DNA barcode of mudskipper based on DNA mini-barcode COI from the North Coast of East Java, Indonesia

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Abstract. Previous research has reported a common phenomenon of ambiguous species in the family Gobiidae, including mudskippers, which have highly similar morphology and phenetics but are genetically different, resulting in taxonomic confusion. Inaccurate identification of cryptic species can lead to taxonomic and conservation problems. The problems posed are multiple names for the same species, an increase in biodiversity, an increase in ambiguous diversity, and two genetically distinct species classified into one species due to morphological similarities. Research on the molecular identification of mudskippers using the COI mitochondrial gene as a DNA mini-barcoding marker in Indonesia, especially in East Java, still needs to be completed. This research aimed to identify and analysis of the genetic relationship of mudskipper based on the COI (cytochrome-c-oxidase subunit I) gene. DNA samples were collected from 5 locations on the North Coast of East Java, Indonesia. DNA samples were collected by cutting the pectoral fins of caught mudskipper fish. The pectoral fins were stored in pure-grade ethanol and subjected to DNA extraction, PCR, and sequence processing analysis using bioinformatics. The result shows that four species based on eight samples from different locations of mudskipper fish were found to have similarity values with *Periophthalmodon schlosseri*, *Periophthalmus argentilineatus*, *Boleophthalmus boddarti*, and *Boleophthalmus pectinirostris*, namely in the range of 98.42-100% genetic similarity. Genetic analysis based on nucleotides showed a site frequency of 66.196%, site parsimony of 24.646%, nucleotide diversity of 0.995, and haplotypes 7 and 11 mutations at the first codon position. Based on the phylogenetic tree topology analysis of the research samples, the bootstrap value range for each Operational Taxonomic Unit (OTU) was between 98 and 100, which indicates that the samples have significant similarities or belong to the same species in one cluster.
Key Words: DNA mini-barcode COI, phylogenetic relationship, genetic identification, mudskipper.

Introduction. Mudskippers belong to the suborder Gobiidae, family Gobiidae, and subfamily Oxudercinae (Rha'ifa et al 2020). Mudskippers inhabit mudflats, sandy beaches, coasts, estuaries, rivers, and mangrove ecosystems (Hartoko et al 2022). This resilience is supported by the morphology and adaptability of mudskippers under various environmental conditions, including pH, salinity, temperature, and dissolved oxygen (Darojat et al 2023). On the other hand, morphological conditions, such as organ respiration in the humid skin mucosal lining in the mouth and throat that support adaptability similar to body appendages in the form of a distinctive pectoral fin also improve maneuverability (Lin et al 2023). Mudskippers exhibit amphibious lifestyles; however, they can be classified into aquatic or terrestrial groups based on habitat preferences, locomotor character states, and gene-based phylogenetic studies (Ghanbarifardi et al 2020; Steppan et al 2022; Zhou et al 2023). The population and biodiversity of the mudskipper also showed a correlation with the condition of the mangrove ecosystem, which is the main habitat for this fish. Organic and inorganic pollutants, the expansion of urban areas, degradation of coastal ecosystems, and natural

disasters significantly impact mudskipper populations. Natural factors and human activities have been identified as causes of damage to the mangrove ecosystem and the biota within it. Conservation of mangrove biota, especially mudskippers, must be conducted to protect mudskipper biodiversity.

Previous research has reported a common phenomenon of ambiguous species in the family Gobiidae, including mudskippers, which have highly similar morphology and phenetics but are genetically different, resulting in taxonomic confusion. Cryptic species are morphologically similar, distinguishing them solely on morphological characteristics is nearly impossible. Delić et al (2017) revealed that cryptic species are the worst-case scenario of taxonomic incompleteness, usually remain undescribed, and cannot be used in conservation practice. Furthermore, the inaccurate identification of cryptic species can lead to taxonomic and conservation problems. Inaccurate identification of a species indicated by an ambiguous species is the cause of this problem, which may lead to erroneous species classification (Leys et al 2016). The problems posed are multiple names for the same species, an increase in biodiversity, and an increase in ambiguous diversity, defined as two or more distinct species and two genetically distinct species classified into one species due to morphological similarities. Misidentification affects mudskipper taxonomy, biodiversity inventory, conservation efforts, and sustainable utilization. Thus, molecular techniques have been widely applied to address the ambiguities. DNA barcoding is a molecular approach for analyzing genetic variability within a specific genomic region, which is designated as a 'DNA barcode', with full-length barcode regions (~650 bp). This analysis was then compared to pre-existing DNA sequence databases for the species of interest, serving as a reference for identifying specific DNA fragments (Venuti et al 2023). The most common genetic region targeted for identifying animal species is a region of the partial sequence mitochondrial gene coding for cytochrome c oxidase subunit I (CO1). However, DNA fragmentation makes it difficult to recover the full-length DNA barcodes highly processed (Zahn et al 2020). DNA mini-barcoding is an alternative approach for molecular marker identification of fragmentation DNA samples. The DNA mini-barcoding extrapolation reliably uses a smaller length of DNA fragments (e.g., 100–200 bp), so it is simpler than conventional DNA barcodes (Xing et al 2019).

In Indonesian waters, few discoveries of cryptic species were reported from members of the family Gobiidae (Rha'ifa et al 2021; Aji & Arisuryanti 2021). Several studies have also differentiated mudskipper species using DNA barcoding and character-based analysis. Tan et al (2020) characterized the genetic diversity of *Periophthalmus novemradiatus* based on the mitochondrial genome. Comprehensive molecular research was conducted on members of the suborder Gobioidae using five mitochondrial and nuclear molecular markers, including the COI gene (Agorreta et al 2013). According to Venuti et al (2023), several mitochondrial genes, such as Cytochrome c Oxidase I-COI, Cytochrome b-Cytb, 16S, and 12S, are currently used as molecular markers for all fish species. The Nigerian mudskipper was identified molecularly using the DNA barcode COI, in conjunction with phylogenetic relationships (Sokefun et al 2022). On the other hand, cryptic species were also observed in mudskippers *Periophthalmus argentilineatus* from Indo-Malayan and East-African using two mitochondrial gene markers (D-loop and 16S rDNA), *Boleophthalmus pectinirostris* from the western Pacific coast of East Asia and the Strait of Malacca in Malaysia, using mitochondrial and nuclear markers (Chen et al 2014), and DNA barcoding was performed using the COI mitochondrial gene on the species *P. argentilineatus* from Bogowonto Lagoon, Yogyakarta, Indonesia.

Research on the molecular identification of mudskippers using the COI mitochondrial gene as a DNA mini-barcoding marker in Indonesia, especially in East Java, is relatively new. Juniar et al (2019) also studied the identification of mudskipper fish species. However, studies are only limited to morphological identification, so there is still an ambiguity when viewed from the perspective of genetic factors. The present research is the first to provide a molecular characterization of the partial sequence of the mitochondrial DNA based on the COI gene from mudskipper fish on the north coast of East Java and to describe the population genetics with their phylogenetic relationships.

Material and Method

Sample collection. This research was conducted from June to October 2023. Mudskippers of several species (Figure 2) were collected from the Kenjeran Beach area, Wonorejo Mangrove Pond, Sedati Pond, Mengare Mangrove, and BJBR Beach, East Java, Indonesia (Figure 1). Eight mudskippers were collected. The tissue sample contained 50-100 mg of muscle tissue. Each tissue sample was dissected with a sterilized surgical scissor, placed into a 1.5 mL tube, preserved in 99% ethanol in the field, and stored at -20°C in the laboratory for further analysis.

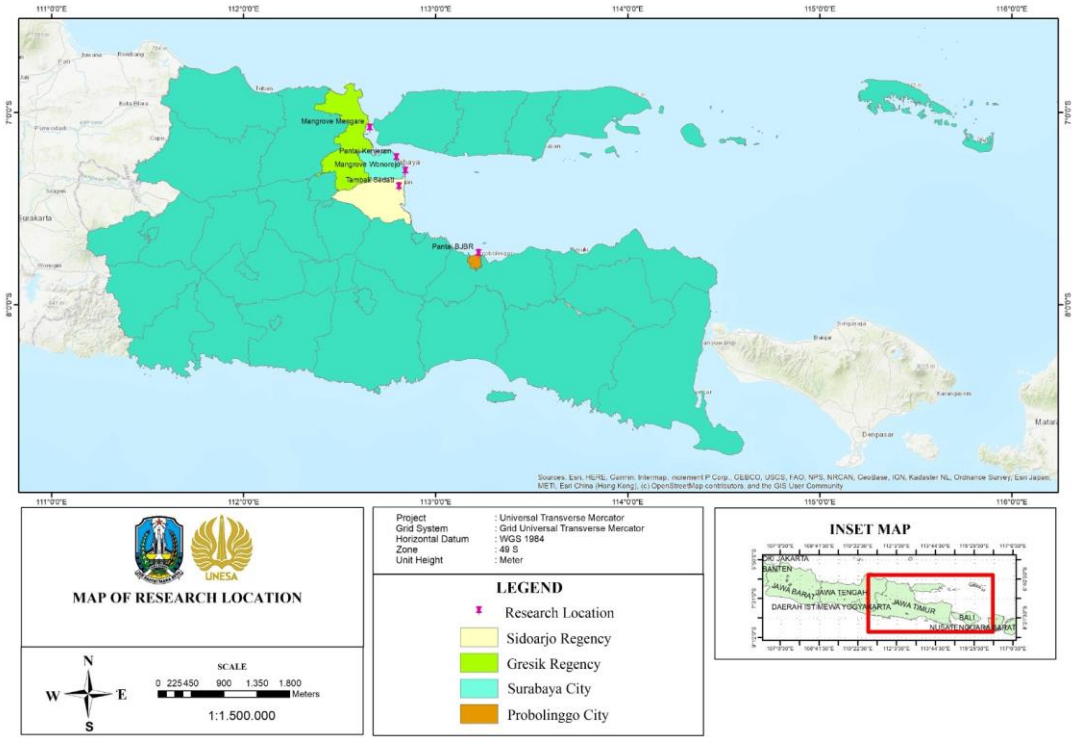


Figure 1. Location of the sampling localities at Kenjeran Beach area, Wonorejo Mangrove Pond, Sedati Pond, Mengare Mangrove, and BJBR Beach (East Java, Indonesia).

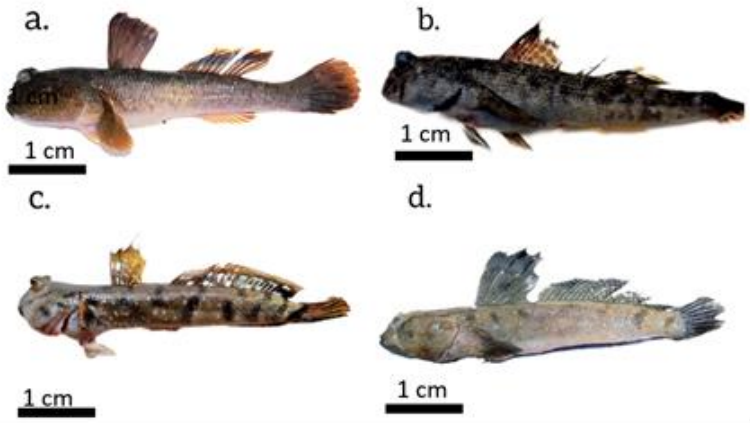


Figure 2. Mudskipper collected from the North Coast of East Java. a. *Periophthalmodon schlosseri,* b. *Periophthalmus argentilineatus,* c. *Boleophthalmus boddarti,* d. *Boleophthalmus pectinirostris.* The black bar is 1 cm.

DNA samples were collected by cutting the pectoral fins of caught mudskipper fish. The pectoral fins were stored in pure-grade ethanol and subjected to DNA extraction, PCR, and sequence processing analysis using bioinformatics.

DNA extraction. DNA extraction was done by grinding a 20 mg mudskipper pectoral fin sample in 1.5 mL tubes. 200 µL of GT1 buffer was pipetted into a 1.5 mL tube, then mixed with and vortexed. 200 µL of GT2 buffer and 20 µL of proteinase K were added and mixed using a vortex. The samples were incubated at 56°C for 10 min and the incubation tube was turned back and forth every 5 min. Absolute ethanol (200 µL) was added, and the mixture was vortexed briefly. The sample was then placed in a spin column and centrifuged for 1 min at 13,000 rpm. The flow-through was discarded, and 500 µL of buffer W1 was added to the spin column and then centrifuged for 1 min at 13,000 rpm. The flow-through was discarded, and 700 µL of W2 buffer (to which ethanol was added) was added and centrifuged for 1 min at 13,000 rpm. The flow-through was discarded and centrifuged again for 2 min at 13,000 rpm. DNA was transferred to a spin column in a new 1.5 mL tube. Elution buffer (50–100 µL) was added, and the mixture was incubated at room temperature for 1 min, followed by centrifugation for 1 min at 13,000 rpm. The DNA spin column was removed, and the DNA was purified for the next step. DNA was stored at -20°C for a few days, at -70°C, for long-term storage.

DNA amplification. The extraction results were then amplified using a Biorad PCR machine in 30 µL of 15 µL solution consisting of PCR Master Mix Nexpro, 3 µL DNA Template samples (100 ng μL^{-1}), 6 µL water, and 3 µL primer (10 pmol each forward primer and reverse). The primers used were LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). Amplification was carried out with the following temperature settings: pre-denaturation at 94°C for 1 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 45°C for 45 s, and extension at 72°C for 1 min 30 s. Next, the post-elongation process was performed at 72°C for 10 min. The PCR results were electrophoresed on 1% agarose.

Sequencing. The PCR results were then sequenced using the sequencing services of the 1stBASE Laboratories Sdn Bhd, Malaysia.

Data analysis. Genetic data analysis was based on the chromatogram reading (Finch TV) results of the COI barcode sequence (±500 bp). The consensus results were matched with BLAST in the laboratory to determine the match of the target gene with the query obtained from GenBank and BOLD Finch TV. Alignment was performed using Clustal W on Mega 6. The results were manually checked using BioEdit version 7.0.9. The alignment results were checked individually for online identification in the BOLD System and similarity checks were performed via GenBank and the samples were compared with their relatives in GenBank. A phylogenetic topology was constructed to determine the relationship between one species and another using the Mega 6 computer program with the Maximum Likelihood (ML) and Neighbor-Joining (NJ) methods with the Kimura-2 parameter algorithmic calculation model. Tree evaluation was performed using bootstrap analysis with 1000 replications. The similarity value was calculated as Similarity Percentage = (1 - Genetic Distance) × 100%. Analysis of variations in nucleotide base sequences and haplotypes was carried out using DnaSP v.5.0, and haplogroups were created based on median-joining network analysis using the Network 4.1.0.8 computer program (Rahayu & Nugroho 2019).

Results

Genetic identification of mudskippers using the COI gene. DNA sequences are nucleotide bases derived from DNA molecules, especially the COI gene, a marker of natural kinship relationships (Kim et al 2017). Genetic identification using the Gelodok DNA sequence was obtained from samples from the North Coast of East Java and Gelodok Fish from GenBank, shown in the sample code and its relatives from GenBank and the BOLD system (Table 1).

Table 1

Sample codes and their relatives from GenBank and BOLD system

No	*Species*	*ACC number gene bank*	*Locality*
1	*Periophthalmodon schlosseri*-BJBR	OR592151	BJBR, Probolinggo
2	*Periophthalmodon schlosseri*-K	OR592220	Kenjeran Beach, Surabaya
3	*Periophthalmodon schlosseri*-MW	OR592231	Wonorejo Mangrove, Surabaya
4	*Periophthalmodon schlosseri*-MS	OR592248	Sedati Mangrove, Sidoarjo
5	*Periophthalmodon schlosseri*-MM	OR592248	Mengare Mangrove, Gresik
6	*Periophthalmus argentilineatus*-MW	OR592251	Wonorejo Mangrove, Surabaya
7	*Boleophthalmus boddarti*-MS	OR592255	Sedati Mangrove, Surabaya
8	*Boleophthalmus pectinirostris*-MM	OR592259	Mengare Mangrove, Gresik
9	*Periophthalmodon schlosseri* voucher 2239	OR053779	Semarang, Central Java
10	*Periophthalmodon schlosseri* voucher 2237	OR053778	Semarang, Central Java
11	*Periophthalmus argentilineatus* voucher BIF1391	KU692745	Semarang, Central Java
12	*Periophthalmus argentilineatus* voucher BIF1390	KU692746	Semarang, Central Java
13	*Boleophthalmus boddarti* voucher BBC6	MF572076	Malaysia
14	*Boleophthalmus boddarti* voucher BBC3	KY754676	Malaysia
15	*Boleophthalmus pectinirostris* voucher CG40G24	KX223892	Malaysia

Identification BOLD system. Among the eight sequenced samples, 611 nucleotide bases were successfully converted into protein without a stop codon in the middle of the sequence (Table 2). The COI sequence obtained was that of a pure COI gene. Figure 3 shows the electropherogram result of the COI amplification of mudskipper fish specimens in a 1% agarose gel. An electropherogram visually represents the electrophoresis results, especially DNA sequencing results. Linkage analysis based on nucleotides showed an unchanged site frequency of 66.196%, site parsimony of 24.646%, nucleotide diversity of 0.995, and number of haplotypes 7 and 11 mutations at the first codon position. The results of the BOLD system analysis (Table 2) show that the four species and eight samples of mudskipper fish were similar to *Periophthalmodon schlosseri*, *P. argentilineatus*, *B. boddarti*, and *Boleophthalmus pectinirostris*, which were in the range of 98.42–100%.

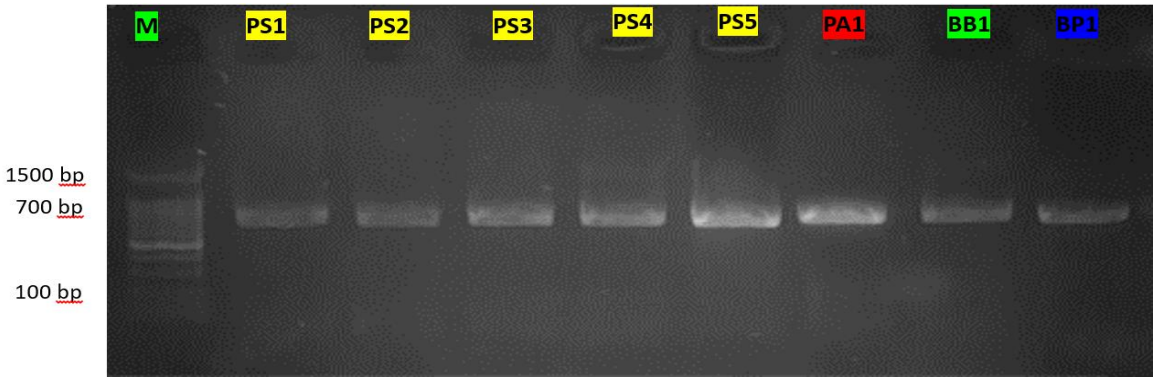


Figure 3. Electropherogram COI M: DNA Ladder 1 kb; PS1: MW; PS2: K; PS3: MS; PS4: BJBR; PS5: MM; PA1: MW; BB1: MS; BP1: MM.

Table 2

The three highest compatibility values from identification through the BOLD System with a representation of the similarity value

Sample name	*Identification BOLD systems (the three highest)*	*Similarity (%)*
Periophthalmodon schlosseri-MW	*Periophthalmodon schlosseri*	100 100 98.42
Periophthalmodon schlosseri-K	*Periophthalmodon schlosseri*	100 99.53 98.58
Periophthalmodon schlosseri-MS	*Periophthalmodon schlosseri*	100 98.58 98.58
Periophthalmodon schlosseri-BJBR	*Periophthalmodon schlosseri*	100 100 98.42
Periophthalmodon schlosseri-MM	*Periophthalmodon schlosseri*	99.9 98.9 98.42
Periophthalmus argentilineatus-MW	*Periophthalmus argentilineatus*	100 99.9 98.42
Boleophthalmus boddarti-MS	*Boleophthalmus boddarti*	100 100 98.42
Boleophthalmus pectinirostris-MM	*Boleophthalmus pectinirostris*	100 100 100

Table 3

Results of relationship analysis based on nucleotides

Parameters	*Position at codon*
	1st
Frequency of invariable sites	66.196%
Frequency of parsimony informative sites	24.646%
Nucleotide diversity (Pi)	0.955%
Nucleotide of haplotypes	7
Total number of mutations	11
ts/tv ratio (k)	Purines = 7.006, Pyrimidines = 0.042

Composition and variation of DNA COI nucleotide. In species identification using COI DNA Barcodes, a significant concern arises from the potential amplification of "COI-like sequences" or pseudogenes originating from nuclear mitochondrial DNA segments (NUMTs) (Buhay 2009). To address this concern, consensus sequence analysis was performed through protein translation to verify the origin of sequences from the mitochondrial DNA COI gene, treating COI as a coding region. The alignment analysis of gelodok fish discovered on the north coast of East Java indicated the absence of insertions or deletions (indels). In addition, to confirm the presence of numbers, the heterozygosity of chromatogram peaks was carefully examined (Bensasson et al 2001). The COI gene's barcode sequence showed that GC's nucleotide base composition was lower than AT's. The GC nucleotide base composition was 43.21-46.48% (Table 4). The average composition of AT was 62.246%, and GC was 44.188%. These findings align with the results of a previous study (Nugroho et al 2017), which reported a higher

number of AT nucleotide bases than GCs in nomei fish found in North Kalimantan waters. Universal primers for partial sequences of the COI gene were developed by Palumbi et al in 1994, carried out through accurate calculations, and successfully applied to the North Coast of East Java. Details of the genetic diversity analysis are summarized in Table 4. The prepared characteristics of The COI gene sequences used for phylogenetic tree reconstruction included sequences from the research and GenBank samples.

Table 4

Nucleotide base composition of the COI gene

Sample code	*T*	*C*	*A*	*G*	*A+T*	*C+G*
MS-OR59225	31.10	25.86	24.22	18.82	55.32	44.68
BJBR-OR592151	30.61	26.35	26.02	17.02	56.63	43.37
K-OR592220	30.77	26.35	25.86	17.02	56.63	43.37
MW-OR592231	30.77	26.35	25.86	17.02	56.63	43.37
MS-OR592248	31.10	26.35	25.53	17.02	56.63	43.37
MM-OR592248	31.26	26.19	25.53	17.02	56.79	43.21
MW-OR592255	29.46	27.99	24.88	17.68	54.34	45.66
MM-OR592259	28.64	27.33	24.88	19.15	53.52	46.48

* A-Adenine; T-Thymine; G-Guanine; C-Cytosine

The analysis results of nucleotide base variations (Table 5) show variations in nucleotide bases between the research samples and GenBank; mutations cause this variation. Based on the alignment stage of all samples (research samples and GenBank), there are: 74 variations of nitrogen bases and 611 bp conserved in *B. boddarti*; 62 variations of nitrogen bases and 611 bp conserved in *P. schlosseri*; 84 variations of nitrogen bases and 611 bp conserved in *P. argentilineatus*, and there are no variations in nitrogen bases in *B. pectinirostris*. It is known that these are unique (diagnostic) nucleotide bases, which are the main requirement for DNA barcoding identification.

Table 5

Nucleotide variation of the COI gene differentiated with data recorded in GeneBank

Species	*Translation*	*Transversion*
Baleopthalmus boddarti	12	6
Periophthalmodon schlosseri	15	10
Periphthalmus argentilineatus	40	10
Boleophthalmus pectinirostris	-	-

Phylogenetic tree of mudskipper. A phylogenetic tree topology analysis was conducted between samples with close relatives from GenBank using the neighbor-joining and maximum likelihood methods with the K2P calculation model (bootstrap 1000 repetitions) (Figure 4, Figure 5). Based on the cladogram, there were two clades with one large clade. *P. schlosseri* in the first apomorphy (species found in five research areas) and the second (species recorded in GenBank) had a bootstrap value of 93. *P. argentilineatus*, between the species found in the Wonorejo Mangrove Forest and the species recorded in GenBank, had a bootstrap value of 100. *B. pectinirostris*, between the species found in the Mengare Mangrove and the species recorded in GenBank, had a bootstrap value of 100. *B. boddarti*, between the species found in Tambak Sedati and the species that have been recorded in GenBank, also had a bootstrap value of 100, and the range of bootstrap values for each OTU was between 98 and 100, indicating that the groups have significant similarities or belong to the same species in one cluster. The neighbor-joining and maximum likelihood methods constantly show genetic relationships with mudskipper species. Hesterberg (2015) revealed that a bootstrap with 1,000 repetitions and a value of more than 80% on a branch indicates very good results as this value shows that the samples in one branch are correct or in the same species.

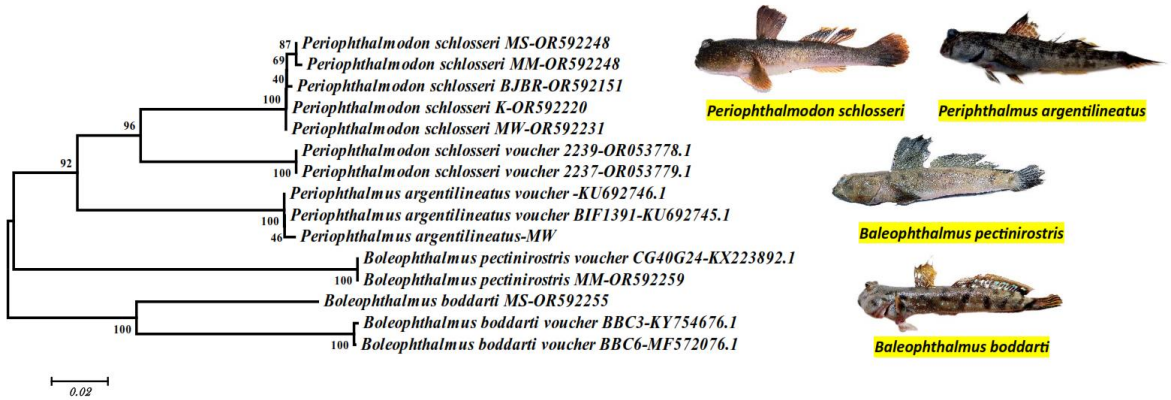


Figure 4. Neighbor-joining method from COI mitochondrial gene sequences.

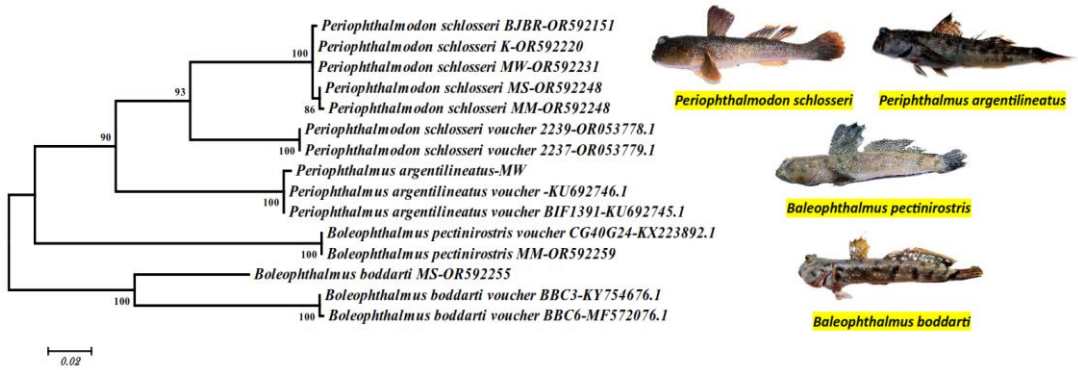
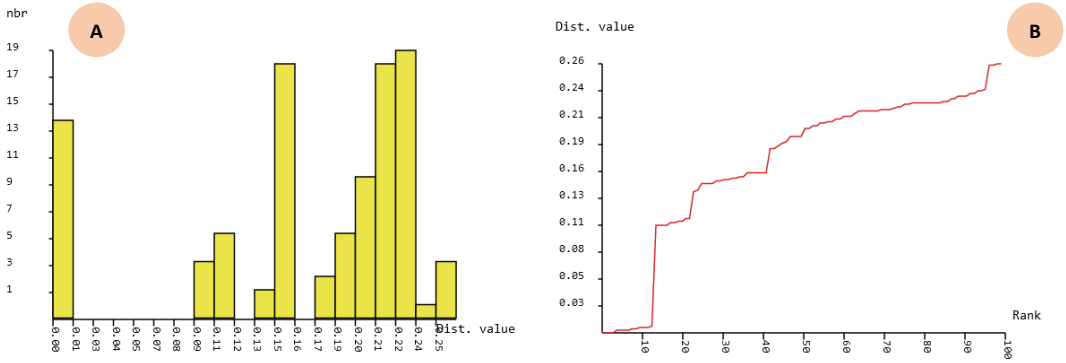


Figure 5. Maximum likelihood method on COI mitochondrial gene sequences with the K2P calculation model (bootstrap 1000 repetitions).

The composition of mitochondrial COI nucleotide (Table 4) for each species showed similar values can only be seen from some of the samples. From Table 4, T, C, A, and G nucleotides' composition was differentiated by 0.0-0.3%, 0-0.76%, 0-0.80%, and 0-99%, respectively. The result revealed the T, C, A, and G nucleotides' divergence in COI mitochondrial genes among the mudskipper samples analyzed in this study. This data revealed a polymorphism in the COI mitochondrial gene of mudskippers in Indonesia, which indicated the intraspecific genetic variation of mudskipper. The novel findings of this research use COI gene correlation with morphology identification. The correct and clear taxonomic status of mudskippers can be applied and a records database for the conservation of this fish species in its habitat. Seven COI haplotypes were found in clade A from 8 individuals (Figure 6).



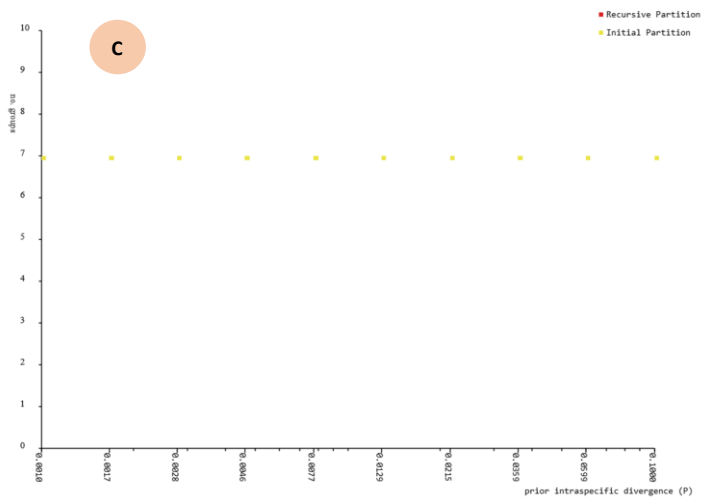


Figure 6. Automatic mudskippers' barcode gap discovery (Puillandre et al 2012). Distribution of K2P distances between each pair of specimens for the COI gene (a) distance histogram, (b) rank distance, and (c) number of PSHS obtained for each previous intraspecific divergence.

Within clade A, the level of divergence among haplotypes ranged from 1 to 20 bp, with seven polymorphic sites detected from the 611 bp sequence. Four of these seven sites were parsimony informative, and transition and transversion were observed. In addition, haplotype diversity and nucleotide diversity were 0.955%, respectively. Figure 6 shows the Automatic Barcode Gap Discovery (ABGD) method identified seven groups for mudskipper specimens with the initial approach. At the same time, the barcode gap threshold was calculated by analyzing the dataset of the partial COI gene sequence using Kimura 2 Parameter models. The value of the barcode gap distance was 0.03, resulting from the ABGD grouping which divided the species into seven groups (Figure 6C). Each was grouped according to its species without overlapping, as demonstrated in Figure 6.

Different haplotypes from the same location indicated that the selected individuals had a heteroplasmic type of mtDNA (Figure 7).

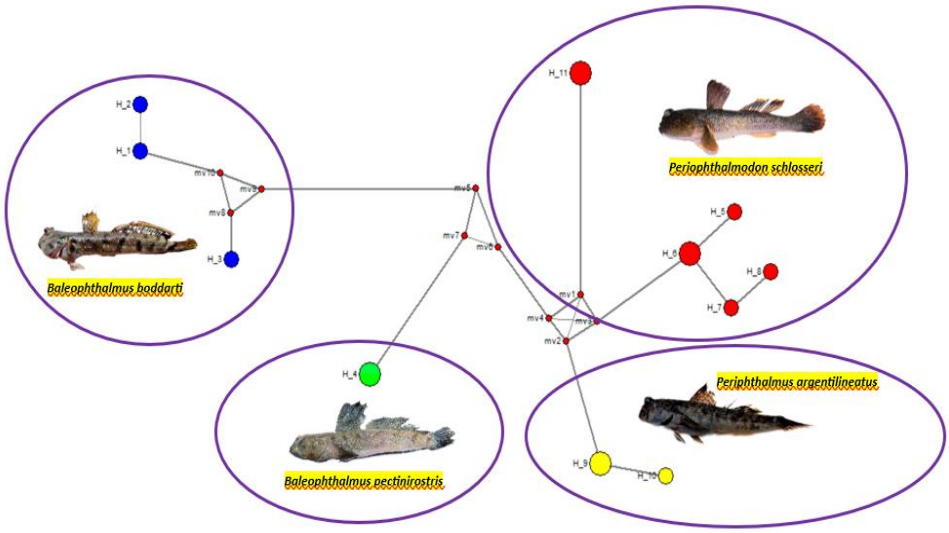


Figure 7. Haplotype network of mudskippers specimens from the North Coast of East Java, Indonesia.

Evolutionary relationships using the Median joining network (MJN) method were further analyzed between the haplotype groups:

Group [1] n:2; id: *B. boddarti voucher* BBC3-KY754676.1 and *B. boddarti* voucher BBC6-MF572076.1
Group [2] n:1; id: *B. boddarti* MS-OR592255;
Group [3] n:2; id: *P. schlosseri* voucher 2239-OR053778.1 and *P. schlosseri* voucher 2237-OR053779.1;
Group [4] n:5; id: *P. schlosseri* BJBR-OR592151, *P. schlosseri* K-OR592220, *P. schlosseri* MW-OR592231, *P. schlosseri* MS-OR592248 and *P. schlosseri* MM-OR592248;
Group [5] n:2; id: *P. argentilineatus* voucher -KU692746.1 and *P. argentilineatus* voucher BIF1391-KU692745.1;
Group [6] n:1; id: *P. argentilineatus*-MW;
Group [7] n:2; id: *B. pectinirostris* voucher CG40G24-KX223892.1 and *B. pectinirostris* MM-OR592259.

Discussion. A total of 8 samples from 5 locations on the North Coast of East Java, Indonesia, belonging to 4 species were successfully identified by morphological characteristics and molecular genetics using the COI gene. This study demonstrated the concordance of morphological identification with the genetic analysis results for about 94% of the investigated species from the North Coast of East Java. The results underlined the reliable species delimitation based on morphological diagnostic features presently applied for most species. However, the COI divergences revealed deep lineage splits within various nominal species. The COI gene partial sequence had been successfully amplified and validated using online database facilities. This amplification is consistent with the results reported by Muhala et al (2024) and Folmer et al (1994), where the developed primer generated partial COI gene sequences of 655 bp in length. The validation results showed that the eight samples were classified as *P. schlosseri*, *P. argentilineatus*, *B. boddarti*, and *B. pectinirostris*. In line with these findings, the sequences determined by Alcudia et al (2020) had a similarity >98%, while Febrianti et al (2023) reported a similarity of 95-100%. All the mudskippers studied are new records in North Java, and their COI barcodes can separate 100% of the four species.

The degree of gene or genome difference between species or populations is referred to as genetic distance (Roesma et al 2020). Based on the results, the samples' genetic distance was proportional between species. The closest values were also found between species, while the greatest values were observed between various classes. This distance demonstrates that the partial COI gene sequence has a high degree of accuracy in distinguishing between Indonesian mudsksippers. The sequence also provides a fast and effective DNA barcode technique for species identification, particularly when addressing morphological identification, which matches to the report of Kuang et al (2018). The variation between species within a genus was 0.024 for Bohadschia and 0.024 for Holothuria, in one class of Holothuroidea. The value obtained for Bohadschia was comparable with the 0.111±0.028 found by Kim et al (2017) and 0.1205 found by Uthicke et al (2010). The current results are in line with Ward et al (2008) and Uthicke et al (2010): the DNA sequences of individuals belonging to the same species have a sequence difference <0.02 or 2%. Finally, the phylogenetic analysis results showed that the samples were differentiated from each other based on relatedness. The divergence between Echinoderms group members had high probabilities, with bootstrapping values of ML=100, and NJ=99. Therefore, the segregation supported that the two clusters should be classified into separate groups, and these results were similar to the genetic distance analysis. No insertions/deletions or codon stops were observed after nucleotide translation, supporting that all amplified sequences denote functional mitochondrial COI sequences. The average length of the amplified sequences was larger than 611 bp, the limit typically observed for nuclear DNA sequences originating from mtDNA (NUMT) (Buhay 2009). All species could be distinguished based on individual DNA barcodes, indicating that this study confirmed the effectiveness of partial COI gene sequence for *Nemacheilus* spp. identification.

The ABGD analysis at a prior maximal distance of 0.955% also delineated the species into a separate partition, and these results supported the phylogenetic tree of Echinoderms in separated groups. Genetic distance, phylogenetics, and ABGD analysis

supported the identification process. In conclusion, the targeted use of DNA barcoding and morphological evidence was confirmed as efficient and reliable tools for identifying mudskipper species. This study becomes the first to report on the genetic identification and phylogenetic reconstruction of mudskippers using a partial COI gene sequence. Furthermore, there is a possibility for conservation management of mudskippers by grouping animal units according to species and genetic entity, as well as the potential of developing cryopreservation for sustainability. A molecular approach using DNA barcoding supports the identification results based on a morphological approach in Echinoderms, and an accession number has been obtained from the GenBank (NCBI) database. Approved and improved identified morphology and molecular characteristics of the samples were found on the Island. This study established a reliable DNA barcode reference library which could be used to assign Echinoderm species by screening sequences against it, in the future. The availability of such a library could help achieve better monitoring, conservation, and management of Echinoderms in Indonesia.

Conclusions. Based on this research, it can be concluded that the results of the BOLD system analysis show that four species and eight samples of mudskipper fish were found to have similarity values with *P. schlosseri*, *P. argentilineatus*, *B. boddarti,* and *B. pectinirostris*, namely in the range of 98.42–100%. Linkage analysis based on nucleotides showed a site frequency of 66.196%, site parsimony of 24.646%, nucleotide diversity of 0.995, and haplotypes 7 and 11 mutations at the first codon position. Based on the phylogenetic tree topology analysis of the research samples, the bootstrap value range for each OTU was between 98 and 100, which indicates that our samples have significant similarities or belong to the same species in one cluster.

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