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Research Article

DNA Barcoding for Selected Mangrove-Based Estuary Fishes from Way Kambas National Park, Lampung Province, Indonesia

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ABSTRACT

Over the past decade, DNA barcoding has provided new insight into fish ecology and biosystematics and led to new species' discovery. DNA barcoding is a method for the recognition and identification of species using short, standardised DNA fragments. The correct taxonomic identification of species is critical for the assessment and monitoring of biodiversity. This study applied DNA barcoding techniques to identify selected fish species from a mangrove-based estuary in Way Kambas National Park, Lampung Province, Indonesia. The gene encoding cytochrome *c* oxidase subunit I (COI) was amplified and bi-directionally sequenced from 22 specimens. The resulting 680 base pairs (bp) sequence was used to identify species, obtain phylogenetic information, and analyse genetic distances. A neighbour-joining tree was constructed based on the mitochondrial COI gene using the Kimura two-parameter model. This study also exhibits conservation status for those identified species. Our findings will facilitate future studies of fish species diversity in mangrove estuary-based ecosystems and provide preliminary data in policymaking in conservation areas such as National Park.

Keywords: Biodiversity, COI, Estuarine, Genetic source, Mangrove

Introduction

DNA barcoding remain a practical tool for species identification due to its rapid, accuracy, cost-effective features, and functionality [1, 2]. DNA barcodes using a standardized DNA region that allows objective and universal comparable results, which can quickly be repeated even by non-taxonomist specialists [3]. Furthermore, this method can analyze inadequate, fragmented specimens and at different life stages [4–6]. The mitochondrial DNA region at the 5' ends of cytochrome oxidase c subunit I (COI) was used as a molecular marker to delineate species; hence, this region is commonly used as a "barcode" [7]. The diversity in the amino acid sequence [about 648 base pairs (bp)] of the COI gene was sufficient to reliably place species into higher taxonomic levels [1]. Over the past decade, DNA barcoding has been used by diverse taxa, including birds [8], fishes [9–11], spiders [12], invertebrates [13], and mammals [14–16]. DNA barcoding has also been used to evaluate food quality and authenticity [17], resolve taxonomic uncertainty [18], and monitoring fish biodiversity [19, 20]. Moreover, this method could provide broader insight into the ecology and biosystematics, for example, by revealing cryptic organisms and discovering new species [21–23].

Way Kambas National Park (WKNP) is located in eastern Lampung province, Sumatera, Indonesia. It covers about 1,300 km^2 of swamp forest and lowland rain forest. Mangrove ecosystem sites can be found in the eastern region of WKNP. Mangrove-based estuarine ecosystems have numerous of finfish species. Around 267 species of fish from 81 families have been reported in two major rivers in India [24]. An estuarine habitat is a partially enclosed, coastal water body where freshwater from rivers and streams mixes with saltwater from the ocean. River mouths, lagoons, and bays often constitute estuarine habitats. The

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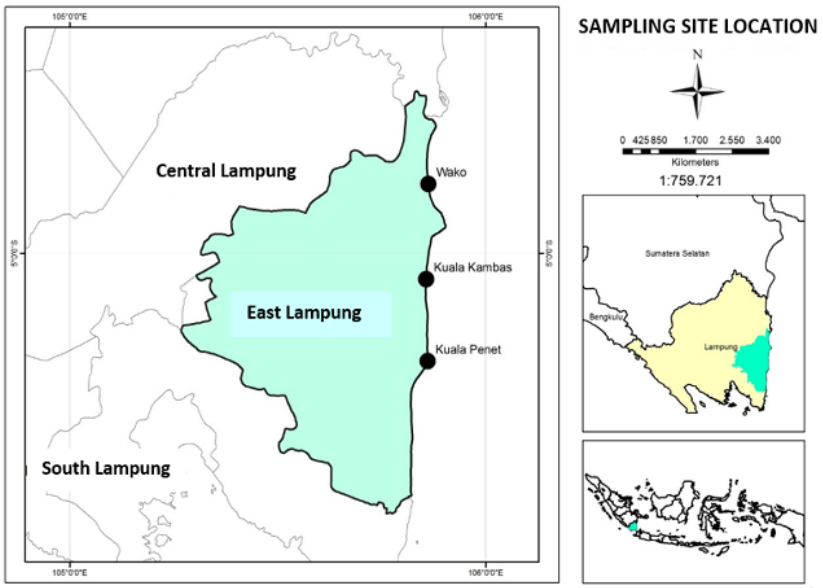

Figure 1. Map of sample collection site in Way Kambas National Park.

mixing of seawater and freshwater provides high levels of nutrients both in the water column and sediment, making estuaries the most productive natural habitat [25]. Estuaries provide habitat for life-cycle completion by various species, including mollusks, crustaceans, and fishes. These unique ecosystems are essential for maintaining biodiversity. The accurate taxonomic identification of species is critical for the assessment and monitoring of biodiversity.

The eastern side of Way Kambas National Park is directly facing the Java Sea, and this area can be accessed freely by many fishermen. Most of them are part of the local community who still often catch fish in the national park estuaries. However, they do not know the conservation status of the various types of fish caught. This study provides information on the conservation status and species of fish originating from the national park area. This information can be used as material for consideration in the making of conservation policies by related agencies. DNA barcode technique was applied to identify and confirm selected fish species in the mangrove-based estuaries in WKNP.

Material and Methods

Study area

Fish were captured with permission from the WKNP Agency (National Park Entrance Permit, sanction order no. SI.990/BTNWK-1/2018, dated 28 July 2018). Ethics approval for this study was unrequired because no endangered or protected fish species were involved. Fish samples were collected from three sampling stations at the following estuarine river mangrove bases: Kuala Penet (5°15'18.01"S 105°51'38.09"E), Kuala Kambas (5° 3'49.43"S 105°51'25.07"E), and Wako (4°50'17.79"S 105°51'45.00"E) (Figure 1). The samples were photographed immediately after collection. Approximately 3–5 g of dorsal muscle tissue or fin were excised and immersed in 96% ethanol for genomic analysis. Voucher specimens were stored in 70% alcohol and morphologically identified based on the FAO Species Identification Guide for Fishery Purposes [26], Kottelat *et al*. [27] and http://www.fishbase.org [28].

DNA isolation and sequencing

Total genomic DNA was extracted from stored epaxial muscle tissue or fins using a Geneaid GT300 Genomic DNA Mini Kit (Tissue) following the manufacturer's protocols. The DNA concentration was estimated using a NanoDrop™ 2000/c spectrophotometer (Thermo Fisher). The target region of COI was amplified by PCR using the primers FishF1 (5′-TCAACCAACCACAAAGACATTGGCAC-3′) and FishR1 (5′-TAGACTTCTGGGTGGCCAAGAATCA-3′) [29]. PCR was performed in a total volume of 25 µL consisting of 1 µL of DNA template, 12.5 µL of Gotaq® Green Master Mix (Promega), 1 µL of each primer, and distilled water. The amplification conditions were as follows: initial denaturation for 2 min at 95°C followed by 35 cycles of 0.5 min at 94°C, 0.5 min at 54°C, and 1 min at 72°C, with a

Table 1. Samples analysed in this study and the GenBank accession numbers of the COI mtDNA sequences

Sample/Genus/Species	No. of specimens (n)	Source	Collection sites	GenBank Accession number	
				References	In this study
Lutjanus argentimaculatus	2	Wild	Wako		MN243478, MN243479
	3	GenBank		MG923374.1	
				EU502685.1	
				KC970482.1	
Tetraodon nigroviridis	1	Wild	Kuala Kambas		MN243476
	3	GenBank		DQ019313.1	
				KC959930.1	
				JQ681838.1	
Photopectoralis bindus	1	Wild	Kuala Kambas		MN243471
	1	Wild	Wako		MN243480
	2	GenBank		KJ013055.1	
				MG677547.1	
Scatophagus argus	1	Wild	Kuala Kambas		MN243469
	3	GenBank		KY634864.1	
				MG923404.1	
				KY634866.1	
Ambassis sp.	1	Wild	Kuala Kambas		MN243472
	3	GenBank		KU692232.1	
				KU692234.1	
				KU692233.1	
Stigmatogobius sadanundio	1	Wild	Wako		MN243486
	2	GenBank		MG495948.1	
				MF594606.1	
Planiliza parmata	1	Wild	Kuala Kambas		MN243475
	2	GenBank		KX977548.1	
				KX977546.1	
Planiliza subviridis	1	GenBank		HQ564490.1	
Mugil cephalus	1	Wild	Wako		MN243481
	1	GenBank		KP856770.1	
Osteochilus hasseltii	1	GenBank		JF915633.1	
Osteochilus vittatus	1	Wild	Wako		MN243482
Osteochilus sp.	1	GenBank		JX074151.1	
Parachela oxygastroides	1	Wild	Wako		MN243487
	1	GenBank		HM224181.1	
Parachela hypophthalamus	2	GenBank		KU692733.1	
				KU692738.1	
Hemibagrus nemurus	2	Wild	Wako		MN243484, MN243488
	3	GenBank		KM213068.1	
				KM213067.1	
				KJ573466.1	
Hemibagrus capitulum	1	GenBank		KP856825.1	
Mystus cavasius	4	GenBank		KT762365.1	
				KU870465.1	
				JX983383.1	
				JX983379.1	
Mystus wolffii	1	Wild	Kuala Kambas		MN243470
	1	Wild	Wako		MN243483
Mystus bleekeri	1	GenBank		KJ936764.1	
Mystus singaringan	1	GenBank		MK448115.1	
Eleutheronema tetradactylum	1	Wild	Kuala Kambas		MN243474
	1	Wild	Wako		MN243477
	3	GenBank		MG923347.1	
				MG923350.1	
				MG923349.1	

Continue…

Table 1. Samples analysed in this study and the GenBank accession numbers of the COI mtDNA sequences

Sample/Genus/Species	No. of specimens (n)	Source	Collection sites	GenBank Accession number	
				References	In this study
Hexanematichthys sagor	1	Wild	Kuala Penet		MN243467
	1	GenBank		JX198212.1	
Netuma cf. *thalassina*	1	GenBank		HQ564482.1	
Arius	1	GenBank		KX211965.1	
Arius microcephalus	1	GenBank		MK604248.1	
Arius maculatus	2	Wild	Kuala Kambas		MN243468, MN243473
	1	Wild	Wako		MN243485
	2	GenBank		KY849505.1	
				KY849504.1	

final hold for 10 min at 72°C. The products were electrophoresed in 1% agarose gels and stained with ethidium bromide; DNA bands were visualised under an ultraviolet transilluminator. Intense bands were sent for sequencing on the BigDye® Terminator v. 3.1 platforms provided by 1st BASE Laboratories (Singapore).

Data analysis

The 22 bi-directional sequences were initially checked by eye using the sequence editor BioEdit v. 7.0.9.0 [30]. Next, the sequences were aligned using ClustalW in MEGA X [31]. The final alignment comprised 680 bp. The sequences were submitted to GenBank. A similarity search of the generated sequences was performed using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/) and the Barcode of Life Data System (BOLD) (http://www.barcodinglife.org/index.php/IDS-OpenIdEngine) [32]. The generated sequences and 44 published sequences of related species were aligned as a dataset (Table 1); equal sequence lengths were used to prevent incongruent outcomes between genetic distances and the neighbour-joining (NJ) tree. The genetic distances within and between species were calculated using the Kimura two-parameter model [33]. A phylogenetic tree was constructed using the NJ method [34] and the maximum parsimony (MP) algorithm. Homologous COI sequences available in GenBank were included in the phylogenetic analysis. The clade confidence in the tree was tested by 1,000-replicate bootstrapping to determine support values for the clade nodes.

Result and Discussion

A total of 68 mitochondrial COI barcode sequences were obtained from 11 families, 15 genera, and 27 species. After editing, the consensus length of all barcode sequences was > 500 bp. Stop codons, insertions, and deletions were undetected in any of the sequences.

The analysis results of the nucleotide pair frequency revealed that 339 of 593 (57.17%) sites were conserved, 254 of 593 (42.83%) sites were variable, 252 of 593 (42.49%) sites were parsimony informative, and 2 of 593 (0.33%) were singleton sites. The transitional substitutions rates are shown in bold and those of transversional substitutions in italics (Table 2).

The transitional substitutions rates ranged from 10.41 to 31.3, while those of transversional substitutions ranged from 2.24 to 3.66. The transition/transversion rate ratios were $k1$ = 10.022 (purines) and $k2$ = 2.862 (pyrimidines). The overall transition/transversion bias for the dataset was R = 2.765. The nucleotide frequencies were 24.65% (A), 28.93% (T/U), 28.72% (C), and 17.70% (G). A base-composition analysis showed that the average T content was highest, and the average G content was lowest. The AT content (53.58%) was higher than the GC content (46.42%), similar to the results for Australian [35], Canadian [36], Cuban [37], and Taiwan Strait fish species [38].

According to Ward *et al*. [35], the result was relevant in mostly marine fishes, in which the content of AT is higher than that of GC. The mean genetic divergence of the dataset was 22%. The intraspecific distance ranged from 0% (mostly species in the studied dataset) to 0.9%; the greatest distance (0.9%) was in *Scatophagus argus*. The interspecific distance ranged from 7.4% to 30.3%. The smallest interspecific distance was between *Netuma* cf. *thalassina* (HQ564482.1) and *Arius arius* (KX211965.1), and the largest was between *Eleutheronema tetradactylum* (MN243474) and *Stigmatogobius sadanundio* (MF594606.1).

The mean within-family distance ranged from

Table 2. Maximum composite likelihood estimates of the nucleotide substitution pattern

	A	T	C	G
A	-	*3.66*	*3.64*	22.47
T	*3.12*	-	**10.41**	*2.24*
C	*3.12*	**10.49**	-	*2.24*
G	**31.3**	*3.66*	*3.64*	-

Notes:
Rates of transitional substitution are in bold and those of transversional substitution in italics.

0.20% to 11.62%, and the mean between-family distance from 12.10% to 28.80%. Species identification by DNA barcoding relies on both intraspecific and interspecific divergence. According to Meier *et al*. [39], the barcode gap can be calculated as the smallest versus the largest intraspecific distance. The within-species genetic distance was < 2%, and that between species was > 5%. The 22 sequences were matched with homologs by BLAST searching using a species identity of > 97%. Phylogenetic and molecular evolutionary analyses were conducted using MEGA X [31]. The NJ tree (Figure 2) shows distinct clustering of all studied species. The unrooted NJ tree comprised three clusters.

Most specimens from the same species were grouped into one cluster. The first cluster consisted of seven families: Lutjanidae, Tetraodontidae, Leiognathidae, Scatopaghidae, Ambassidae, Gobiidae, and Mugilidae. All members of the first cluster were typically found in marine to brackish water. The second cluster consisted of Cyprinidae; this cluster was placed with confidence between the other two clusters. The third cluster consisted of three families: Bagridae, Polynemidae, and Ariidae. The Cyprinidae are a family of freshwater fishes known as cyprinids and commonly called carp. Cyprinidae is the largest and most diverse fish family and the most abundant vertebrate family (~3,000 species) [27, 40].

Osteochilus and *Parachela* are cyprinid fish genera mainly found in Southeast Asia. Both inhabit freshwater habitats, including rivers [41]. Two families, Bagridae and Ariidae, are catfishes. Ariids are found in shallow temperate and tropical seas around the coastlines of North and South America, Africa, Asia, and Australia. Ariid catfish species mainly live in freshwater and brackish water. Bagridae (naked catfish) are freshwater cat fish native to Africa and Asia (from Japan to Borneo).

Hexanemathycthys sagor (MN243467) was genetically close (0.002) to the other Ariid catfish species, *H. sagor* (JX198212.1). The two *Mystus* sp. sequences (MN243470 and MN243483) had an identity of ~88%, meaning that species are missing from our reference library. Those sequences matched (99%) *Mystus wolffii* according to a sequence alignment using the BOLD ID database (http://www.barcodinglife.org/index.php/IDS-OpenIdEngine). Figure 3 shows the phylogenetic tree constructed from the BOLD ID database sequences; the *Mystus* sp. was confidently grouped with *M. wolffii*. Two *M. wolffii* sequences were added to GenBank. The sequences added for these species could previously only be found in the BOLD database as private records.

The eastern coast of Sumatera has a vast area of mangrove swamp. This study was conducted in a mangrove ecosystem within the protected area of WKNP. Data on species diversity, especially fish, originating from the mangrove ecosystem in this area, is still lacking. Barcode data for most of the studied species are available in GenBank. However, the library still lacks suitable COI homologous sequences for some species.

Based on BOLD ID results, three sequences (*Osteochilus vittatus, M. wolffii*, and *Ambassis* sp.) had 100% similarity scores. However, one *Ambassis* sp. was unnamed to the species level due to a lack of appropriate specimens and a reference sequence in GenBank or BOLD ID. There were eight species with the Least Concern status according to the IUCN Red List—*Lutjanus argentimaculatu, S. argus, Ambassis* sp., *Mugil cephalus, O. vittatus, Parachela oxygastroides, Hemibagrus nemurus,* and *M. wolffii. Photopectoralis bindus* and *Arius maculatus* were categorised as Data-Deficient species due to insufficient information for a proper assessment, while the remaining species were categorised as Not Evaluated [42–50] (Table 3).

Intriguingly, one species of the family Polynemidae, *E. tetradactylum*, was classified as endangered under criterion A (EN A4d). Based on the previous assessment (2014), this species has likely declined by ~50% in the Persian Gulf. The population of this species is likely to decline by 50–87.5% over a three-generation period [51]. *Eleutheronema tetradactylum* possesses several diagnostic traits, including four pectoral filaments;

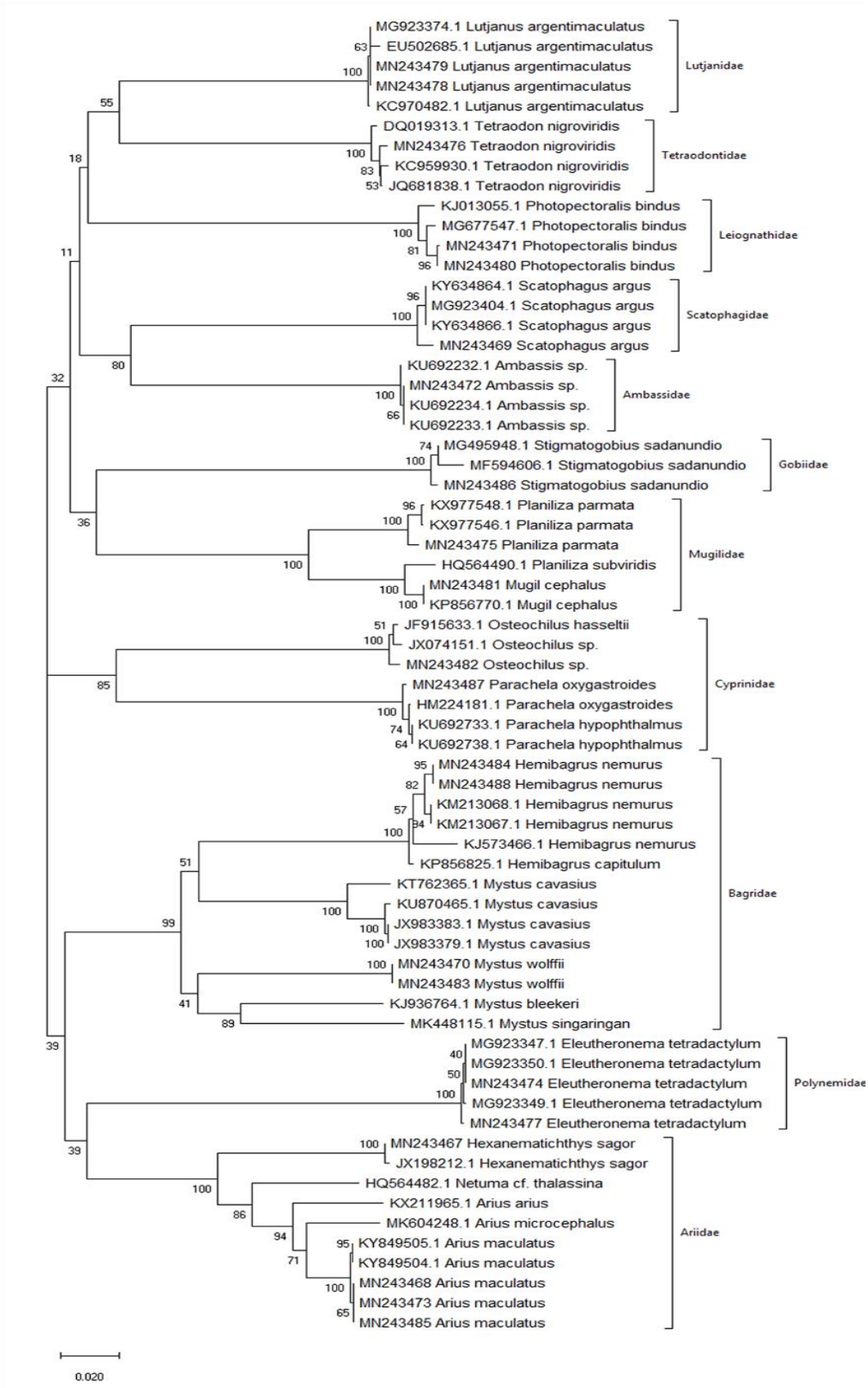

Figure 2. NJ tree constructed based on COI sequences using Kimura two-parameter distances. Scale bar, 0.020 substitutions per nucleotide position.

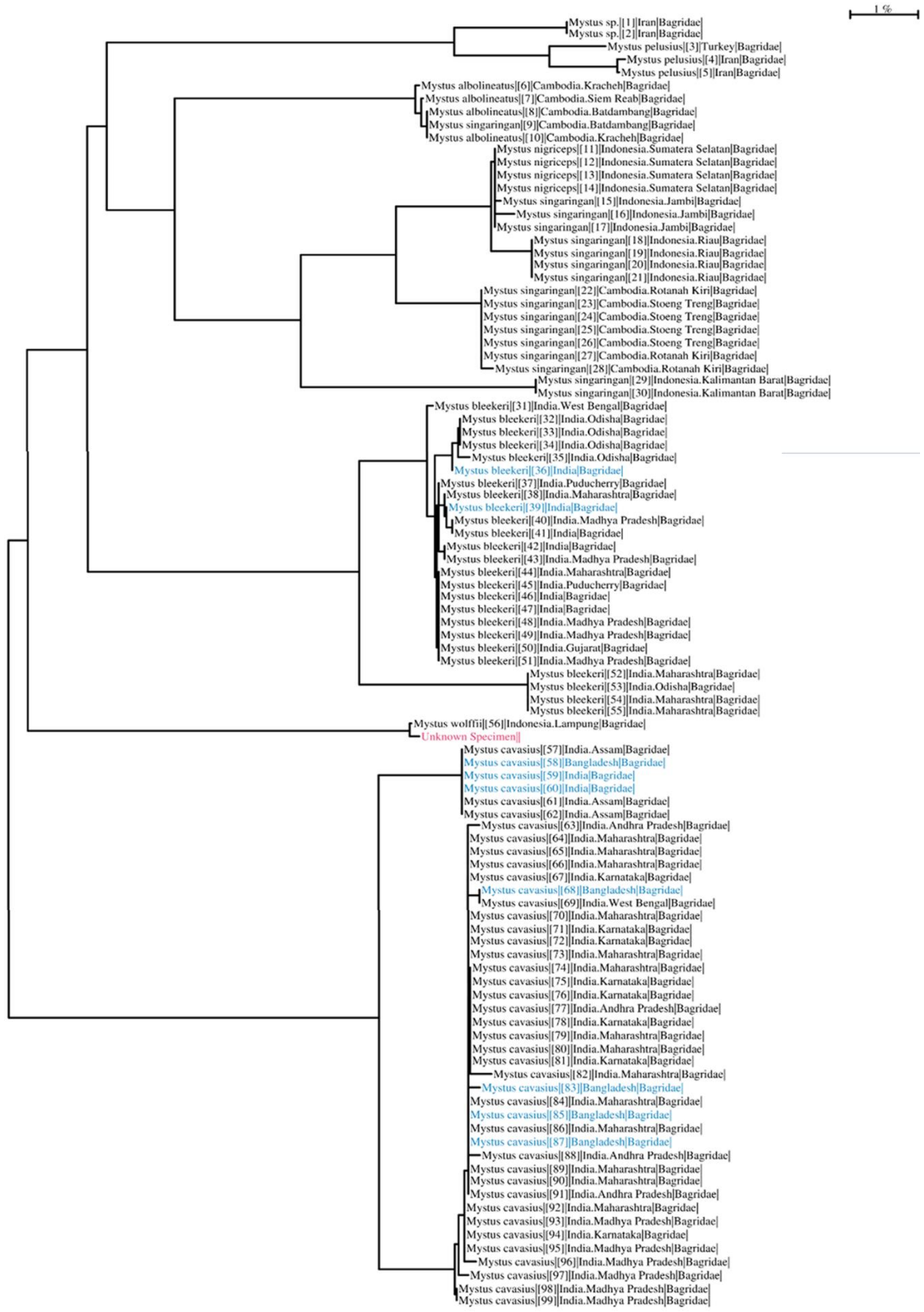

Figure 3. NJ tree constructed based on the BOLD ID data. Red, position of *Mystus wolffii* within the BOLD phylogenetic tree

Table 3. Details of the studied fish species from the mangrove ecosystem in WKNP and their IUCN conservation status.

Order	Family	Genus/Species	IUCN status
Perciformes	Lutjanidae	*Lutjanus argentimaculatus*	LC
Tetraodontiformes	Tetraodontidae	*Tetraodon nigroviridis*	NE
Perciformes	Leiognathidae	*Photopectoralis bindus*	DD
Perciformes	Scatophagidae	*Scatophagus argus*	LC
Perciformes	Ambassidae	*Ambassis* sp.	LC
Perciformes	Gobiidae	*Stigmatogobius sadanundio*	NE
Mugiliformes	Mugilidae	*Planiliza parmata*	NE
		Mugil cephalus	LC
Cypriniformes	Cyprinidae	*Osteochilus vittatus*	LC
		Parachela oxygastroides	LC
Siluriformes	Bagridae	*Hemibagrus nemurus*	LC
		Mystus wolffii	LC
Perciformes	Polynemidae	*Eleutheronema tetradactylum*	E A4d*
Siluriformes	Ariidae	*Hexanematichthys sagor*	NE
		Arius maculatus	DD

16–18 (mode 17, rarely 15 or 19) pectoral fins rays; 14 (rarely 13 or 15) second dorsal fin soft rays; a vomer with deciduous tooth plates on both sides, except in juveniles; and pectoral fin membranes that are vivid yellow in life, except in large specimens. This species is widely distributed in the Indo-West Pacific and is extant in Bahrain, Iran, Iraq, Kuwait, Qatar, Saudi Arabia, and the United Arab Emirates [52]. However, there is no policy on the susceptibility of these species in the Indo-West Pacific; thus, they should focus on conservation efforts, particularly in Indonesia.

The total number of barcoded Chordata from Indonesia was 20,217, of which 15,631 were Actinopterygii. Based on the Fish Barcode of Life Initiative (FISH-BOL), there are about 12,140 of fish species from Southeast Asia have been barcoded in 2019. The Scientific Committee on Antarctic Research–Marine Biodiversity Information Network in 2012 reported that < 20% of fish species in Southeast Asia have a barcode [53]. The development of comprehensive DNA barcode reference libraries, especially in Indonesian freshwater fishes, was initiated by Hubert *et al*. [9] several years ago.

DNA barcode techniques have been widely used to reveal the diversity of fish in Indonesia, for example, in the endangered species of sharks [54], the substantial economic value of reef fish [55], and fish originating from the peat swamp environment of New Guinea island, Indonesia [56]. Wibowo *et al*. [56] found something unusual, about 68% of the fish larvae sequences could not determine into species level due to the lack of a suitable COI sequence in the reference dataset. The vast region and various habitats are become challenging to reveal the diversity of fish in Indonesia. In summary, the present study contributes to form a complete DNA barcode library, especially for teleost fish originating from the mangrove ecosystem.

Conclusion

The DNA Barcoding technique enabled to discriminate of selected fish from WKNP into species level. Mitochondrial COI barcode for 22 mangrove-based estuarine fish species from WKNP has been submitted to GenBank. These findings will facilitate future studies on the diversity of fish species in mangrove estuary-based ecosystems and provide valuable preliminary data in policy-making in conservation areas such as National Parks.

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