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Short Communication

DNA Barcoding of Freshwater Eels *Anguilla* spp from Kuari River Based on Cytochrome C Oxidase Subunit I (COI) Gene

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Abstract

One Freshwater eels (*Anguilla spp.*) are classified in the family Anguillidae, and included in the catadromous group. This study aimed to determine the COI gene sequences of the mitochondrial DNA, analyze the genetic distances, and phylogenetics, and characterize the physical and chemical parameters of freshwater eels habitat in the Kuari River Bengkulu. This research was conducted from November 2020 – April 2021. The methods used in barcoding eel species were DNA isolation, DNA amplification using PCR (polymerase chain reaction), electrophoresis, and sequencing of COI gene regions in mtDNA. The COI mtDNA gene fragments were obtained from PCR results with an annealing temperature of 50°C for 30 seconds in 35 cycles. BLASTn analysis of eel samples AM3 and AM4 had the highest similarity of 99.82%-100% to Anguilla marmorata, and samples AB2-AB5 indicated the highest identity of 99.84%-100% to *Anguilla bengalensis*. Phylogenetic species indicated that *Anguilla marmorata* and *Anguilla bengalensis* form two different sub-clusters. The water qualities in the Bengkulu Kuari River were temperatures 26.5-27.5°C, pH 7.1-8.7, dissolved oxygen 6.19-9.54 mg L-1, brightness 21-47 cm, ammonia 0.16-0.41 mg L-1, total alkalinity 20–52 mg L-1, TDS 33–49 mg L-1, salinity 0.3-0.4 ppt and water velocity 0.5-0.8 ms-1. The COI gene in DNA barcoding is very appropriate to be used for the identification of *Anguilla spp* species by comparing the DNA sequence of the COI gene with the existing database in the Genbank.

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1. Introduction

Freshwater eels (*Anguilla* spp.) are a type of fish from the family Anguillidae which is included in the catadromous group, meaning that eels will forage for food and grow into adults in rivers and will migrate back to sea waters when spawning (Kardin *et al.*, 2016). Eels can be cultivated in earthen ponds, concrete ponds, and floating net cages (Mulis, 2015). *Anguilla bengalensis* (Wibowo *et al.*, 2021) and *Anguilla marmorata* have been found in the waters of Bengkulu Province (Fahmi, 2015). One of the rivers that provide a habitat for eels is the Kuari River, which is part of the Luas Watershed in the District of Luas, Kaur Regency, Bengkulu Province. Nonmolecular identification of freshwater eels was conducted based on morphometric and meristic characteristics (Saanin, 1984). Morphologically, the species *A. bengalensis* and *A. marmorata* have different skin types, and colors of the back and abdomen. *A bengalensis* has a skin type that is not patterned (plain), the back is black and the abdomen silver (Fishbase, 2021). Meanwhile, *A. marmorata* has patterned skin (striped) (Silfvergrip, 2009), the back is black with a pattern and the abdomen is white. However, species identification using a morphological approach is subjective, resulting in overlapping information regarding the characteristics of adjacent taxa (Rasmussen and Kellis, 2011). Morphological assessment of the genus Anguilla is challenging because of species similarities, particularly in the young eel stage (elvers) (Watanabe *et al.*, 2008). Four species of *Anguilla* spp. from river estuaries from Central Java have been identified morphologically as *Anguilla bicolor bicolor*, *Anguilla bicolor pacifica*, *Anguilla obscura*, and *Anguilla australis*, but molecularly they were confirmed as *Anguilla bicolor bicolor* with an identity percentage > 98% (Falah *et al.*, 2023).

Given the intersecting morphological differences between species, it is necessary to have more precise genetic markers for species identification and to determine the relationship between freshwater eel species.. For this reason, a study of molecular genetic identification is needed. This can be done by DNA barcoding (Peninal *et al.*, 2017; Putra *el al.*, 2024; Syaifudin *et al.*, 2017a). DNA barcoding is used to identify genetic diversity between species (Laudien *et al.*, 2003; Syaifudin *et al.*, 2021). DNA barcoding is also carried out for domestication in fish farming activities (Rasmussen *et al.*, 2009), species identification (Marnis *et al.*, 2024; Syaifudin *et al.*, 2017b, 2023; Zan *et al.*, 2020); phylogenetic studies (Anjarsari *et al.*, 2021, Basith *et al.*, 2021, Syaifudin *et al.*, 2021), and species mislabelling (Barendse *et al.*, 2019; Han *et al.*, 2021).

The *cytochrome-c oxidase I* (*COI*) gene is a molecular marker that has a nucleotide base sequence that maintains genes for genetic conservation (Hebert *et al.*, 2003) and is a barcoding marker that is often used in animals. The use of the *cytochrome-c oxidase I (COI)* gene in eels has been carried out in several studies, such as DNA barcodes of *A. bengalensis* and *A. bicolor* in Peninsular Malaysia (Arai *et al.*, 2015), species diversity of eels from Aceh waters (Muchlisin *et al.*, 2017), phylogenetic analysis of *A. marmorata* population in Thua Thien Hue, Vietnam (Huyen and Linh, 2020), eels in Africa (Hanzen *et al.*, 2020), eels in Kedurang River Bengkulu (Wibowo *et al.*, 2021) and elver stadia in Central Java (Falah *et al.*, 2023). However, DNA authentication of freshwater eels and water quality characteristics in the Kuari River has not been determined. Therefore, it is necessary to do DNA barcoding research using the *COI* gene of eels and analyze water physical and chemical parameters from the Kuari River, Luas District, Kaur Regency, Bengkulu Province. This study aims to determine the *COI* gene sequence of eels' mitochondrial DNA, and the percentage of species similarity, and to analyze the genetic and phylogenetic distances between eel species from the results of research using the DNA database at the Genbank data center.

2. Materials and Methods

2.1 Material

Fish and water samples were collected from Kuari River, District Luas, Kaur Regency, Bengkulu Province (Indonesia) on 21st November 2020 at the rainy season. The research sites are presented in Figure 1. A sample of six freshwater eels, with total length of 23.7-44 cm, were captured with the help of fish farmers using traps. Sample identification of eels based on morphological indicators is shown in Table 1. Fish fins were cut at the research site, then put into a 2 ml tube filled with 96% ethanol solution, labeled, and stored at 4ºC until DNA isolation was carried out. The materials used in the molecular work are aquadest, aquabidest, 96% ethanol, DNA extraction kit, reverse2 and forward2 primers, 1x TAE buffer, agarose, DNA template, 10X Taq buffer, dNTP Mix, taq DNA polymerase, Mg2+,ddH2O, DNA marker, loading dye, and diamond dye.

The tools used are scissors, tweezers, freezer, incubator, vortex, centrifuge, microcentrifuge, thermocycler, analytical balance, DNA marker, micropipette, 1.5 ml tube, PCR tube, electrophoresis, gel documentation, thermometer, pH meter, DO meter, spectrophotometer, secchi disk, titrimeter, TDS

meter, refractometer, current ball, and stopwatch.

2.1.1 Ethical approval

This study does not require ethical approval because it does not use experimental animals.

2.2 Method

2.2.1 DNA extraction

Each fin sample was cut to a size of about 2 mm^2. Total genomic DNA was extracted using a mini genomic DNA extraction kit for animal tissue (*Geneaid Biotech* Ltd). In general, DNA extraction consists of six stages, namely DNA sample preparation, cell lysis, RNAse treatment, DNA precipitation, washing and dissolving DNA. The DNA genome was visualized for the DNA band integrity through electrophoresis and the DNA samples were then stored in a freezer at -20ºC.

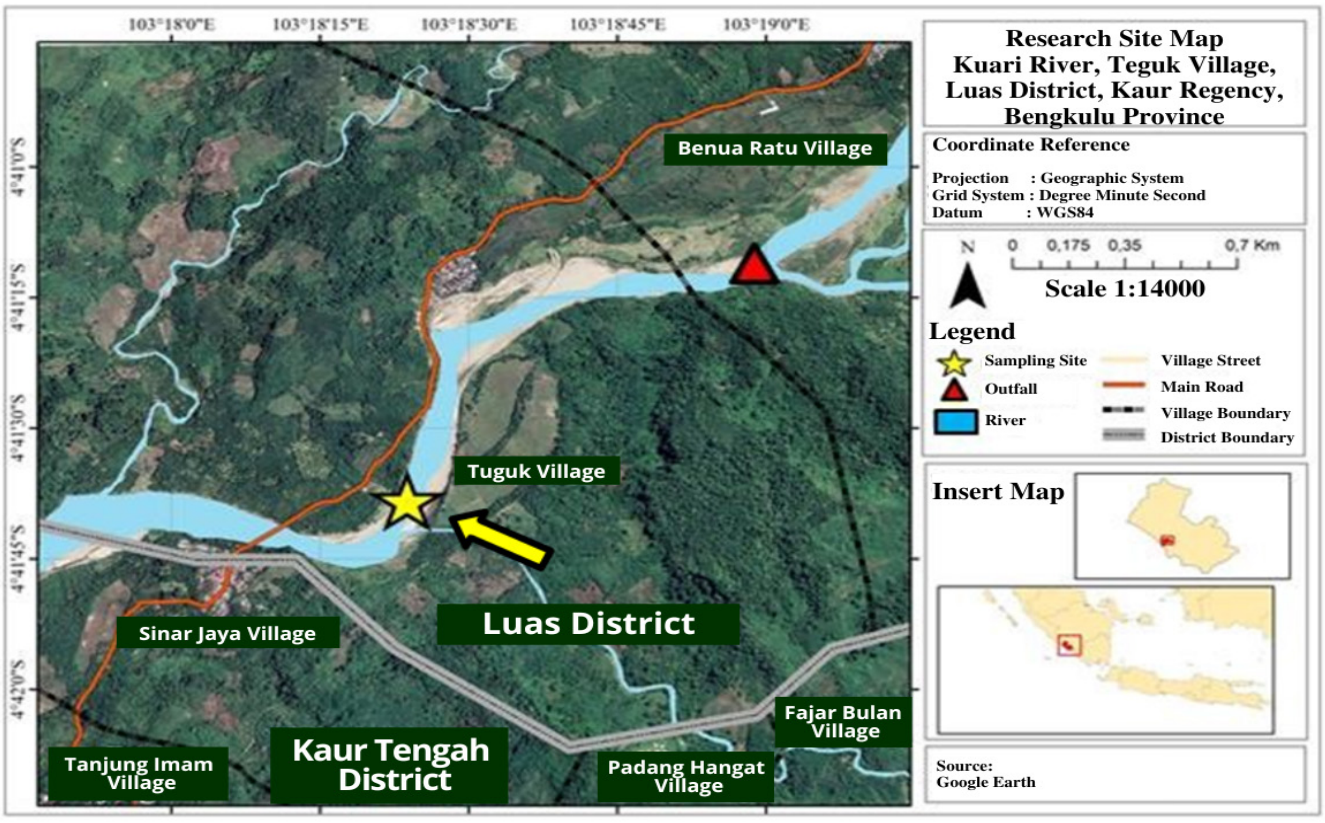

Figure 1. Research sites in the Bengkulu Kuari River.

2.2.2 DNA amplification

DNA amplification was conducted using the polymerase chain reaction (PCR) method. The *COI* gene of eel mitochondrial DNA target was derived from PCR with primer pairs FishF2-5' TCGACTAATCATAAAGATATCGGCAC3' and FishR25'ACTTCAGGGTGACCGAAGAATCAGAA 3'(Ward *et al.*, 2005). PCR was performed in a final volume of 50 µl. Each reaction contained 5 µl 10X Taq Buffer, 1 µl dNTP Mix, 1 µl FishF2 primer, 1 µl Fish R2 primer, 1 µl DNA template, 1 µl Taq DNA Polymerase, 8 µl Mg^{2+} and 32 µl ddH_2O. DNA amplification was carried out in stages: initiation cycle at 95°C for 1 minute, denaturation at 95°C for 30 seconds, annealing or primer attachment at 50°C for 30 seconds, extension or elongation at 72°C for 1 minute in 35 cycles and final elongation at 72°C for 7 minutes. Furthermore, the PCR products were visualized by electrophoresis using 1% agarose gel for 25 minutes. PCR products were visualized using a 1 kb marker. The eel DNA samples that were successfully amplified in the *COI* gene region by the PCR method were then sequenced in the Apical Scientific Sdn. Bhd. Malaysia through the services of the Institute of Genetics Science in Jakarta.

2.2.3 Water quality

Water quality samples were measured in three replications in each station, including temperature

(ºC) using a thermometer, pH media using a pH meter, dissolved oxygen (mg L^{-1}) with the DO meter, transparency (cm) using a secchi disk, ammonia (mg L^{-1}) using spectrophotometer, total alkalinity (mg L^{-1}) using titrimetric method, total dissolved solids (TDS) with TDS meter, salinity (ppt) using digital salinometer, and water current using a current ball. The water temperature, pH media, dissolved oxygen, transparency, total dissolved solid, salinity, and water current were measured in situ, meanwhile ammonia and total alkalinity were analyzed ex-situ in the Fisheries Basic Laboratory, Aquaculture Study Program, Universitas Sriwijaya.

2.3 Analysis Data

The *COI* sequences were saved in FASTA format, and were then manually edited and assembled using MEGA X.0 software. All the sequences have been deposited in the BOLDSYSTEMS in a project code ABM (Barcode Index Number BOLD:AAD2092 and BOLD:AEF5765) and GenBank Accession Numbers PP906109-PP906114. The similarity percentage of DNA sequences was determined based on BLAST (Basic Local Alignment Search Tool) analysis in the NCBI Genbank (National Center for Biotechnology Information). Furthermore, the phylogenetic tree between freshwater eels obtained from the Kuari River was constructed using the Neighbor-Joining (NJ) method of maximum composite likelihood model and the genetic distance was analyzed using the pairwise distance method p-distance model (Stecher *et al.*, 2020; Tamura *et al.*, 2021). Water quality data were analyzed descriptively in tabular form.

3. Results and Discussion

3.1. Results

3.1.1 Nucleotide Similarity

Two species of freshwater eels from Kuari River in Southern Sumatra were identified using the *COI* gene DNA marker. The nucleotide length of the COI gene of freshwater eel was 572 – 644 bp. The percentage of nucleotide similarity in BLASTn is presented in Table 2. Based on BLASTn, samples of AM 3 and AM 4 had the highest similarity of 99.82% and 100% to *Anguilla marmorata* from Manna River, Bengkulu (JQ665824.1). Meanwhile, samples AB 2, AB 3, AB 4, and AB 5 were found to be 100% similar to the species *Anguilla bengalensis* from the Ghats River, India (MF612058.1). The four samples also have high similarities with *Anguilla bicolor* from the Cisolok River, West Java (KU692247.1) by 99.22%-99.37%, and Kongsi River Malaysia (99.40%).

Table 1. Sample identification of eels based on morphological indicators.

No	Morphological indicators	*A. marmorata*	*A. bengalensis*
A	Morphology		
1	Fin length	long fin	long fin
2	Skin pattern	mottled skin	plain skin
3	Color of the back	mottled black	black
4	Color of the stomach	white	silver
B	Morphometric		
1	Total length (cm)	24.1; 44	29.3 - 35.7
2	Weight (g)	155; 200	161 - 189
C	Meristic		
1	Number of pectoral fin rays (P)	34; 68	30 - 54
2	Number of dorsal fin rays (D)	370; 410	398 - 438
3	Number of anal fin rays (A)	274; 374	318 - 382
4	Number of ventral fin rays (V)	0	0
5	Number of caudal fin rays (C)	28; 52	34 - 68

3.1.2 Genetic Distance and Phylogenetic

The genetic distance of the freshwater eels

from the Bengkulu Kuari River with other species is presented in Table 3. The genetic distance of samples AM 3 and AM 4 from the Kuari River Bengkulu showed a value of 0.002 (0.2%) and a range value of 0.002-0.007 (0.2-0.7%) to *Anguilla marmorata* as the data in Genbank. The genetic distance of samples AB 2, AB 3, AB 4, and AB 5 showed a value of 0.000 (0%)-0.008 (0.8%), and 0.003-0.012 (0,3-0.1.2%) to *Anguilla bengalensis* as per the data in Genbank. The phylogenetic tree of *Anguilla marmorata* and *Anguilla bengalensis* from the Kuari River Bengkulu are presented in Figure 2.

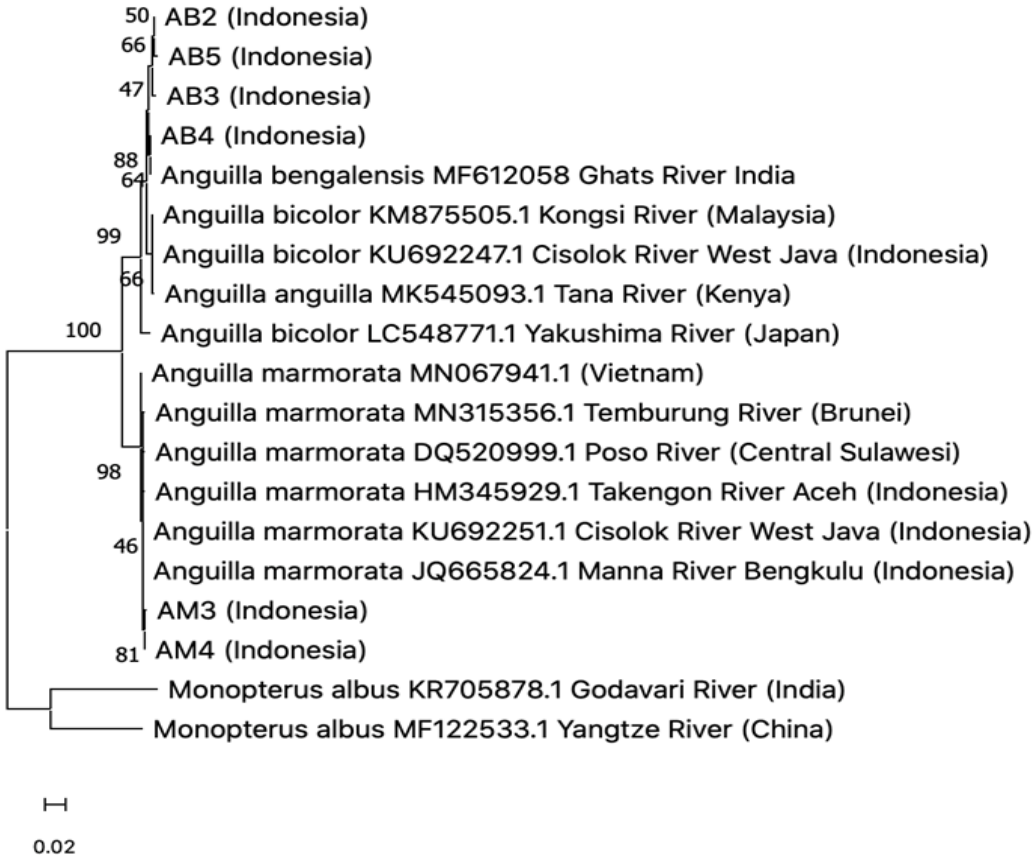

Figure 2. The phylogenetic of freshwater eels from the Kuari River Bengkulu.

3.1.3 Water Quality

Water quality measurements of freshwater eels habitat in the Bengkulu Kuari River are presented in Table 4. It is indicated that water temperature was in the range of 26.6-27.6°C, the pH value was 7.1-8.7, the transparency of 21-47 cm, with a dissolved oxygen content of 6.20-9.54 mg. L^{-1}. Ammonia measurements in the Kuari River ranged from 0.16-0.41 mg L^{-1}, total alkalinity of 20–52 mg. L^{-1}, the TDS value of 33–49 mg. L^{-1}, salinity 0.3-0.4 ppt, and the current speed of the river was 0.1-0.8 m. s^{-1}.

3.2. Discussion

The morphology characteristics and *COI* gene sequence of eels' mitochondrial DNA denoted two species of *A. marmorata* and *A. bengalensis* at Kuari River. Morphologically, the species *A bengalensis* has a plain skin, while, *A. marmorata* has patterned skin (striped) type. Based on the AD/TL value, *Anguilla bengalensis* eels has a long fin type and *Anguilla bicolor* has a short-finned type. Furthermore, *A. bengalensis* has skin with variegated markings, narrow maxillary bands of teeth and long dorsal fins, while *A. bicolor* has skin without variegated markings and short dorsal fins (Arai *et al.,* 2015). Differences in morphological and molecular identification may occur because morphological characteristics of the genus *Anguilla* are challenging due to species similarities, particularly in the young eel phase (elvers) (Watanabe *et al.,* 2008), for instance between *A. australis* with *A. obscura* and *A. bocolor bicolor* (Falah *et al.,* 2023).

Genetic distance is used to investigate a genetic relationship between species at Kuari River. This study reported that genetic distance between *A. marmorata* and *A. bengalensis* were 0.049-0.057 (4.9-5.7%). Another study reported the genetic divergence between *A. bicolor* and *A. marmorata* was 5.0%, *A. bicolor* and *A. bengalensis* was 6.7%, and between *A. marmorata* - *A. bengalensis* was 4.0% (Muchlisin *et al.,*

2017). *A. marmorata* and *A. bengalensis bengalensis* might have a panmictic-population structure (Arai and Taha, 2021), where all individuals are potential partners. The lower genetic distance denotes that the level of relationship is closer, whereas the higher the value of the genetic distance indicates that the level of relationship is farther away.

along with *Anguilla marmorata* from Manna River Bengkulu (JQ665824.1), Cisolok River West Java KU692251.1), Takengon River Aceh (HM345929.1), River Poso Central Sulawesi (DQ520999.1), Kongsi River Malaysia (KM875505.1), Temburung River Brunei (MN315356.1), and Thua Thien Hue RiverVietnam (MN067941.1) with bootstrap value of 99%.

Table 2. The highest percentage of nucleotide similarity of freshwater eels (*Anguilla* spp.) from the Kuari River Bengkulu.

No.	Sample Code	Description	Identity (%)	Access Code	Sample Origin
1.	AM 3	*Anguilla marmorata*	99.82	JQ665824.1	Manna River, Bengkulu
			99.82	KU692251.1	Cisolok River, West Java
2.	AM 4	*A. marmorata*	100	JQ665824.1	Manna River, Bengkulu
			100	KU692251.1	Cisolok River, West Java
3.	AB 2	*A. bengalensis*	100	MF612058.1	Ghats River,India
		A. bicolor	99.37	KU692247.1	Cisolok River, West Java
4.	AB 3	*A. bengalensis*	100	MF612058.1	Ghats River,India
		A. bicolor	99.37	KU692247.1	Cisolok River, West Java
5.	AB 4	*A. bengalensis*	99.84	MF612058.1	Ghats River, India
		A. bicolor	99.22	KU692247.1	Cisolok River, West Java
6.	AB 5	*A.bengalensis*	100	MF612058.1	Ghats River, India
		A. bicolor	99.40	KM875505.1	Kongsi River, Malaysia

The phylogenetic construction indicated two main clusters, namely the first cluster consisted of *Anguilla* spp., and the second cluster consists of *Monopterus albus*, a species outgroup, with a bootstrap value of 100%. A gene tree is the phylogeny of alleles or haplotypes for any specified stretch of DNA, either derived from components of population trees or species trees (Avise, 1989). The outgroup had more outlying related organisms to determine the evolutionary relationship of freshwater eels. The first cluster had two sub-clusters. AB2, AB3, AB4 and AB5 were in the first sub-cluster along with *Anguilla bicolor* from Kongsi River Malaysia (KM875505.1), Cisolok River West Java (KU692247.1), Yakushima River Japan (LC548771.1), *Anguilla anguilla* from Tana River Kenya (MK545093.1) and *A. bengalensis* from Ghats River India (MF612058). *Anguilla bicolor-Anguilla anguilla* and *A. bengalensis* were in a separate sub-cluster; however, the bootstrap value (bv) was low (64%). The greater the bootstrap value, the higher the level of confidence resulting from the phylogenetic tree reconstruction (Hillis and Bull, 1993). AM3 and AM4 were in a second sub-cluster

Anguilla marmorata kinship was associated with the area that borders the sea. *A. marmorata* has spread in areas bordering the sea in Indonesia, such as on the West Coast of Sumatra Island, South Coast of Java Island, East Coast of Kalimantan Island, the entire coast of Sulawesi, Maluku Islands, West Nusa Tenggara, Southeastern East and North Coast of Papua (Affandi, 2005). In Thua Thien Hue Vietnam, the population of *Anguilla marmorata* eels is divided into two separate groups that are guided by the migration process and specific ecological (Huyen and Linh, 2020). Based on the strontium to calcium (Sr:Ca) ratio analyses, *A. marmorata* prefer freshwater rather than I marine water (Briones *et al.*, 2007). Another study indicated all localities of *A. marmorata* in Indonesia, Japan and Vietnam showed various migratory patterns (Arai and Chino, 2018), while *A. bengalensis bengalensis* would settle in the freshwater environment for 6–9 years until starting their downstream migration to the open ocean. The *Anguilla bengalensis* species from this study was closely related to the *A. bengalensis* from the Indian

Ghats River (MF612058.1). This kinship was due to the fact that, in the western part of Bengkulu Province, waters are bordered by Indian Ocean waters (Bengkulu Provincial Government, 2021). The very close genetic relationship between species is also caused by the migration pattern of most tropical eels, which is different from eels in other climates (Arai and Taha, 2021). *Anguilla bengalensis* has spread in Indonesian waters, throughout the Indian Ocean and the Pacific Ocean (Wibowo *et al.*, 2021). *A. bengalensis* has been found in upstream of northwestern part of Peninsular Malaysia with no tidal effect, where salinity ranged from 0.01 to 0.03 psu, water temperatures ranged from 23.6 to 28.1°C (Arai *et al.*, 2020). Meanwhile, *A. marmorata* are estuarine residents (Arai and Chino, 2018); however, in the Philippines and Taiwan it was more than 70% freshwater-oriented (Briones *et al.*, 2007). The water quality observed, i.e. temperature, pH values, brightness, dissolved water oxygen, ammonia, total alkalinity and TDS value, were still within good tolerance for fish survival and growth based on the water quality standard in Table 4. Meanwhile, the current speed was classified as a slow to fast river current. In this study, there is no extreme value of water quality; therefore, it might not influence the genetic disruption in the freshwater eels. The extreme changes in water quality can result in damage to the DNA structure caused by an increase in harmful chemicals in waters (Kultz, 2005).

Table 3. The highest percentage of nucleotide similarity of freshwater eels (*Anguilla* spp.) from the Kuari River Bengkulu.

No	Spesies	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	AM3 (Indonesia)																			
2	AM4 (Indonesia)	0.002																		
3	AB2 (Indonesia)	0.049	0.047																	
4	AB3 (Indonesia)	0.049	0.047	0.008																
5	AB4 (Indonesia)	0.049	0.057	0	0.006															
6	AB5 (Indonesia)	0.049	0.057	0.002	0.006	0.018														
7	*Anguilla marmorata* JQ665824.1	0.003	0.002	0.047	0.047	0.047	0.047													
8	*Anguilla marmorata* KU692251.1	0.003	0.002	0.047	0.053	0.044	0.052	0												
9	*Anguilla bengalensis* MF612058	0.049	0.046	0.003	0.009	0	0.012	0.047	0.044											
10	*Anguilla bicolor* KU692247.1	0.051	0.048	0.009	0.016	0.006	0.017	0.048	0.046	0.006										
11	*Anguilla marmorata* MN067941.1	0.003	0.002	0.048	0.055	0.045	0.056	0	0	0.046	0.048									
12	*Anguilla marmorata* DQ520999.1	0.005	0.003	0.049	0.055	0.045	0.054	0.002	0.002	0.046	0.048	0.002								
13	*Anguilla marmorata* HM345929.1	0.005	0.003	0.05	0.056	0.046	0.057	0.002	0.002	0.047	0.049	0.003	0.003							
14	*Anguilla marmorata* MN315356.1	0.007	0.005	0.05	0.056	0.047	0.057	0.003	0.003	0.047	0.049	0.003	0.005	0.005						
15	*Anguilla anguilla* MK545093.1	0.052	0.058	0.008	0.008	0.022	0.009	0.05	0.048	0.008	0.002	0.049	0.049	0.051	0.051					
16	*Anguilla bicolor* KM875505.1	0.051	0.05	0.009	0.016	0.009	0.018	0.048	0.046	0.006	0	0.048	0.048	0.05	0.049	0.003				
17	*Anguilla bicolor* LC548771.1	0.051	0.048	0.019	0.025	0.016	0.026	0.048	0.047	0.015	0.02	0.048	0.048	0.049	0.05	0.022	0.02			
18	*Monopterus albus* KR705878.1	0.229	0.229	0.229	0.231	0.226	0.233	0.225	0.224	0.228	0.229	0.22	0.225	0.22	0.223	0.231	0.228	0.228		
19	*Monopterus albus* MF122533.1	0.217	0.219	0.221	0.226	0.219	0.227	0.215	0.211	0.217	0.221	0.206	0.21	0.208	0.212	0.226	0.22	0.219	0.169	

Description: The number of base differences per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). This analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 933 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Stecher *et al.*, 2020; Tamura *et al.*, 2021)

Table 4. Water quality characteristic of the Bengkulu Kuari River.

No.	Parameter	Value	Tolerable range
1	Temperature (°C)	26.6-27.6	24-30 (Fekri et al., 2018
2	pH (unit)	7.1-8.7	6-8 (EFSA, 2009)
3	DO (mg L^{-1})	6.20-9.54	> 5 (Mallya, 2007)
4	Transparency (cm)	21-47	42 (Government Regulation of Republic Indonesia No. 82, 2001)
5	Ammonia (mg L^{-1})	0.16-0.41	<0.5 (Sadler,1981)
6	Total alkalinity (mg L^{-1})	20-52	5-500 (Lawson, 1995)
7	TDS (mg L^{-1})	33-49	< 1,000 (Government Regulation of Republic Indonesia No. 82, 2001)
8	Salinity (ppt)	0.3-0.4	0-35 psu (Han et al., 2012)
9	Current (m s^{-1})	0.1-0.8	0.6-1.2 (Jellyman & Arai, 2016)

4. Conclusion

The nucleotide sequences of the COI gene of freshwater eels from the Kuari River Bengkulu (AM3 and AM4) indicated 100% similarity to *A. marmorata* from Manna River Bengkulu with close genetic distance 0.003, meanwhile AB 2, AB 3, AB 4 and AB 5 were 100% similar to *A. bengalensis* from the Indian Ghats River (MF612058.1) with genetic distance 0.003. Phylogenetic freshwater eels from the Kuari River form two separate sub-clusters with genetic distance of 0.047 between two species. The first sub-cluster consists of AM 3, AM 4, with *A. marmorata* from the Manna River Bengkulu (JQ665824.1) and the Cisolok River, West Java (KU692251.1). The second sub-cluster consists of AB 2, AB 3, AB 4, and AB 5 with *A. bengalensis* from the Indian Ghats River (MF612058.1).

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Authors' Contributions

The conception and design of the study: MSF; the acquisition of data, or the analysis and interpretation: AM, MW; Funding Acquisition: MSF, writing-original draft: AM, MW; writing-review and editing: all authors.

Conflict of Interest

The authors declare no competing interests.

Declaration of Artificial Intelligence (AI)

The author(s) affirm that no artificial intelligence (AI) tools, services, or technologies were employed in the creation, editing, or refinement of this manuscript. All content presented is the result of the independent intellectual efforts of the author(s), ensuring originality and integrity

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