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Cryptic diversity of mudskipper genus *Boleophthalmus* (Gobiiformes: Oxudercidae) from the north coast of East Java, Indonesia

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Abstract. *Shabrina FN, Wibowo K, Arisuryanti T. 2024. Cryptic diversity of mudskipper genus* Boleophthalmus *(Gobiiformes: Oxudercidae) from the north coast of East Java, Indonesia. Biodiversitas 25: 412-420.* Mudskippers of genus *Boleophthalmus* are commonly found in the Indo-Pacific area. This study aimed to examine the diversity of the genus *Boleophthalmus* from the north coast of East Java Province, Indonesia by employing the mitochondrial COI gene for DNA barcoding and morphological characterization. Ten individuals of this genus were analyzed. According to the similarity percentage evaluated using Basic Local Alignment Search Tool (BLAST) and Barcode of Life Data (BOLD) identification engines, all the individuals were identified as *Boleophthalmus boddarti* Pallas, 1770. However, phylogenetic analysis revealed that three of the individuals were identified as *B. boddarti*, and seven were identified as *Boleophthalmus pectinirostris* Linnaeus, 1758 originated from East Java, which is separated from the clade of *B. pectinirostris* from East Asia population. These results were supported by the genetic distance of 8.93%-13.81% and the morphological characteristics determined by principal component analysis (PCA). PCA revealed that *B. boddarti* and *B. pectinirostris* are primarily distinguished by the number of (longitudinal) scales (69-75 versus 113-123), length of second dorsal fin base (42.3-43.9 versus 38.1-49.0), and caudal fin length (18.1-22.4 versus 17.5-21.6). Further detailed studies are needed to clarify whether the seven individuals belonging to *B. pectinirostris* complex should be regarded as a new species or a new subspecies.

Key words: *Boleophthalmus boddarti*, *Boleophthalmus pectinirostris* complex, COI, DNA barcoding, morphology, mudskipper

INTRODUCTION

Boleophthalmus Valenciennes, 1837 is a taxonomic genus of amphibious gobies, commonly referred to as mudskippers. This genus belong to Gobiiformes order and Oxudercidae family (Polgar et al. 2013; McCraney et al. 2020) and is characterized by the presence of a unique rectangular cartilage anterior to the pelvic spines and the extremely thickened skin on the cranium and nape. It consists of six species, namely, *Boleophthalmus boddarti* Pallas, 1770; *Boleophthalmus caeruleomaculatus* McCulloch and Waite, 1918; *Boleophthalmus dussumieri* Valenciennes, 1837; *Boleophthalmus birdsongi* Murdy, 1989; *Boleophthalmus pectinirostris* Linnaeus, 1758; and *Boleophthalmus poti* Polgar, Jaafar and Konstantinidis, 2013 (Murdy 1989; Polgar et al. 2013). Mudskipper fish are frequently observed in soft-bottom ecosystems, such as mudflats and mangroves, and they are an essential species for this habitat (Ghanbarifardi et al. 2016). *Boleophthalmus* is among the three most terrestrialized genera in the Oxudercidae family, owing to its epithelial, locomotory, vertebral, and appendicular specializations that facilitate transcontinental excursions (Steppan et al. 2022). Adult individuals of all species of *Boleophthalmus* are commonly seen inhabiting open non-vegetated regions within the lower intertidal zones. *Boleophthalmus* is widely distributed in tropical and temperate intertidal environments within The Persian Gulf, The coast of Mozambique, Southern Japan in the north, Indo-Pacific, and Northern Australia in the south (Muhala et al. 2023; Polgar et al. 2013). Oxudercidae comprises many species that are difficult to distinguish from each other due to their morphological similarities (Febrianti et al. 2023). In Indonesian waters, this genus is represented by two species, *B. boddarti* and *B. pectinirostris* (Polgar et al. 2013; Dahruddin et al. 2016).

Previous studies showed separation in the phylogenetic tree and genetic distance indicating there were two distinct groups of *B. dussumieri*. The first group was originated from Iran, and the second group was originated from Mozambique and India (Muhala et al. 2023). Morphological assessments also suggested that populations of the great blue spotted mudskipper *B. pectinirostris* from East Asia and the Malacca Strait, Malaysia are of the same species (Murdy 1989; Polgar et al. 2013). However, genetic analysis indicated that the Malaysian populations are likely a distinct species separate from *B. pectinirostris* found in East Asia (Chen et al. 2014). The lack of exact identification of cryptic species can give rise to taxonomic challenges and affect conservation efforts that are aimed at protecting these elusive species. One potential conservation strategy involves the utilization of genomic analysis to assess the genetic diversity within cryptic species (Delić et al. 2017). DNA barcoding analysis reveals the hidden differences between them, allowing for the accurate identification of these species despite the difficulty in

morphologically distinguishing them.

DNA barcoding is a rapid and efficient technique that analyzes selected short-target genes to properly identify the species that are difficult to describe by traditional morphology (Hebert et al. 2003). COI mitochondrial gene is extensively used to identify fish (Ward et al. 2005; Polgar et al. 2017; Abdulmalik-Labe et al. 2022). The selection of the COI depends on its DNA-level variation patterns and the comparatively straightforwardness of sequence retrieval. It has been demonstrated that the region in animals is adequately conserved among species, but also sufficiently variable between species to permit dependable taxonomic identification (Bingpeng et al. 2018; Pentinsaari et al. 2016). In Indonesia, DNA barcoding with COI mitochondrial gene has been used to identify freshwater and marine fish species (Dahruddin et al. 2016; Fadli et al. 2020; Aji and Arisuryanti 2021; Rhaifa et al. 2021;). A hybrid technique of genetic and morphological characteristics has been widely employed and verified for the taxonomic categorization of various fish species, with special emphasis on Gobiiformes (Islam et al. 2021; Kovačić et al. 2022; Thacker et al. 2022).

East Java is a province in Indonesia that has a significant number of mangrove habitats located in its northern region. The wide mangrove area in East Java Province is located in the Brantas River Delta, specifically in Gresik, Surabaya, Sidoarjo, Pasuruan, and Probolinggo (Hakim et al. 2017). Mangrove areas with high productivity serve as feeding and breeding grounds for various species of fish (Hasan et al. 2023). This study aimed to identify 10 specimens of the mudskipper genus *Boleophthalmus* collected from the mangrove ecosystems in the north coast of East Java Province, Indonesia by conducting molecular and morphology analyses.

MATERIALS AND METHODS

Specimen collection

Ten mudskipper specimens of *Boleophthalmus* genus were collected by local fishermen using large fishing nets from three mangrove ecosystems (Table 1) in the North Coast of East Java Province, Indonesia (Figure 1). Muscle tissues of about 50 mg were obtained from the right side of the body and preserved in 1.5 mL tubes with 99% pure ethanol. Fish and muscle tissues were labeled and frozen at -20°C for DNA analysis and morphological characterization at the Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Table 1. Coordinates and description of sampling location

Point	Sites	Coordinates	Site description
1.	Wonorejo Mangrove Area, Surabaya	7°18'27.65"S, 112°49'57.31"E	Mangrove area near the estuary
2.	Panteguran Mangrove Area, Pasuruan	7°42'12.91"S, 113° 5'32.31"E	Mangrove area near the estuary
3.	Bahak Indah Beach, Probolinggo City	7°44'10.62"S, 113°13'25.88"E	Muddy beach with mangrove vegetation

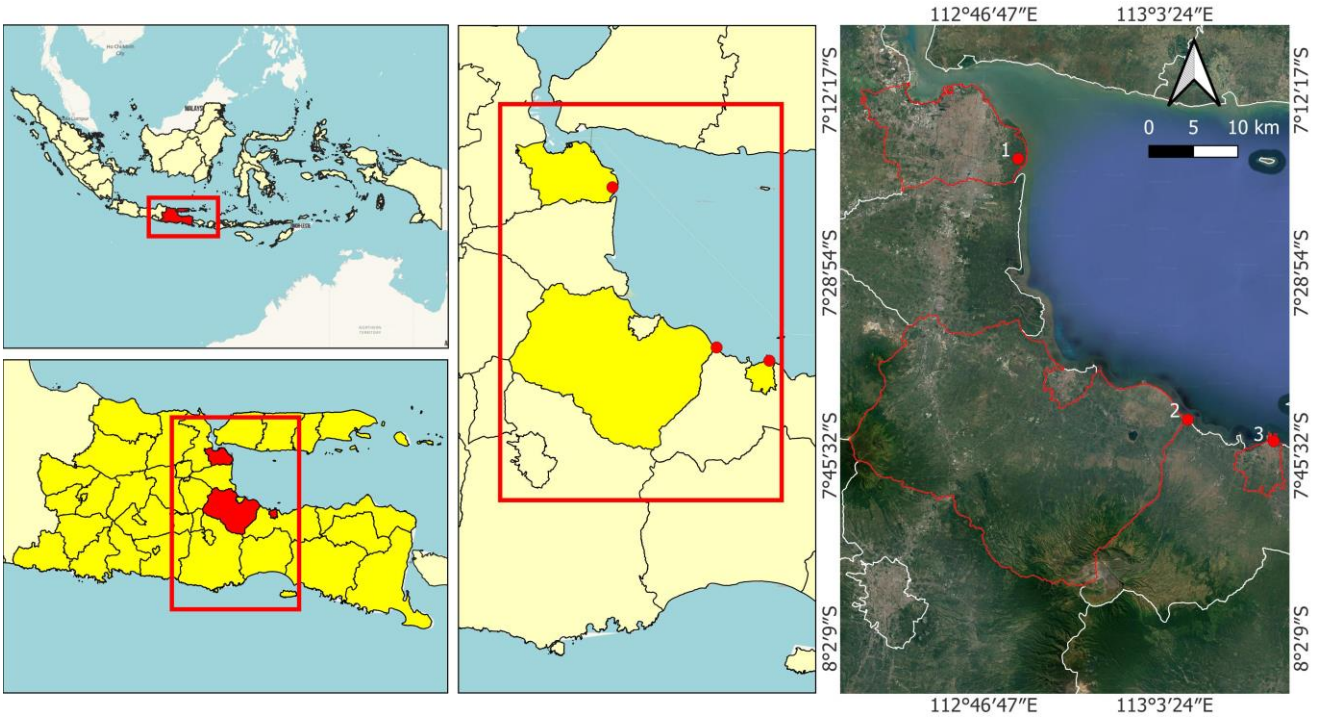

Figure 1. Map showing the sampling sites of mudskippers genus *Boleophthalmus* in the North Coast of East Java Province, Indonesia. Point 1: Surabaya, point 2: Pasuruan , Point 3: Probolinggo City

DNA isolation, DNA amplification and electrophoresis

DNA was isolated from the fish with Qiagen DNEasy Blood and Tissue kit following the manufacturer's instructions. The isolated COI fragment was amplified using a T100 Thermal Cycler (Biorad) Polymerase chain reaction (PCR) machine. Primer barcodes FishF2 and FishR2 (5′-TCGACTAATCATAAAGATATCGGCAC-3′ and 5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′) were used for this study (Ward et al. 2005). Polymerase chain reaction (PCR) was performed in a total volume of 25 µL, consisting of 5 µL isolated DNA, 12.5 µL of My Taq HS Red Mix PCR, 1 µL of $MgCl_2$, 1.5 µL of the two sets of COI primers, and 5.5 µL of ddH_2O. The PCR machine was programmed as follows: predenaturation at 95°C for 1 minute; 35 cycles of primer annealing at 50°C for 30 seconds and elongation at 72°C for 30 seconds; and a final elongation at 72°C for 1 minute before the temperature was held at 4°C (Arisuryanti et al. 2020). The PCR products were then electrophoresed for 20 minutes at 50 volts on a 1% dyed agarose gel with FluoroSafe and Tris-acetate EDTA buffer. The electrophoresed gels were placed on a UV transilluminator and gel documentation system to observe the DNA bands. The amplified samples were then sent to the UGM LPPT service for bidirectional COI gene sequencing with the ABI 3730XL Genetic Analyzer.

Morphological observation

Morphometric and meristic characterization was performed following Murdy (1989) and Polgar et al. (2013). Seventeen morphometric and 10 meristic characteristics were examined: standard length (SL), total length (TL), head length (HL), head depth (HD), body depths (BD1 & BD2), predorsal length (PREDL), length of first dorsal fin base (DF1BL), length of second dorsal fin base (DF2 BL), gap between first dorsal fin & second dorsal fin (Gap D1-D2), distance between end of second dorsal fin to origin of caudal fin (DF2-CF), anal fin base length (AFBL), caudal peduncle length (CPL), caudal peduncle depth (CPD), caudal fin length (CFL), pectoral fin base height (PcBH), pectoral fin length (PcFL), pelvic fin length (PvFL), number of first dorsal fin rays (DF1R); number of second dorsal fin rays (DF2R); number of anal fin rays (AFR); number of pectoral fin rays (PFR); number of segmented caudal fin rays (CFR), longitudinal scales (LS), transverse scales from origin of second dorsal fin ventroposteriorly (TRDB), transverse scales from anal fin origin dorsoposteriorly (TRB), transverse scales from anal fin origin dorsoanteriorly (TRF), and predorsal scales (PRED). All measurements were obtained using dial calipers in straight-line distances to the nearest tenth of a millimeter. The measurements were expressed as percentages relative to the standard length. All specimens examined in this study were deposited in Museum Biologi Yogyakarta (MBY), Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Data analysis

The sequencing results were processed using GeneStudio program and then verified using the DNASTAR program. The consensus sequence was analyzed using the Identification Engine tool available on the Barcode of Life Data Systems (BOLD) website and Basic Local Alignment Search Tool (BLAST) program to determine the percentage of identity. Sequence data were aligned using Opal by Mesquite v.3.51 (Maddison and Maddison 2018) and ClustalW by MEGA X software (Kumar et al. 2018). Genetic distance calculation and phylogenetic tree reconstruction were conducted with the MEGA X tool. Kimura 2 Parameter (K2P) model was used in genetic distance calculation (Hebert et al. 2003). The phylogenetic tree was reconstructed using three different methods: neighbor joining and maximum likelihood, both with 1,000 bootstrap repeats (Kumar et al. 2018), and Bayesian inference implemented through the BEAST program (Suchard et al. 2018). The substitution model on Bayesian tree reconstruction was GTR + G based on the Bayesian Information Criterion. In this analysis, 12 sample sequences of *B. pectinirostris* and 7 sample sequences of *B. boddarti* from BOLD and GenBank were obtained for comparison. Two sample sequences of mudskippers *Periophthalmus argentilineatus* Valenciennes, 1837 and *Periophthalmus kalolo* Lesson, 1831 were obtained from GenBank and used as outgroups. The morphological data were analyzed by principal component analysis (PCA) using PAST 4 (PAleontological STatistics) program to identify the grouping characteristics and determine the diagnostic characteristics (Hammer et al. 2001).

RESULTS AND DISCUSSION

Sequence alignment, sequence identification, and PCR amplification

The result showed that the amplification of COI mitochondrial gene in mudskipper genus *Boleophthalmus* produced a fragment length approximately 700 bp (Figure 2). The COI mitochondrial gene in the 10 specimens were identified as *B. boddarti* with 99.69% to 100% similarity from BLAST and BOLD SYSTEM identification engine (Table 2). The 582 bp length aligned COI sequence of 10 *Boleophthalmus* specimens from the north coast of East Java, 7 *B. boddarti* sequences from Indonesia, and 12 *B. pectinirostris* sequences from other Asian regions registered in GenBank and BOLD SYSTEM were used for phylogenetic tree analysis.

Phylogenetic analysis

All three reconstructed phylogenetic trees produced identical topologies, with high bootstrap values (99%-100%) for neighbor-joining and maximum likelihood and a high posterior probability value (1.00) for Bayesian inference (Figure 3). The consensus tree presented three distinct clades. The first clade (A) was formed by *B. pectinirostris* mitochondrial COI gene from GenBank and BOLD, the second clade (B) was formed by seven sample from the north coast of East Java that identified as *B. boddarti* by BLAST and BOLD SYSTEM identification engine, and the third clade (C) was formed by *B. boddarti* from the north coast of East Java and from other Indonesian locations reported by GenBank and BOLD.

Table 2. Identification of genus *Boleophthalmus* using BLAST and BOLD identification

Sample code	% Similarity	% Query cover	Accession Number	Identified species	Location	References
MBI 01	100	92	MW591022.1	*B. boddarti*	Malaysia	Jamaluddin et al. (2022)
MBI 02	100	90	MW591022.2	*B. boddarti*	Malaysia	Jamaluddin et al. (2022)
MBI 07	98.4	90	BIFD557-13.*COI*-5P	*B. boddarti*	Cilacap	Dahruddin et al. (2016)
MBI 09	99.3	90	BIFD557-13.*COI*-5P	*B. boddarti*	Cilacap	Dahruddin et al. (2016)
MPS 14	100	92	MW591022.2	*B. boddarti*	Malaysia	Jamaluddin et al. (2022)
MPS 15	100	94	KY754666.1	*B. boddarti*	Malaysia	Jamaluddin et al. (2022)
MPS 16	99.69	94	MW591022.2	*B. boddarti*	Malaysia	Jamaluddin et al. (2022)
MPS 17	99.85	93	MW591022.1	*B. boddarti*	Malaysia	Jamaluddin et al. (2022)
MPS 18	100	93	MW591022.1	*B. boddarti*	Malaysia	Jamaluddin et al. (2022)
MWN 02	99.84	98	BIFD557-13.*COI*-5P	*B. boddarti*	Cilacap	Dahruddin et al. (2016)

Notes: MWN=Sample from point 1 (Surabaya); MPS=Sample from point 2 (Pasuruan); MBI=Sample from point 3 (Probolinggo)

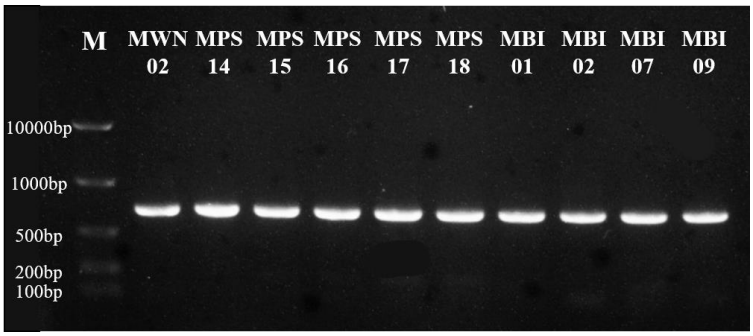

Figure 2. PCR amplification results of the *COI* mitochondrial gene in the specimens of *Boleophthalmus* from the north coast of East Java Province in 1% agarose electrophoresis. M=DNA marker ladder 1 kb; MWN=Sample from point 1 (Surabaya); MPS=Sampel from point 2 (Pasuruan); MBI=Sample from point 3 (Probolinggo)

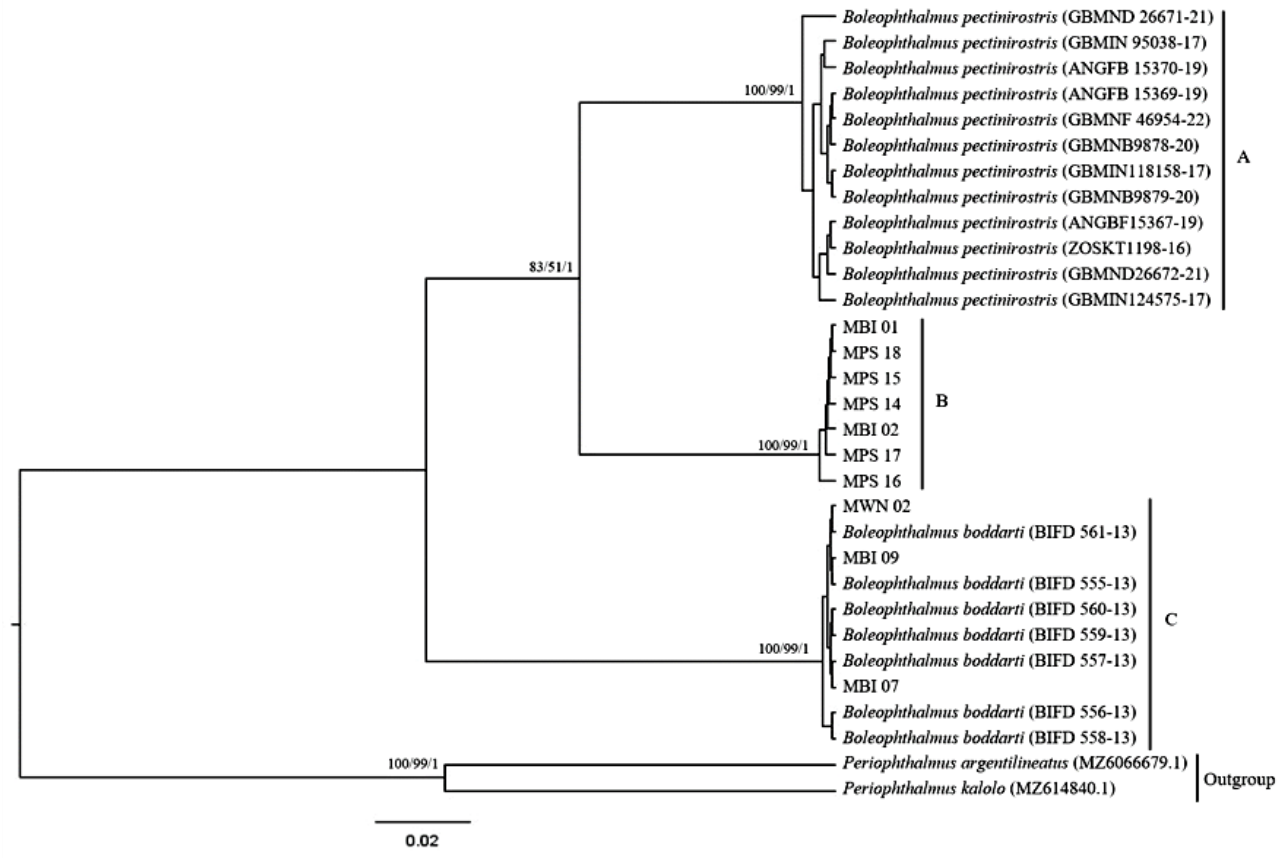

Figure 3. Phylogenetic trees constructed by neighbor-joining, maximum-likelihood, and Bayesian inference for the COI mitochondrial gene sequence of the genus *Boleophthalmus* and outgroup (528 bp)

Genetic distance

Phylogenetic analysis revealed the genetic distance (measured as percentage) between the formed clades (Table 3). *B. pectinirostris* from GenBank and BOLD and seven sample from the north coast of East Java that identified as *B. boddarti* by BLAST and BOLD SYSTEM identification engine had genetic distance between each other with an average of 9.67% (8.93%-10.05%). Similar genetic distance values were found in the genetic distance between *B. boddarti* (Clade C) and *B. pectinirostris* from East Java (13.57 - 13.81%) and *B. pectinirostris* from the GenBank and BOLD databases (13.01-13.71%).

Morphological characterization

Morphological identification of mudskipper genus *Boleophthalmus* from the north coast of East Java Province revealed that 3 specimens are *B. boddarti* and 7 specimens are *B. pectinirostris* by the diagnostic characteristics as reported by Murdy (1989). The specimens of *B. boddarti* were characterized by the following features: DF2R 24-25, with the first ray unsegmented and unbranched; LS 69-75; PRED 25-29; CFL 18.1-22.4% of SL; HL 25.5-26.3% of SL; DF2 BL 42.3-43.9% of SL; and lower jaw teeth notched. Meanwhile, the specimens of *B. pectinirostris* were characterized by the following features: DF2R 23-26, with the first ray segmented and branched; LD 113-123; PRED 27-36; CFL 17.5%-21.6% of SL; HL 24.7-26.7% of SL; DF2 BL 38.1-49.0% of SL; and lower jaw teeth notched (Table 4; Figure 4).

Figure 4. A. *Boleophthalmus boddarti*, MBY_BB_MBI 07_2023; B. *B. pectinirostris*, MBY_BP_MBI 02_2023 from north coast of East Java. Scale bars=10mm

Table 3. The genetic distance percentages among and between species of genus *Boleophthalmus*

	Clade_C	Clade_B	Clade_A
Clade_C			
Clade_B	13.78 (13.57-13.81)		
Clade_A	13.52 (13.01-13.71)	9.67 (8.93-10.05)	

Table 4. Morphometric (expressed as percentages of SL) and meristic characters of *Boleophthalmus boddarti* and *B. pectinirostris*

	B. boddarti (3)				*B. pectinirostris* (7)			
	Mean	**Range**	**Sd**	**Murdy (1989)**	**Mean**	**Range**	**Sd**	**Murdy (1989)**
SL	110.9	98.9-117.7	10.4	-	144.4	126.3-159.1	12.1	-
Morphometrics								
TL	127.5	123.9-129.6	3.1	-	120.7	112.8-124.3	4.8	-
HL	26.0	25.5-26.3	0.4	25.0-30.4	25.5	24.7-26.7	0.6	24.3-28
HD	16.1	15.4-17.2	1.0	15-17	15.8	14.6-17.8	1.1	13.5-15.9
BD1	16.4	15.6-16.8	0.7	14.5-18.4	15.8	14.7-17.8	1.1	12.2-17.6
BD2	15.2	14.3-16.5	1.2	-	16.0	14.8-18.5	1.5	-
PRED	34.3	34.2-34.4	0.1	-	33.2	31.1-37.2	2.2	-
DF1 BL	11.2	10.5-11.9	0.7	9.9-14.3	11.6	9.3-14.1	1.9	9.9-14.3
DF2 BL	42.9	42.3-43.9	0.9	40.2-46.4	42.4	38.1-49.0	3.6	41.5-46.1
DF 1&2 Gap	6.3	5.2-7.3	1.1	-	2.6	0-5.1	1.6	-
DF2-CF	14.0	10.4-19.5	4.8	-	9.3	6.0-11.1	1.9	-
AFBL	39.5	38.3-41.7	1.9	38.1-42.4	39.9	37.9-40.7	1.0	36.6-40.9
CPL	11.2	10.6-11.7	0.6	-	10.7	8.3-12.7	1.5	-
CPD	9.2	9.1-9.3	0.1	8.3-9.8	9.0	7.9-10.4	0.7	7.8-9.3
CFL	20.6	18.1-22.4	2.2	17.9-23.3	20.1	17.5-21.6	1.6	18.3-22.2
PvBL	14.5	13.5-15.2	0.9	12.8-16.3	14.8	13.4-15.7	0.7	13.4-15.2
PcFH	9.6	9.0-9.9	0.5	-	10.2	8.2-13.4	1.6	-
PcFBL	18.5	13.9-21.2	4.0	16.5-22.1	20.0	17.3-23.3	1.9	16.0-22.3
Meristic								
DF1R	V	V		V	V	V		V
DF2R	24.6	24-25	0.6	24-26	25.1	23-26	1.1	23-26
AFR	24.7	24-25	0.6	24-27	24.9	24-25	0.4	23-25
PFR	19.0	18-21	1.7	17-20	18.7	18-20	1.1	18-20
CFR	15.7	15-16	0.6	-	17.9	15-24	3.2	-
LS	71.3	69-75	3.2	61-79	120.3	113-123	3.4	84-123
TRDB	21.0	19-25	3.5	18-27	35.0	31-37	2.2	22-37
TRB	19.7	18-22	2.1	18-23	27.9	26-29	1.3	23-36
TRF	19.7	19-21	1.2	19-27	24.1	21-27	2.4	22-39
PRED	26.7	25-29	2.1	25-35	32.0	27-36	3.7	26-48

The morphometric and meristic data were assessed by PCA to investigate the effect of dominating features on the variance of grouping patterns across individuals of *Boleophthalmus* (Figure 5). The eigenvalues and percentage variances indicated that two components collectively accounted for more than 75% of the total variations in quantitative features (Table 5). PCA revealed the sample clustering pattern based on the function of each variable in the clustering procedure. The loading plots for Components 1 and 2 can be spotted, with two species-specific groups, *B. boddarti* and *B. pectinirostris*, formed from the 10 analyzed mudskipper individuals (Figure 5).

Table 5. Eigenvalue and % variance

PC	Eigenvalue	% variance
1	773.998	91.446
2	31.0686	3.6707
3	14.3102	1.6907
4	10.6142	1.254
5	6.65242	0.78597
6	4.06858	0.48069
7	3.43812	0.4062
8	1.29528	0.15303
9	0.953963	0.11271

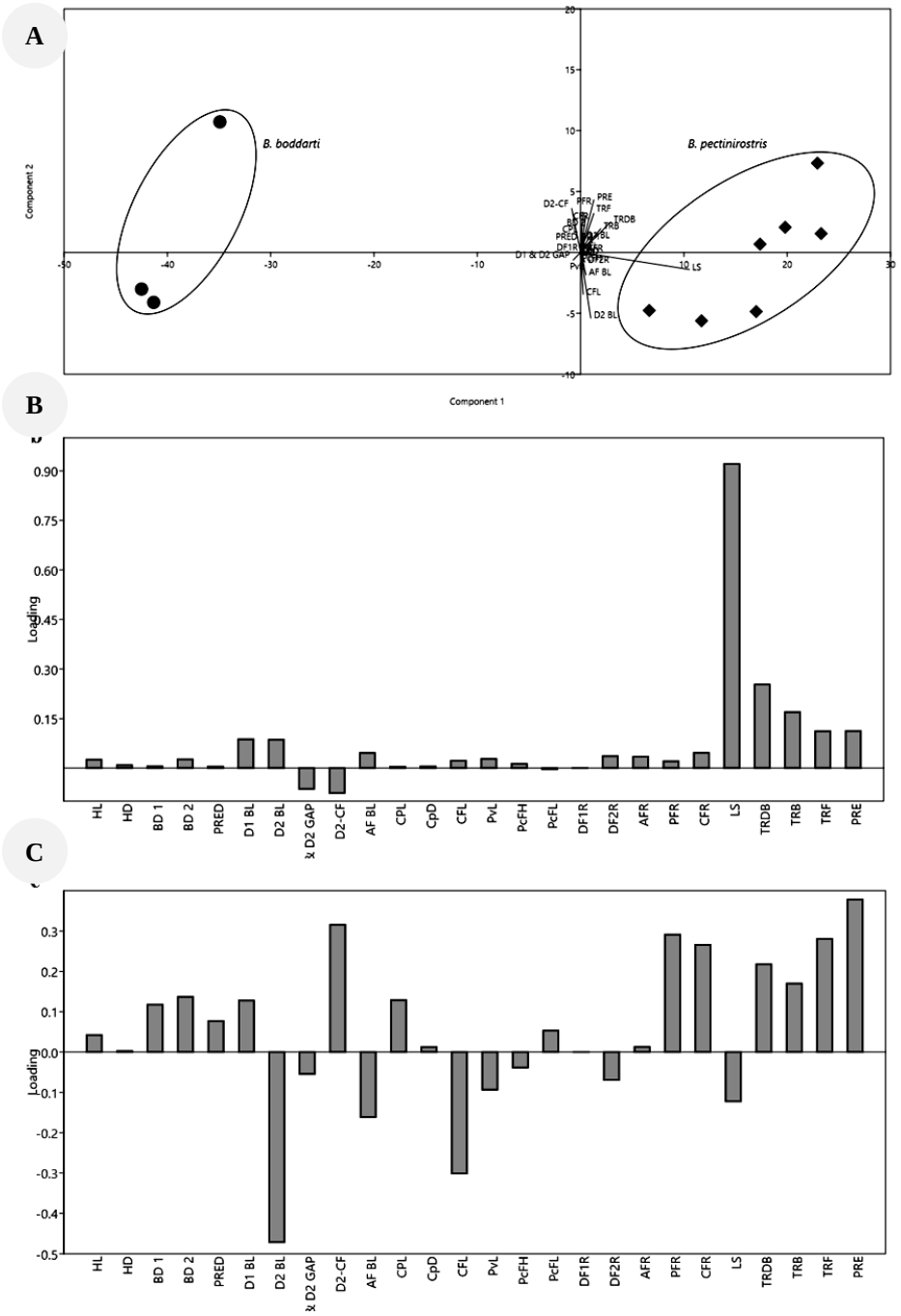

Figure 5. A. Scatter plot results of principal component analysis (PCA) for genus *Boleophthalmus;* B. Component 1 loading plot.; c. Component 2 loading plot

Discussion

DNA barcoding is useful in identifying cryptic diversity within species, particularly when combined with other taxonomic methods such as morphology (Imtiaz et al. 2017). BLAST NCBI and BOLD identification revealed that all 10 individuals of mudskipper from the north coast of East Java were identified as *B. boddarti*. However, phylogenetic tree reconstruction generated three different clades for the 10 individuals of mudskipper genus *Boleophthalmus* from the north coast of East Java and other sequences from GenBank and BOLD.

The *B. boddarti* specimens from the north coast of East Java were grouped as one clade with the *B. boddarti* from GenBank and BOLD that originated from Cilacap, Indonesia (BIFD555-13, BIFD556-13, BIFD557-13, BIFD558-13, BIFD559-13, BIFD560-13, BIFD561-13). The phylogenetic tree topology was supported by the genetic distance results (Table 5). The genetic distance between *B. boddarti* (Clade C) and *B. pectinirostris* (Clades A and B) was more than 3.5%, which is the intraspecific genetic distance threshold for fish species (Zemlak et al. 2009). The genetic distance between the *B. boddarti* populations in the north coast of East Java and Cilacap, Indonesia further confirmed the grouping of *B. boddarti* and indicated a significant genetic relationship among them. Therefore, the *B. boddarti* specimens collected from the north coast of East Java and the South of Java form a monophyletic group and exhibit a close genetic relationship. The seven specimens previously identified as *B. boddarti* based on BLAST results and the BOLD identification engine are now believed to be more closely related to *B. pectinirostris*. This is due to their clustering in the phylogenetic tree (Clade B and Clade C) and their large genetic distances (13.57-13.81%) with B. boddarti populations from East Java and Cilacap.

Boleophthalmus pectinirostris showed two separated clades. The first clade was 12 COI mitochondrial gene sequences of *B. pectinirostris* from GenBank and BOLD originated from China (ANGBF15367-19, ANGBF15369-19, ANGBF15370-19, ANGBF2670-12, GBMIN118158-17, GBMIN118159-17, GBMIN124575-17, GBMIN95038-17, GBMNF46954-22), Taiwan (ZOSKT1198-16), Shanghai (GBMND26671-21, GBMND26672-21), and Korea (GBMNB9878-20, GBMNB9879-20, JX679027.1, MN206502.1). The second clade was the sample that suspected to be *B. pectinirostris* from the north coast of East Java. All the phylogenetic trees constructed by neighbor-joining, maximum-likelihood, and Bayesian inference methods (Figure 3) have a consistent topology. The genetic distance of greater than 3.5% (8.93%-10.05%) between the two clades of *B. pectinirostris* also validated that they considered to be different species. This phenomenon can be attributed to the separate populations of *B. pectinirostris* in East Asia and Indonesia, which are considered sister groups. Chen et al. (2014) reported that the *B. pectinirostris* found on the Pacific Northwest region is a cryptic species encompassing the East Asian and Malaysian populations based on the GMYC and Bayesian tree reconstruction ND5 marker. They reported that the differentiation of species and the existence of the cryptic species of mudskipper *B. pectinirostris* could be due to the geographical isolation during the Late Pliocene in South China Sea and Malacca Straits. This isolation is believed to have been produced by minimum glacial sea level and interglacial sea current patterns, which in turn led to genetic isolation.

The East Java specimens were identified as *B. boddarti* (3 specimens) and *B. pectinirostris* (7 specimens). Their morphological characteristics agreed well with the diagnostic characteristics reported by Murdy (1989). However, several morphometric and meristic characteristics of the present specimens of *B. boddarti* and *B. pectinirostris* slightly differed from those reported by Murdy (1989), including the head depth and pectoral fin base length in *B. boddarti* and head depth, first body depth, second dorsal fin base length, caudal peduncle depth, pelvic fin length, pectoral fin base length, and transverse scales from origin of second dorsal fin ventroposteriorly in *B. pectinirostris* (Table 4). These differences in *B. boddarti* are regarded as intraspecific variations (no genetic differences between the specimens from East Java and those from other regions; Figure 3). However, the differences for *B. pectinirostris* indicated that the *B. pectinirostris* from East Java is a different species from the *B. pectinirostris* from East Asia, supporting the clade of two distinct populations (Figure 3).

Boleophthalmus boddarti and *B. pectinirostris* have been documented in Indonesian waters, specifically along the east coast of Sumatra and the Java Sea for *B. boddarti* (Dahruddin et al. 2016; Ridho et al. 2019) and in the Java Sea and West Papua for *B. pectinirostris* (Polgar et al. 2013; Sunarni et al. 2019). *B. boddarti* and *B. pectinirostris* can be distinguished from each other by the first ray of second dorsal fin (spinous in the former versus segmented in the latter), number of longitudinal scales (less than 80 versus greater than 80), five to seven prominent blackish bars from the dorsal surface of body (extending well below the midline versus never extending ventrally beyond the lateral midline), and pectoral fin (pale, dorsal edge black versus uniformly blackish) (Murdy 1989; this study).

This study also employed PCA to determine the similarities in morphometric and meristic characteristics. The results clearly distinguished the *B. boddarti* and *B. pectinirostris* samples as different species. According to PCA, the most important characteristics for the distinction between the two clusters of *B. boddarti* and *B. pectinirostris* are the lateral line scales, followed by DF2 BL and CFL. The prevalent characteristics are revealed by the length of the resulting line (Figure 4) and the images of loading plot of component (Figures 5 and 6). Morphometric and meristic analysis showed that *B. boddarti* and *B. pectinirostris* can be distinguished from each other by their lateral line scales, transverse scale counts, and predorsal scales. Meanwhile, the morphometric characteristics of the two species are relatively similar.

In conclusion, DNA barcoding and morphological analyses successfully identified two species of genus *Boleophthalmus* from the north coast of East Java Province, e.g., *B. boddarti* and *B. pectinirostris*. However, *B. pectinirostris* was regarded herein as a cryptic species

because of the high genetic distance and forming a separated clade of *B. pectinirostris* originating from East Asia and the north coast of East Java. Further DNA and morphological analyses are required to confirm its taxonomic status.

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