.31.03.

Nishimaki T, Sato K. 2019. An extension of the Kimura two-parameter model to the natural evolutionary process. J Mol Evol 87 (1): 60-67. DOI: 10.1007/s00239-018-9885-1.

Nugroho ED, Ardiansyah R, Kurniawan N, Widodo, Rahayu DA. 2023. Species identification of echinoderms from Gili Ketapang Island by combining morphology and molecular data. AACL Bioflux 16 (1): 135-150.

Page LM, Pfeiffer JM, Suksri S, Randall ZS, Boyd DA. 2020. Variation in the arrow loach, *Nemacheilus masyae* (Cypriniformes: Nemacheilidae), in mainland southeast asia with description of a new species. Copeia 108 (2): 392-402. DOI: 10.1643/ci-19-305.

Panijpan B, Laosinchai P, Senapin S, Kowasupat C, Ruenwongsa P, Kühne J, Phiwsaiya K. 2015. Mitochondrial COI and nuclear RAG1 DNA sequences and analyses of specimens of the three morphologically established species in the genus *Trichopsis* (Perciformes: Osphronemidae) reveal new/cryptic species. Meta Gene 4: 17-28. DOI: 10.1016/j.mgene.2015.02.003.

Panprommin D, Soontornprasit K, Tuncharoen S, Pithakpol S, Keereelang J. 2019. DNA barcodes for the identification of species diversity in fish from Kwan Phayao, Thailand. J Asia-Pac Biodivers 12 (3): 382-389. DOI: 10.1016/j.japb.2019.05.003.

Pappalardo AM, Federico C, Sabella G, Saccone S, Ferrito V. 2015. A COI nonsynonymous mutation as diagnostic tool for intraspecific discrimination in the European Anchovy *Engraulis encrasicolus* (Linnaeus). PLoS One 10 (11): e0143297. DOI: 10.1371/journal.pone.0143297.

Quenouille B, Bermingham E, Planes E. 2004. Molecular systematics of the damselfishes (Teleostei: Pomacentridae): Bayesian phylogenetic analyses of mitochondrial and nuclear DNA sequences. Mol Phylogenet Evol 31: 66-88. DOI: 10.1016/S1055-7903(03)00278-1.

Radulovici AE, Vieira PE, Duarte S, Teixeira MAL, Borges LMS, Deagle BE, Majaneva S, Redmond N, Schultz JA, Costa FO. 2021. Revision and annotation of DNA barcode records for marine invertebrates: Report of the 8th IBOL Conference Hackathon. Metabarcoding Metagenom 5: e67862. DOI: 10.3897/mbmg.5.67862.

Rahayu DA, Kuntjoro S, Budijastuti W, Winarsih W, Ambarwati R, Nugroho ED, Basith A, Kurniawan N, Haryono. 2023. Further study on two species of Loach fishes (Cypriniformes: Nemacheilidae: Nemacheilus) based on morphology and molecular data. Biotropia 30 (3): 329-345. DOI: 10.11598/btb.2023.30.2.1942.

Reid BN, Le M, McCord WP, Iverson JB, Georges A, Bergmann T, Amato G, Desalle R, Naro-Maciel E. 2011. Comparing and combining distance-based and character-based approaches for barcoding turtles. Mol Ecol Resour 11: 956-967. DOI: 10.1111/j.1755-0998.2011.03032.x.

Riyadini AA, Widodo MS, Fadjar M. 2020. Cytochrome Oxidase C Subunit I (COI) for identification and genetic variation of Loaches (*Nemacheilus fasciatus*). Res J Life Sci 7 (3): 142-154. DOI: 10.21776/ub.rjls.2020.007.03.4.

Sari DWK, Achmad H, Rahman H, Bimasuci H. 2023. Molecular identification of several morphologically distinct flowerhorn fish (family) using mitochondrial gene marker. J Trop Biodivers Biotechnol 8 (2): jtbb78459. DOI: 10.22146/jtbb.78459.

Sari SYP, Ambarwati R, Rahayu DA. 2021. Molecular characteristics of *Donax faba* (Bivalvia: Donacidae) from Nepa Beach, Madura, based on cytochrome oxidase subunit I gene sequences. AACL Bioflux 14 (4): 2416-2426.

Sember A, Bohlen J, Šlechtová V, Altmanová M, Symonová R, Ráb P. 2015. Karyotype differentiation in 19 species of river loach fishes (Nemacheilidae, Teleostei): Extensive variability associated with rdna and heterochromatin distribution and its phylogenetic and ecological interpretation. BMC Evol Biol 15: 251. DOI: 10.1186/s12862-015-0532-9.

Sgouros K, Page LM, Orlofske SA, Jadin RC. 2019. A revised molecular phylogeny reveals polyphyly in Schistura (Teleostei: Cypriniformes: Nemacheilidae). Zootaxa 4559 (2): 349-362. DOI: 10.11646/zootaxa.4559.2.8.

Šlechtová V, Bohlen J, Tan HH. 2007. Families of Cobitoidea (Teleostei; Cypriniformes) as revealed from nuclear genetic data and the position of the mysterious genera *Barbucca*, *Psilorhynchus*, *Serpenticobitis* and *Vaillantella*. Mol Phylogenet Evol 44 (3): 1358-1365. DOI: 10.1016/j.ympev.2007.02.019.

Šlechtová V, Musilova Z, Tan HK, Kottelat M, Bohlen J. 2021. One northward, one southward: Contrasting biogeographical history in two

benthic freshwater fish genera across Southeast Asia (Teleostei: Cobitoidea: Nemacheilus, Pangio). Mol Phylogenet Evol 161: 107139. DOI: 10.1016/j.ympev.2021.107139.

Sulung CAVT, El Chair N, Rahayu DA, Nugroho ED, Rusdianto. 2024. Morphology and COI barcodes reveal local loaches (*Nemacheilus* Spp.) from Pasuruan River, East Java, Indonesia. Biodiversitas 25 (7): 2806-2819. DOI: 10.13057/biodiv/d250702.

Tornow MA, Skelton RR. 2011. A priori assumptions about characters as a cause of incongruence between molecular and morphological hypotheses of primate interrelationships. Primates 53 (1): 83-96. DOI: 10.1007/s10329-011-0282-2.

Trivedi S, Aloufi AA, Rehman H, Saggu S, Ghosh SK. 2016. DNA Barcoding: Tool for assessing species identification in reptilia. J Entomol Zool Stud 4 (1): 332-337.

Venera-Pontón DE, Driskell AC, De Grave S, Felder DL, Scioli JA, Collin R. 2020. Documenting decapod biodiversity in the Caribbean from DNA barcodes generated during field training in taxonomy. Biodivers Data J 8: e47333. DOI: 10.3897/bdj.8.e47333.

Vieira C, De Ramon N'Yeurt A, Rasoamanendrika FA, D'Hondt S, Tran L-AT, Van den Spiegel D, Kawai H, De Clerk O. 2021. Marine macroalga biodiversity of Northern Madagascar: Morpho-genetic systematics and implications of Anthropic impacts for conservation. Biodivers Conserv 30: 1501-1546. DOI: 10.1007/s10531-021-02156-0.

Yan J, Pal C, Anderson D, Vétek G, Farkas P, Burne A, Fan Q-H, Zhang J, Gunawardana DN, Balan RK, George S, Li D. 2021. Genetic diversity analysis of brown marmorated stink bug, *Halyomorpha halys* based on Mitochondrial COI and COII haplotypes. Res Sq 2021: 1-19. DOI: 10.21203/rs.3.rs-59349/v1.

Yedier S, Bostanci D. 2021. Intra- and interspecific discrimination of *Scorpaena* species from the Aegean, Black, Mediterranean and Marmara seas. Sci Mar 85: 197-209. DOI: 10.3989/scimar.05185.018.

Yedier S, Yalçınkaya SK, Bostanci D, Polat N. 2022. Morphologic, morphometric and contour shape variations of sagittal otoliths of *Lepidorhombus* spp. in the aegean sea. Anat Histol Embryol 52 (2): 279-288. DOI: 10.1111/ahe.12881.

Wolf J, Ellegren H. Making sense of genomic islands of differentiation in light of speciation. Nat Rev Genet 18: 87-100 DOI: 10.1038/nrg.2016.133.