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Research Article

Cryptic Diversity of Barred Mudskippers, *Periophthalmus argentilineatus* (Valenciennes, 1837), from the Southern Coast of Java and East Lombok, Indonesia inferred by *COI* Mitochondrial Gene

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ABSTRACT

The Barred Mudskipper (*P. argentilineatus*) is an amphibious fish species that displays fully terrestrial behaviour during low tides. Previous studies have indicated the existence of cryptic species of the barred mudskipper, leading to difficulties in taxonomic identification due to similarities in morphological characteristics. Therefore, this study aimed to generate DNA barcodes for Indonesian barred mudskipper populations. We collected ten specimens from Clungup Beach and Kondang Bandung Beach, representing our samples. Additionally, we incorporated 25 previously collected *COI* sequences from Indonesia into our analysis. The mitochondrial *COI* gene was amplified using PCR and analysed using various bioinformatic programs. This study provides evidence for the presence of three genetically distinct clades (A, B, and C) within the *P. argentilineatus* population in Indonesia, with a deep genetic divergence of 2.41% to 6.12%. Clade A showed a high genetic divergence of 5.51-6.12%, suggesting the presence of a cryptic species consistent with previous studies. The high level of haplotype diversity and low nucleotide diversity observed in each clade suggest a population bottleneck followed by a rapid expansion. The lack of geographical separation in the haplotype network analysis indicates that gene flow between populations may have been facilitated by glaciation events in the past. These findings contribute to a better understanding of the biodiversity of the barred mudskipper species in Indonesia and will aid in the accurate identification of cryptic species. This study highlights the importance of using molecular techniques to complement morphological identification in understanding the evolution and diversity of mudskipper fish species.

INTRODUCTION

Mudskipper is an amphibious fish that exhibits fully terrestrial behaviour during low tides (Polgar et al. 2017; Xinxin et al. 2018). This definition applies to genera: *Periophthalmus* Bloch & Schneider, 1801, *Boleophthalmus* Valenciennes, 1837, *Scartelaos* Swainson, 1839, *Periophthalmodon* Bleeker, 1874, and *Zappa* Murdy, 1989 (Murdy 1989; Polgar et al. 2010). These genera were classified into the subfamily Oxudercinae based on morpho-

logical, osteological, and eco-ethological characteristics (Murdy 1989). Several molecular studies, however, have indicated that Oxudercinae is paraphyletic relative to the gobiid of subfamily Amblyopinae (worm-eel gobies) and that both subfamilies are members of the '*Periophthalmus* lineage' of gobionelline-like gobies (Agorreta & Rüber 2012; Agorreta et al. 2013). As a result, both subfamilies previously included in the family Gobiidae underwent a major revision and both subfamilies are placed in a separate family, Oxudercidae (Nelson et al. 2016; Kuang et al. 2018; McCraney et al. 2020).

The genus *Periophthalmus* Bloch & Schneider, 1801 is widely recognized as the most diverse genus among mudskipper genera. In recent years, several new species within the genus *Periophthalmus* have been discovered, resulting in a total of 20 valid species (Murdy 1989; Murdy & Takita 1999; Darumas & Tantichodok 2002; Larson & Takita 2004; Jafaar & Larson 2008; Jaafar et al. 2016; Fricke et al. 2023). One such species is *Periophthalmus argentilineatus* (Valenciennes, 1837), commonly known as the barred mudskipper, which is distributed throughout a wide range of geographical regions, including the Red Sea and the east coast of Africa, and extending eastward to Southern Japan, Australasia, and Oceania, up to the Samoa Islands (Murdy 1989). In Indonesia, this fish species can be found on seven main islands, such as Sumatera, Java, Kalimantan, Sulawesi, Lesser Sunda, Moluccas, and Papua (Pormansyah et al. 2019).

Barred mudskippers generally inhabit mangrove swamps, tidal mudflats, and estuaries. This fish is a carnivorous and opportunistic feeder on various types of prey such as insects, crustaceans, fish eggs, and polychaetes worms (Kruitwagen et al. 2007). Barred mudskipper possesses two distinct dorsal fins and pelvic fins that are not interconnected or fused for less than 1/3 the length of the inner rays. Additionally, the presence of a pelvic frenum is either absent or only visible under magnification, as reported by Murdy (1989) and Polgar (2014). These fish are also widely recognized for their remarkable abilities to move, climb, and skip around in the water, a characteristic attributed to their powerful pectoral fins (Murdy 1989; Khaironizam & Norma-Rashid 2002; Kottelat 2013).

The molecular study conducted by Polgar et al. (2014) using both nuclear (*rag1*) and mitochondrial markers (*D-loop* and *16S* rRNA) suggested the existence of at least three distinct cryptic or pseudo-cryptic species in *P. argentilineatus*. Furthermore, according to the personal observation by Polgar (2014), these three cryptic species include one that is morphologically consistent with *P. sobrinus* Eggert (found in the Red Sea and South Africa), another that is consistent with *P. vulgaris* Eggert (observed from Sri Lanka to West Sumatra, the Sunda Islands, Sulawesi, the Philippines, the Moluccas, West Papua, and Northern Australia), and a third that consistent with *P. argentilineatus* Valenciennes (found from West Sumatra to the Sunda Islands, Southeast Borneo, and the Moluccas). Another study conducted by Aji and Arisuryanti (2021) using *COI* mitochondrial gene as a DNA barcoding marker also showed a suspected cryptic species of *P. argentilineatus* with a genetic divergence of 5.46-5.96% from Baros Beach, Special Region of Yogyakarta, Indonesia.

Due to the limitations of morphological identification methods of cryptic species, molecular genetic approaches for species identification have been used and developed in recent years. The primary purpose of DNA barcoding is to provide for the rapid identification of potentially unknown species, including cryptic species (Hebert et al. 2003a). DNA barcoding method can facilitate the discovery of cryptic species that are

still difficult to analyse using traditional approaches. The mitochondrial cytochrome c oxidase subunit I (*COI*) gene has been widely accepted as a reliable, universal animal species-level barcode for the vast majority of the animal kingdom (Hebert et al. 2003b). The *COI* gene has been used to detect and differentiate multiple cryptic species of fish such as the Swamp eel (Arisuryanti et al. 2016), Neotropical fish (Melo et al. 2016), Pearl cichlid (Souza et al. 2017), Cardinalfish, Pilot fish, Pacific rudderfish (Huo et al. 2017), and Pomfret (Li et al. 2019).

This study aimed to generate DNA barcodes for the barred mudskipper populations from Clungup and Kondang Bandung Beach in East Java, Indonesia, using the cytochrome c oxidase subunit I (*COI*) gene. We also aimed to investigate the genetic diversity and relationships among the barred mudskipper from these two populations and other regions in Indonesia, providing valuable insights into the evolutionary history and conservation status of this species.

MATERIALS AND METHODS

Sample Collection

Ten barred mudskipper fish specimens were collected from two different locations, Clungup Beach and Kondang Bandung Beach (Figure 1). The sampling of the barred mudskipper fish was obtained using a hand net. The collected samples were then thoroughly cleaned and documented. The documentation of the collected barred mudskipper fish specimens involved the use of a digital camera to photograph each sample. The digital photographs served to record essential details such as size and any notable external features. Each mudskipper fish sample was placed in a zip-lock bag, stored in a cool-box, and then transported to the Laboratory of Genetics and Breeding, Faculty of Biology, UGM. The samples were preserved in 99% ethanol and stored at -20°C for further analysis. To gain a more comprehensive analysis, we also included 25 previously registered *COI* sequences of the barred mudskipper from various locations in Indonesia. These locations included Bogowonto Lagoon (MT439598-MT439600) (Arisuryanti et al. 2018), Tekolok Estuary (MW514015-MW514024) (Rha'ifa et al. 2021), Baros Beach (MZ606679-MZ606687) (Aji & Arisuryanti 2021), and Pasir Mendit Beach (OQ804359-OQ804361) (Febrianti et al. 2023) (Table 1). The inclusion of these samples aimed to enhance the analysis of the genetic diversity and relationships among the barred mudskipper populations from different regions in Indonesia.

DNA Extraction

DNA extraction from fish tissue muscle (fillet) was performed using the Qiagen DNEasy Blood and Tissue kit (QIAGEN, Valencia, CA, USA). Each sample was mixed with 180 µL of ATL buffer in a 1.5 ml tube. The fillet was mechanically disrupted with ethanol-sterilized scissors and then added with 20 µL Proteinase K (600 mAU/mL). The sample was then thoroughly mixed and underwent an overnight incubation at 50°C, with periodic tube inversion during the initial three hours. After incubation, the samples were vortexed, and a solution consisting of 200 µL of AL buffer and 200 µL of cold absolute ethanol was added. After further mixing and centrifugation, the resulting mixture was processed through a spin column and successively washed with 500 µL of AW-1 buffer and 500 µL of AW-2 buffer. Following centrifugation, the spin column was transferred to a new microtube, subjected to a 2-minute incubation at 50° C, and eluted with 250 µL of AE buffer. The eluted DNA solution was then stored at -20°C for the following analysis.

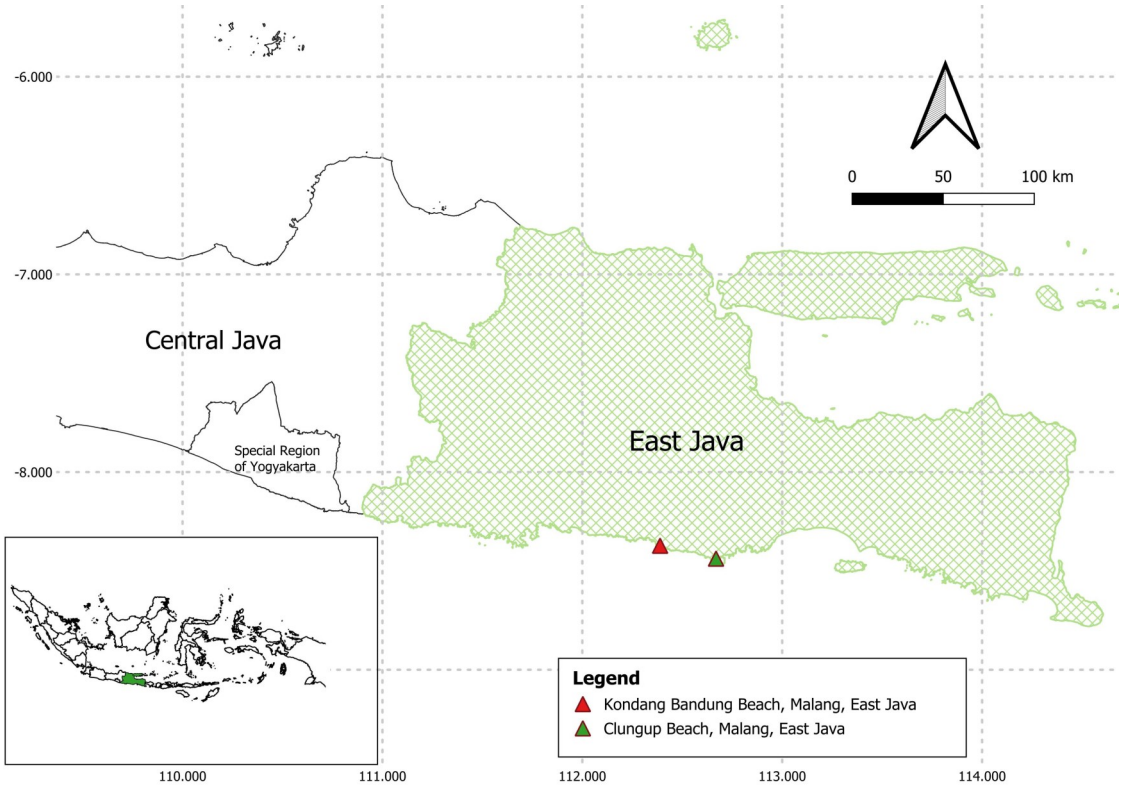

Figure 1. Sampling sites of barred mudskipper populations.

Table 1. Sample location, sample code, geographic reference, and sample size of Indonesian barred mudskippers used in this study.

Location	Sample Code	Latitude (S)	Longitude (E)	Sample Size (N)	References
Kondang Bandung Beach, East Java	MSK	8°22'20.0"	112°23'19.5"	4	This Study
Clungup Beach, East Java	MSC	8°26'15.1"	112°40'07.0"	6	This Study
Pasir Mendit Beach, Yogyakarta	MSP	7°53'39.6"	110°01'10.1"	3	Febrianti et al. 2023
Bogowonto Lagoon, Yogyakarta	MSB	7°53'58.1"	110°01'54.2"	3	Arisuryanti et al. 2018
Baros Beach, Yogyakarta	MBR	8°00'27.4"	110°17'02.2"	9	Aji & Arisuryanti 2021
Tekolok Estuary, West Nusa Tenggara	MSL	8°20'30.0"	116°42'31.0"	10	Rha'ifa et al. 2021

Barcode Marker Amplification

The *COI* mitochondrial gene was amplified in the T100 Thermal-Cycler (Biorad) using two universal primers for fish, FishF2 (5'-TCGACTAATCATAAAGATATCGGCAC-3') and FishR2 (5'-ACTTCAGGGTGACCGAAGAATCAGAA-3') (Ward et al. 2005). The PCR reaction was carried out in a 25 µL reaction volume containing 5-50 ng of genomic DNA, 12.5 µL MyTaq HS Red Mix (Bioline), 1 mM $MgCl_2$, 0.6 µM of forward primer and 0.6 µM of reverse primer, and 5.5µL double distilled water (ddH_2O), and 3 µL DNA template. Negative control was established by omitting the template DNA from the reaction mixture to assess the effectiveness of the DNA amplification. The PCR thermal profile followed Arisuryanti et al. (2020) and consisted of: 2 min of pre-denaturation at 95°C, followed by 35 cycles of denaturation at 95° C for 15 sec, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec, with a final extension of 5 min at 72°C.

Electrophoresis and Sanger Sequencing

The electrophoresis of PCR products was performed on a 1% agarose gel stained with Florosafe (Bioline) and buffered with Tris-acetate EDTA (TAE) at 100 volts for 25 minutes. The gel was visualized under ultraviolet light. All amplification products were then transported to LPPT UGM for purification and sequencing. Sanger sequencing reactions were performed on each specimen using both forward and reverse primers.

Sequence Editing & Verification

The DNA sequencing results were edited in GeneStudio and verified using the DNASTAR program with SeqMan and EditSeq (DNASTAR Inc. Madison, USA). The consensus sequencing results were examined using the Nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Identification Engine in BOLD (https://www.boldsystems.org/index.php/IDS_OpenIdEngine). The species verification of barred mudskipper was determined by achieving a high BLAST identity percentage paired with the E-value 0.0 and integrating similarity results from the BOLD database to ensure a comprehensive similarity assessment.

Sequence Alignment, Genetic Diversity & Genetic Distance

The *COI* sequence alignment of the barred mudskipper was completed using Opal on Mesquite v.3.51 program (Maddison & Maddison 2018) and ClustalW on the MEGAX program (Kumar et al. 2018). The DnaSP 6.12.01 program was used to calculate genetic diversity (Rozas et al. 2017). The divergences among the major clades were calculated using MEGAX program (Kumar et al. 2018) under the Kimura-2 Parameter (K2P) model.

Phylogenetic Relationship

The phylogenetic tree was constructed using the Neighbor-Joining and Maximum Likelihood methods with Kimura-2 Parameter (K2P) substitution model and 1000 bootstraps replications in MEGAX program (Kumar et al. 2018) and Bayesian Inference using the BEAST program (Suchard et al. 2018). The Akkaike Information Criterion (AIC) implemented in jModelTest 2.1.10 (Darriba et al. 2012) was used to determine the best fit evolutionary model. The most suitable sequence substitution model for this research is HKY with gamma (HKY + G) on the Akaike Information Criterion (AIC). To estimate the posterior probability distribution, the Markov Chain Monte Carlo (MCMC) method was used for 10 million generations with a sampling frequency of every 1,000 generations. The consensus trees were then visualized in FigTree 1.4.4 (Rambaut 2019).

Haplotype Network & Principal Coordinate Analysis

The haplotype network was generated using PopART v1.7. (Leigh & Bryant 2015) and the Principal Coordinate Analysis (PCoA) was carried out in GenAlEx 6.5 (Peakall & Smouse 2012).

RESULTS

Sequence alignment

A fragment length of 579 bp was successfully generated from the *COI* sequence of this research. The length of the *COI* sequences remained at 579 bp even after adding two additional sequences i.e. *P. novemradiatus* (KU692765) and *B. boddarti* (KU692378) as the outgroups of the phylogenetic tree. The *COI* sequences of *P. argentilineatus* from Clungup Beach

have been deposited in GenBank under accession number PP593608-PP593613 whereas the *COI* sequences of *P. argentilineatus* from Kondang Bandung Beach have been deposited in GenBank under accession number PP593621-PP593624.

Nucleotide Composition

The nucleotide composition of six populations of the barred mudskipper was found to be different, except for the populations found at Pasir Mendit Beach and Bogowonto Lagoon, which had the same composition of C and A (as shown in Table 2). There were small variations in the percentages of T, C, A, and G nucleotides among the populations, with differences ranging from 0.60% to 0.26%. Additionally, the total composition of A and T was found to be greater than the composition of G and C. The GC content, or the proportion of guanine and cytosine, varied slightly among all populations, ranging from 42.31% to 42.70%.

Table 2. Mean of nucleotide composition (%) of *COI* mitochondrial gene among six populations of Indonesian barred mudskippers in this study.

Population	T	C	A	G	A+T	G+C
Pasir Mendit (MSP)	32.47	25.79	25.22	16.52	57.69	42.31
Bogowonto (MSB)	32.41	25.79	25.22	16.58	57.63	42.37
Baros (MBR)	31.95	26.25	25.41	16.39	57.36	42.64
Kondang Bandung (MSK)	31.87	26.38	25.43	16.32	57.30	42.70
Clungup (MSC)	32.30	26.05	25.22	16.44	57.51	42.49
Tekolok (MSL)	32.38	25.98	25.09	16.55	57.48	42.52

Phylogenetic Tree and Genetic Distance

A total of 37 sequences were used in the phylogenetic analysis, including 2 samples from GenBank as outgroups: *P. novemradiatus* (KU692765) *and B. boddarti* (KU692378). The phylogenetic tree topology of the NJ, ML, and BI trees was found to be identical, therefore only the BI tree that was presented in this study (Figure 2). The phylogenetic tree showed three distinct clades, Clade A (n=18), Clade B (n=9), and Clade C (n=8). The lowest genetic distance was 2.41% between clade B and C, while the highest was 6.12% between clade A and C (Table 3).

Table 3. Mean percentage nucleotide sequence divergence of a 579 bp fragment of the *COI* mitochondrial gene among Indonesian barred mudskippers in this study.

	Clade A	Clade B	Clade C
Clade A			
Clade B	5.51 (5.24-6.00)		
Clade C	6.12 (5,62-6,97)	2.41 (1.76-2.84)	

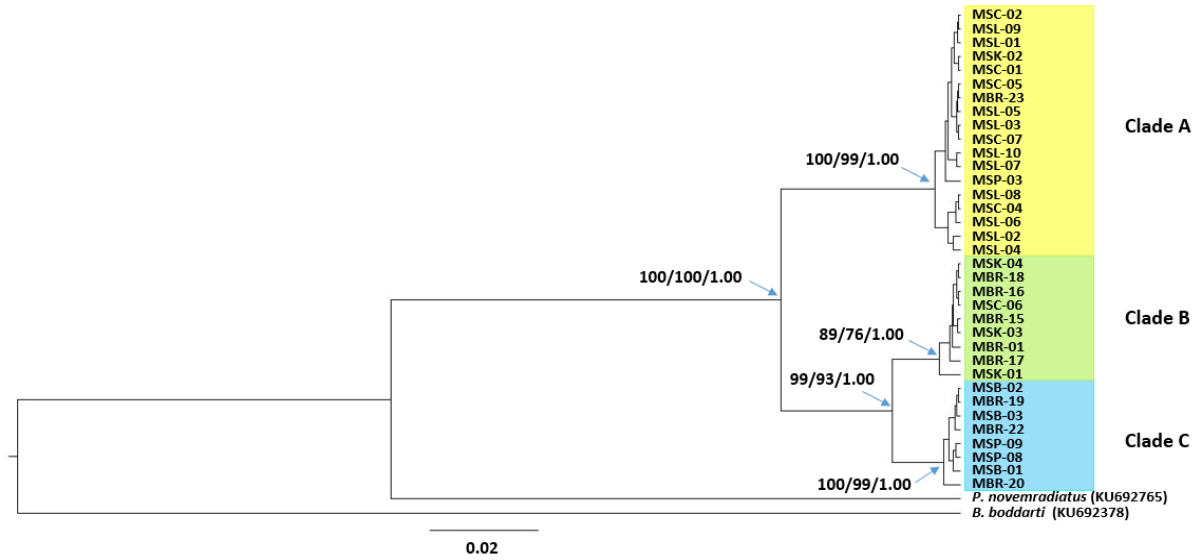

Figure 2. Phylogenetic tree of Indonesian barred mudskippers (*P. argentilineatus*) and outgroup inferred from *COI* mitochondrial gene sequences (579 bp) based on Neighbour-Joining (NJ), Maximum-Likelihood (ML), Bayesian Inference (BI) topology. The number of each node represents bootstraps for NJ and ML and posterior probabilities for Bayesian Inference.

Genetic diversity of Indonesian barred mudskippers based on the *COI* gene

The thirty-five barred mudskipper *COI* sequences revealed 49 variable sites, 40 parsimony informative sites, and 18 haplotypes. No insertions, deletions, or stop codons in the sequences were detected. The transitional pairs (si= 43) were more frequent than trans-versional pairs (sv= 8). The overall mean of haplotype diversity (Hd) was 0.884±0.043 and nucleotide diversity (π) was 0.03223±0.00158. The highest haplotype diversity was found in Clade C (0.884±0.043), while the lowest was found in Clade A (0.634±0.127). For the nucleotide diversity, the highest was detected in Clade A (0.00293±0.00073), whereas the lowest was in Clade B (0.00192±0.00063) (Table 4). The polymorphic sites were visualized in three distinct colours, representing three different clades: Clade A (green), Clade B (yellow), and Clade C (blue) (Table 5-6). A unique polymorphic site, number 297, was detected with different nucleotides in each clade: Clade A (nucleotide C), Clade B (nucleotide A), and Clade C (nucleotide G).

Haplotype Network and Principal Coordinate Analysis (PCoA)

Both the haplotype network and PCoA (Figure 3 & 4) revealed three distinct clusters and haplogroups representing three different clades, but there was a lack of clear separation by geographical region, indicating overlap among the three genetically divergent lineages. Haplogroup A consisted of 7 haplotypes (HA1-HA7), Haplogroup B consisted of 5 haplotypes (HB1-HB5), and Haplogroup C consisted of 6 haplotypes (HC1-HC6). Haplogroup A and B were separated by 27 mutation points, while Haplogroup B and C were separated by 10 mutation points. The most widespread haplotype, HA1 (n = 11), was shared among 4 populations (Tekolok Estuary, Baros Beach, Bogowonto Lagoon, and Clungup Beach).

DISCUSSION

In this study, we present evidence of the existence of three genetically distinct monophyletic clades (A, B, and C) within the *P. argentilineatus* species from six different populations in Indonesia. Using maximum sup-

Table 4. The genetic diversity of barred mudskippers based on the *COI* mitochondrial gene.

Sample	Number of Samples	Haplotype Number	Haplotype Diversity	Nucleotide Diversity
Clade A	18	7	0.634±0.127	0.00293±0.00073
Clade B	9	5	0.722±0.159	0.00192±0.00063
Clade C	8	6	0.893±0.111	0.00271±0.00052
All Populations	35	18	0.884±0.043	0.03223±0.00158

Table 5. Polymorphic sites of Indonesian barred mudskippers inferred from *COI* gene (site 06 to 297).

	Nucleotide sites number	06	19	26	34	54	63	66	87	93	111	114	147	180	219	222	228	234	237	246	252	261	279	288	297
CLADE A	MSC-01	G	C	G	T	T	C	T	A	C	C	A	T	A	C	G	T	T	T	T	A	C	G	A	C
	MSC-02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSC-04	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	MSC-05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSC-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSK-02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-01	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-02	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-04	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-06	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	MSL-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	MSL-09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-10	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MBR-23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSP-03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CLADE B	MSK-01	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	A
	MSK-03	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MSK-04	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MBR-01	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MBR-15	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MBR-16	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MBR-17	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MBR-18	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MSC-06	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
CLADE C	MSB-01	A	.	T	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MSB-02	A	.	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MSB-03	A	G	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MSP-08	A	.	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MSP-09	A	.	.	.	C	T	C	.	T	T	.	C	G	T	A	C	C	A	.	G	T	A	C	.	G
	MBR-19	A	.	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MBR-20	A	.	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MBR-22	A	.	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G

port from bootstraps (100% in NJ/ML) and posterior probability (1.00 in BI), we were able to construct an accurate phylogenetic tree. Our analysis revealed a deep genetic divergence of 2.41-6.12% among these clades, with Clades B and C being considered conspecific based on the intraspecies genetic distance threshold of 3.5% proposed by Zemlak et al. (2009). Clade A, however, was found to have a genetic divergence of 5.51-6.12%, suggesting the presence of a cryptic species. This finding is consistent with previous genetic studies of *P. argentilineatus* from East African and Indo-Malayan populations using nuclear markers (*rag1*) and mitochondrial markers (*D-loop* and *16S* rRNA) which revealed the existence of at

Table 6. Polymorphic sites of Indonesian barred mudskippers inferred from the *COI* gene (site 333 to 573).

	Nucleotide sites number	333	335	336	337	339	399	432	438	441	450	453	456	457	478	489	498	513	516	517	528	531	543	561	573
C	MSC-01	A	T	T	C	A	T	T	C	T	C	A	C	C	C	T	G	C	A	T	T	T	T	T	T
L	MSC-02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A	MSC-04	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.
D	MSC-05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E	MSC-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSK-02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A	MSL-01	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-02	.	.	C	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.
	MSL-03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-04	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.
	MSL-05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-06	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	.	A	.	.	.	.	.	.	.
	MSL-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-08	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.
	MSL-09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MBR-23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSP-03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.
C	MSK-01	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
L	MSK-03	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
A	MSK-04	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
D	MBR-01	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	.	.	.	C
E	MBR-15	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	G	C	.	.	C
	MBR-16	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
B	MBR-17	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	C	C
	MBR-18	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
	MSC-06	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
C	MSB-01	G	C	.	T	G	.	C	T	.	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
L	MSB-02	G	C	.	T	G	.	C	T	C	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
A	MSB-03	G	C	.	T	G	.	C	T	C	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
D	MSP-08	G	C	.	T	G	.	C	T	.	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
E	MSP-09	G	C	.	T	G	.	C	T	.	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
	MBR-19	G	C	.	T	G	.	C	T	C	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
C	MBR-20	G	.	.	T	G	.	C	T	.	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
	MBR-22	G	C	.	T	G	.	C	T	C	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C

least three molecularly distinct cryptic or pseudo-cryptic species (Polgar et al. 2014). Furthermore, Polgar (2014) proposed that one of these cryptic species is morphologically consistent with *P. sobrinus* Eggert, one is morphologically consistent with *P. vulgaris* Eggert, and one is morphologically consistent with *P. argentilineatus* Valenciennes. Our discovery highlights the occurrence of cryptic species with preserved morphologies in the genus *Periophthalmus*, a genus with a long evolutionary history of more than 30 million years under strong stabilizing selection in *Periophthalmus* habitats (Polgar et al. 2014). Additionally, the use of a different molecular marker in this study (*COI*) compared to previous studies by Polgar et al. (2014) using *rag1*, *D-loop*, and *16S* markers and personal observation of Polgar (2014) yielded the same conclusion, further demonstrating the robustness of our findings. This highlights the importance of using multiple markers and approaches in species delimitation for a more comprehensive understanding of the diversity within a genus.

We discovered an overall high level of haplotype diversity (Hd ≥ 0.5) and nucleotide diversity ($\pi \geq 0.01$) in six different populations of Indonesian barred mudskipper. In contrast, each clade of A, B, and C sepa-

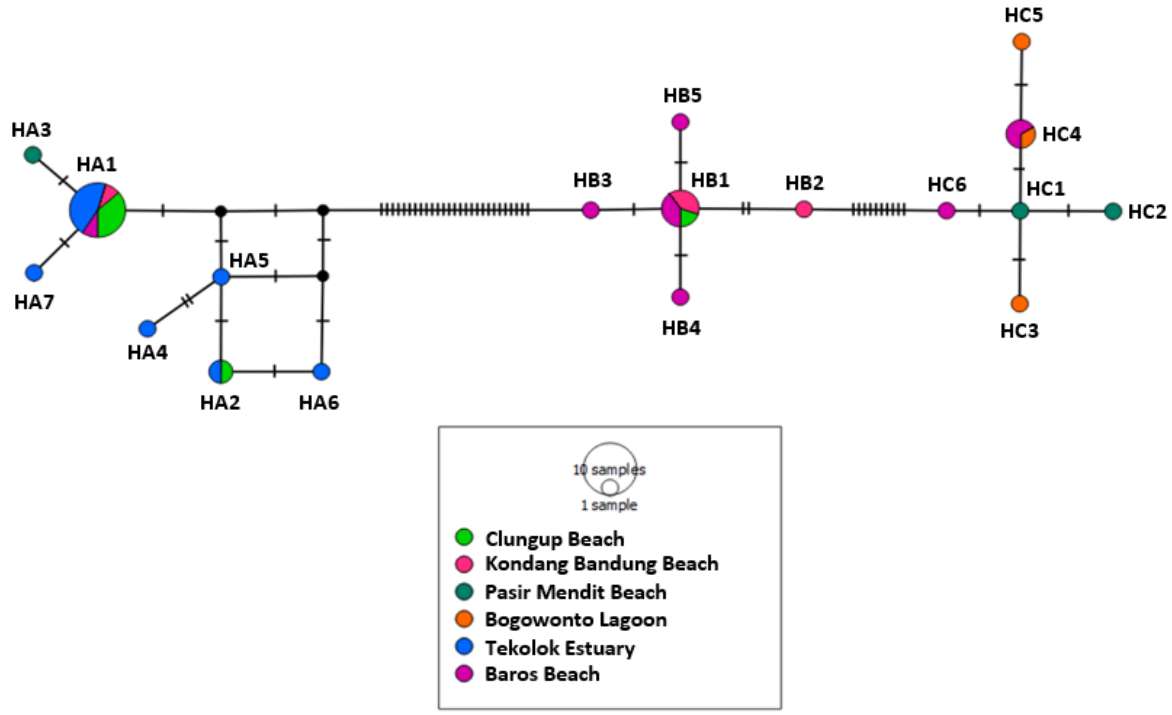

Figure 3. Median-joining haplotype network of Indonesian barred mudskippers based on *COI* mitochondrial gene sequences (579 bp). The size of the circles reflects the number of samples. Lines connecting haplotypes show evolutionary routes between haplotypes, whereas short stripes represent mutation points between haplotypes. Each colour represents six different populations of barred mudskipper in this study.

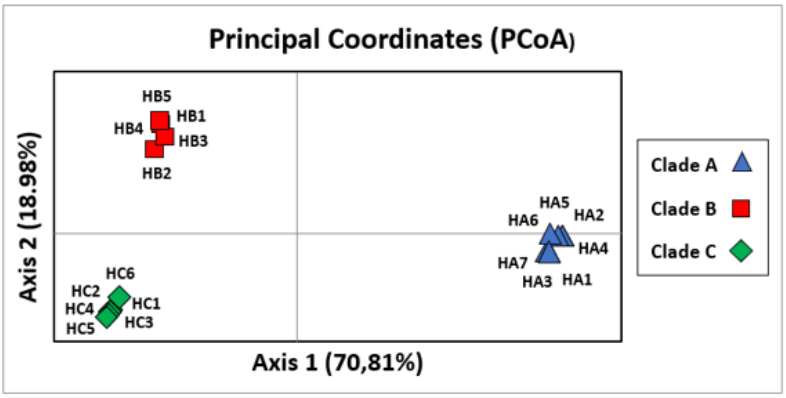

Figure 4. Principal Coordinate of Analysis (PCoA) of Indonesian barred mudskippers based on *COI* mitochondrial gene sequences (579 bp).

rately showed a high level of haplotype diversity (Hd ≥ 0.5) and low nucleotide diversity ($\pi \leq 0.01$), which is consistent with the pattern of a population bottleneck followed by a rapid expansion (Grant & Bowen 1998) and may reflect the evolutionary history of the barred mudskipper. The ratio of transitions to transversions in this study is similar to that observed in other mtDNA studies of teleost fish (Bingpeng et al. 2018; Wu et al. 2018), which suggests that the molecular evolution of the *COI* gene in barred mudskippers is consistent with that of other fish. The T>C>A>G nucleotide composition pattern is also consistent with other fish families that have been studied using the *COI* gene as a DNA barcoding marker (Bingpeng et al. 2018; Wu et al. 2018; Linh et al. 2019). An interesting finding of our study was the identification of a unique polymorphic site (number 297) in the *COI* gene, which could serve as a genetic marker for each barred mudskipper clade.

The haplotype network analysis revealed a lack of clear separation by geographical region, indicating an overlap of the three genetically divergent lineages. Haplotype HA1, included in haplogroup A, was the most widespread haplotype (n = 11) and was shared among four populations (Tekolok Estuary, Baros Beach, Bogowonto Lagoon, and Clungup Beach). These four populations are relatively far apart, with the greatest distance between Bogowonto Lagoon (Java) and Tekolok Estuary (Lombok, Lesser Sunda) being over 700 km, which implies that there should be little or no gene flow between these populations. Based on this result, we hypothesize that in the past, there was a spatial linkage that connected all of these populations. There are many possibilities that could be influenced, for example by historical events, such as sea level or ocean currents, which facilitate the dispersal and gene flow between distant populations (Miller et al. 2005; García-De León et al. 2018). Additionally, the Sunda Shelf, which covers a large area of western Indonesia, may have played a crucial role in this process. This shallow marine shelf has been known to have experienced significant changes in sea level in the past and may provide a suitable habitat for the mudskippers. The amphibious nature of the mudskipper populations would have allowed them to migrate and establish themselves on both islands during periods of low sea level. Once sea levels rose again, the connection was lost, but the mudskipper population had already spread and established itself on both islands, leading to the shared genetic haplotype observed in our study. This hypothesis has been proposed by multiple studies to explain the gene flow between the mainland and the Sunda Islands of various fish populations (Dodson et al. 1995; Nelson et al. 2000; McConnell 2002). Further studies, such as population genetic modelling and historical demographic analysis, are needed to reveal this hypothesis and to gain a deeper understanding of the evolutionary history of the Indonesian barred mudskipper.

CONCLUSIONS

This study provides evidence for the presence of three genetically distinct clades (A, B, and C) of barred mudskipper populations in Indonesia, with a deep genetic divergence of 2.41% to 6.12% among the clades. This genetic divergence further suggests the presence of cryptic species within the genus *Periophthalmus*, which is consistent with previous studies on the genus. The high level of haplotype diversity and low nucleotide diversity observed in each clade is indicative of a population bottleneck followed by a rapid expansion. Furthermore, the lack of clear separation by geographical region in the haplotype network analysis suggests that historical events, such as sea level changes or oceanic currents, may have facilitated gene flow between distant populations in the past. These findings highlight the importance of using molecular approaches in species delimitation and the need for further studies to gain a deeper understanding of the evolutionary history of the Indonesian barred mudskipper.

AUTHOR CONTRIBUTION

The laboratory work for this study was conducted by a team of researchers including KWA, HH, IPS, FAR, and DF. They were responsible for collecting samples, extracting DNA, amplifying it using PCR, analysing the results using agarose gel electrophoresis, and writing the manuscript. The overall design and planning of the study, and writing the manucript were led by TA and DSP, who also supervised the entire process.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest. The authors are responsible for the article's content and writing.

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