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DNA Barcoding, Phylogenetic Analysis, and Haplotype Characteristic of Mungkus Fish (Genus *Sicyopterus*) Existing in Eight Main Rivers in Bengkulu Province, Indonesia

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ABSTRACT

Mungkus fish is a culturally and economically important fish in Bengkulu. However, the accurate species name of the existing Mungkus fish in Bengkulu province remains unclear. Our research aimed to accurately identify the species of Mungkus fish by using a molecular approach. The DNA Barcoding method, using the Cytochrome c oxidase subunit 1 (COI) gene sequence, was employed for species identification and phylogenetic analysis. The DNA of 12 fin tissue samples, chosen from eight main rivers, was extracted using the Phenol-Chloroform method. The target DNA was amplified using the paired primers Fish-BCL and Fish-BCH before sequencing. The sequence data were then analyzed by using bioinformatic tools. This study successfully identifies three species of Mungkus fish in Bengkulu (*Sicyopterus lagochepalus, Sicyopterus squamossissimus,* and *Sicyopterus cynocephalus*), with similarity to GenBank data> 99.00%. ASAP analysis supports the species delimitation, which clustered into three species. Bayesian Inference phylogenetic analysis clearly resolved all samples into three distinct clades. The analysis also unveiled clear haplotype divergence among *Sicyopterus* species, indicating both shared and species-specific genetic lineages. Close genetic affinity was observed among *S. lagocephalus* populations from Bengkulu, Maluku, and the Philippines, whereas *S. cynocephalus* showed regional differentiation from Gorontalo populations. These findings provide taxonomic clarity that is fundamental for future sustainable management and conservation planning of Mungkus fisheries in Bengkulu Province.

1. Introduction

The mungkus fish, a local name of the genus *Sicyopterus* (subfamily Sicydiinae), inhabits fast-flowing rivers and streams in Bengkulu. It can be found in Sumatra (Anggraini *et al.* 2018; Lestari *et al.* 2021; Putra *et al.* 2021), Java and Bali (Keith *et al.* 2014), Sulawesi (Nurjirana *et al.* 2019; Muthiadin *et al.* 2020; Astuti *et al.* 2022), and Papua (Keith *et al.* 2011). These are amphidromous gobies that reproduce in a freshwater environment (Valade *et al.* 2009; Pouil and Colsoul 2021). They have a unique reproductive strategy in which they spawn within rivers, with eggs attaching to the undersides of large rocks (Nurjirana *et al.* 2021). Adult fish live in the rivers, but larvae are carried to the estuary after hatching (Anggraini *et al.* 2018). The post-larvae migrate back to the river to grow and reproduce. Local people capture these post-larval fishes as a distinctive and traditional food source. Consequently, they hold high economic value for local

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people living near the river (Pangemanan *et al.* 2020; Lestari *et al.* 2021; Putra *et al.* 2021). Our direct survey in the traditional market in South Bengkulu found that the price reached Rp. 25,000.00/group (5-7 adult fish) and Rp. 20,000.00/small cup of post larvae. The local people did not sell them by weight. Mungkus fish has also become a mascot of Kaur Regency, known as a source of high-quality fish in Bengkulu Province. Local people introduced the cuisine named "Gulai Mungkus" as the traditional cuisine from South Bengkulu and Kaur (Mentari *et al.* 2025). Therefore, mungkus has become economically and culturally important in Bengkulu.

Specific studies on the diversity of Mungkus fish in Bengkulu Province have not been well developed. Fundamental information about Mungkus fish, including species diversity, distribution, population structure, reproduction, and other ecological aspects, remains very limited. Therefore, such studies are essential for developing effective conservation strategies and sustainable fisheries management to prevent them from the population decline that is caused by overfishing and massive fishing of larval stage fish, locally called "ipun" (Anggraini *et al.* 2018). A previous study about species identification of Mungkus fish in Indonesia revealed several species exist, which include *S. cynocephalus, S. longifilis, Sicyopterus squamosissimus, S. microcephalus, S. lagocephalus, S. micrurus,* and *S. hageni* (Keith *et al.* 2005, 2015; Olii *et al.* 2019; Sahami and Habibie 2021). Additionally, a study by Keith *et al.* discovered five new species from Papua and Papua New Guinea: *S. calliochromus, S. erythropterus, S. lengguru, S. ocellaris,* and *S. stiphodonoides* (Keith *et al.* 2011). Most of them were identified morphologically.

Morphological analysis has been traditionally used for species identification. However, it often failed to report cryptic species and intraspecific variation (Bingpeng *et al.* 2018). Thus, molecular identification becomes essential to the current demand for species diversity assessment through DNA barcoding. The application of DNA barcoding, particularly focusing on the cytochrome oxidase I (COI) gene fragment, can be highly beneficial for accurate species identification (Coissac *et al.* 2016; Antil *et al.* 2023). This method involves sequencing a small fragment of the mitochondrial COI gene, approximately 655 base pairs in length, which has become a standard marker for identifying eukaryotic species, including fish (Hebert *et al.* 2003; Chac and Thinh 2023). To date, DNA barcoding using COI is widely used by researchers for species identification (Sadurudeen *et al.* 2017; Bingpeng *et al.* 2018; Dailami *et al.* 2019; Fadli *et al.* 2020; Praveenraj *et al.* 2022; Roesma *et al.* 2023; Indrayani *et al.* 2024). DNA barcoding is characterized by low intraspecific variation and high interspecific variation, which is crucial for assessing genetic diversity within and between populations. Additionally, the method's ability to identify cryptic species that are morphologically similar but genetically distinct could be vital for unveiling the diversity within fishes (Lord *et al.* 2012a). Therefore, incorporating DNA barcoding and haplotype characterization into this research could significantly enhance the accuracy and reliability of species identification, providing deeper insights into the genetic relationships and diversity of Mungkus fish in Bengkulu.

Although it was assumed that there are several species of Mungkus fish in Bengkulu Province, there is no accurate information to support this assumption. The present study will address this ambiguity by providing comprehensive genetic profiles of Mungkus fish populations across several rivers in Bengkulu to support future conservation and aquaculture, thereby preventing the scarcity of Mungkus fish caused by overfishing and habitat destruction.

2. Materials and Methods

2.1. Taxon Sampling

Adult fish samples were collected from eight main rivers along Bengkulu Province that are commonly found with Mungkus fish, based on the information from local people: Batang Muar River (BMR), Nokan River (NKN), Lubuk Banyau River (LBY), Kedurang River (KRD), Nipis River (NPS), Padang Guci River (PGC), Kinal River (KNL), and Maras River (MRS) (Table 1). Those are located from north to south in Bengkulu (Figure 1, the sampling site map). Samples were preserved in 95% ethanol and then stored in the Laboratory of Biomolecular at the University of Bengkulu for further work.

2.2. DNA Extraction and PCR Amplification

We extracted Genomic DNA from 12 fish fin samples using the phenol–chloroform method. Fin tissue was digested in TNES urea buffer containing Proteinase K, incubated at 55°C, then extracted with phenol-chloroform-isoamyl alcohol, and centrifuged to separate phases. DNA in the aqueous phase was precipitated with ethanol in the presence of sodium acetate, air-dried, and resuspended in Tris-EDTA (TE) buffer for storage at 4°C. For molecular identification,

Table 1. List of sampling sites and coordinate information

Sample code	River name	Coordinate	Regency
MKS_1 BMR	Batang Muar	S 02° 58.451, E 101° 35.343	Muko-Muko
MKS_2 LBY	Lubuk Banyau	S 03° 21.186, E 102° 04.241	North Bengkulu
MKS_3 NKN	Nokan	S 03° 24.223, E 102°10.408	North Bengkulu
MKS_4 MRS	Maras	S 04° 15.242, E 102° 47.110	Seluma
MKS_5 KDR	Kedurang	S 03° 24.225, E 102° 10.409	South Bengkulu
MKS_6 KDR	Kedurang	S 03° 24.225, E 102° 10.409	South Bengkulu
MKS_7 NPS	Nipis	S 04° 26.089, E 103° 01.100	South Bengkulu
MKS_8 NPS	Nipis	S 04° 26.089, E 103° 01.100	South Bengkulu
MKS_9 KNL	Kinal	S 04° 39.802, E 103°14.930	Kaur
MKS_10 KNL	Kinal	S 04° 39.802, E 103°14.930	Kaur
MKS_11 PGC	Padang guci	S 04° 35.010, E 103° 08.556	Kaur
MKS_12 PGC	Padang guci	S 04° 35.010, E 103° 08.556	Kaur

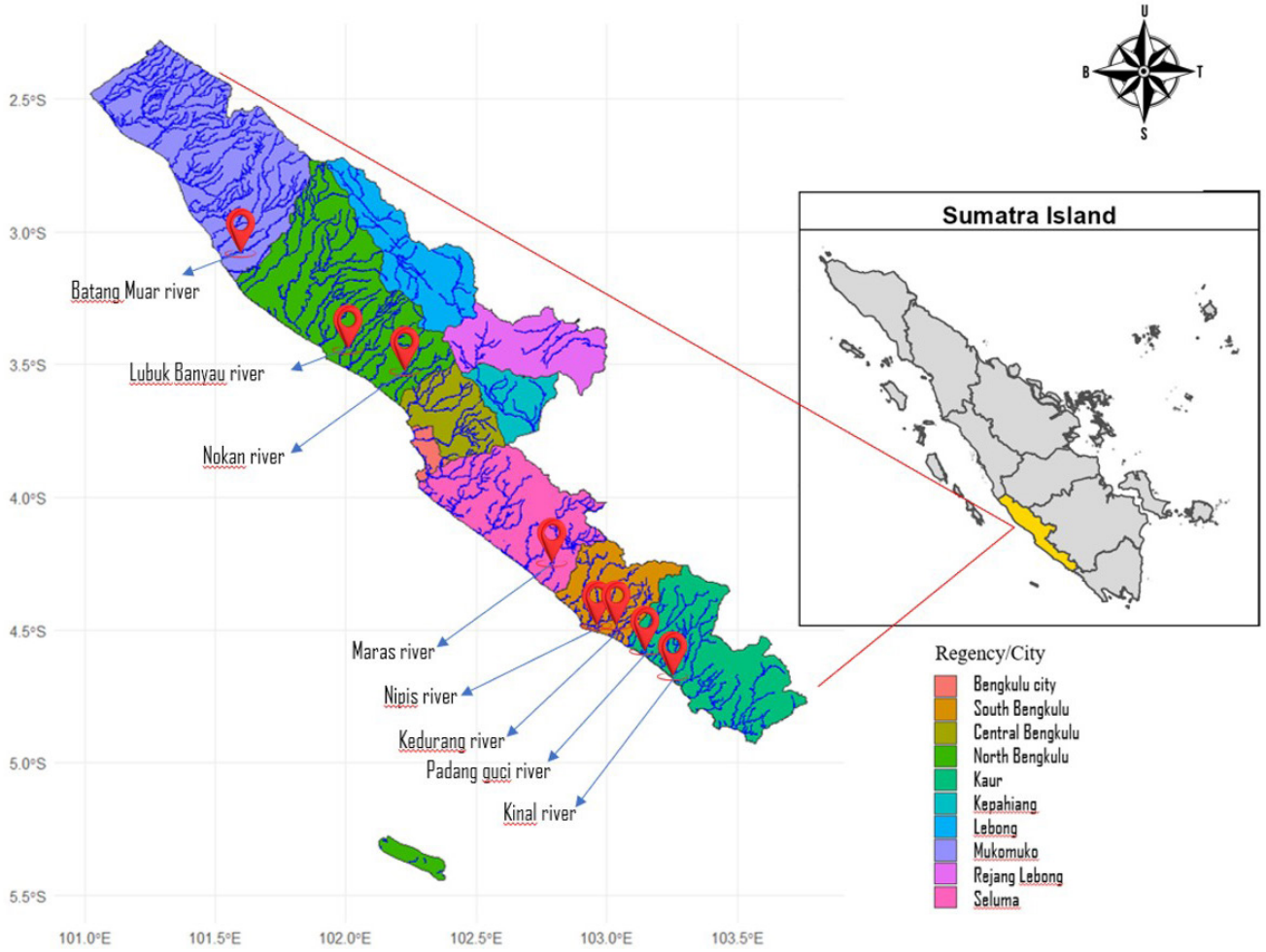

Figure 1. Sampling site map of mungkus fish in Bengkulu Province (created in R Studio)

the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified by PCR using the forward Fish-BCL (5'-TCAACCAACCACAAAGACATTGG CAC-3') and reverse Fish-BCH (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') primer pair (Dailami *et al.* 2019; Suryani *et al.* 2021). PCR amplification was carried out in a thermal cycler with the following cycling conditions: an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 2.5 minutes. A final extension was performed at 72°C for 10

minutes to ensure complete elongation of all amplified products. PCR products were held at 4°C and verified by agarose gel electrophoresis (2% agarose in 0.5× TBE) with ethidium bromide staining and UV visualization.

2.3. Sequencing and Data Analysis

The PCR product was sent to a sequencing company (PT. Genetika Sains Indonesia). The forward and reverse sequences were checked and assembled in Geneious Prime 2025.0.1 and identified via BLASTn against the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov). The sequences were deposited in GenBank under accession numbers PX091156.1–PX091163.1 and PX091362.1–PX091365.1 (Table 2). All sequences were aligned using Geneious Alignment with the default settings. The final alignment was then imported into MEGA 11 (Tamura *et al.* 2021) to calculate conserved sequences, sites of variation, and pairwise genetic distances under the Kimura 2-parameter (K2P) model. Gaps and missing data were completely deleted to maintain consistency across comparisons. These distance values were then used to evaluate genetic divergence within and between species. MEGA K2 thresholds (2% rule) and the ASAP tool were used for species delimitation (Puillandre *et al.* 2021). Phylogenetic analysis was performed with Bayesian Inference (BI) in BEAST v1.10.4 (Hill and Baele 2019). Model Finder IQ-TREE v2.3.6, based on Maximum Likelihood estimation, determined HKY+G+I as the evolutionary model. BEAUti v1.10.4 was employed to prepare an input file for BEAST (speciation: Yule process, uncorrelated relaxed clock, and MCMC chains 100 million generations) (Drummond *et al.* 2012). Convergence and ESS were assessed in Tracer v1.7.2, and the maximum clade credibility (MCC) tree was generated in Tree Annotator with 10% burn-in and visualized in FigTree v1.4.4 (Rambaut *et al.* 2018; Rambaut 2018). In addition, the haplotype characteristics of fish were identified using the DnaSP program (Librado and Rozas 2009) and COI sequence references from the GenBank database (Attachment).

3. Results

The 12 samples from eight main rivers have been successfully extracted by using the Phenol-Chloroform technique. Then, PCR amplification of the COI genes showed that the amplicons were well-enriched and produced visible bands under UV light, with a size of approximately 700 bp (Figure 3). It means that the Fish BCL-fish BCH primer successfully amplified the COI sequences of Mungkus fish. This step is essential before sequencing, as it verifies that the COI amplicons are of high quality and concentration for accurate downstream analysis.

3.1. BLASTn Result

The BLASTn analysis of the 12 sequences produced clear and consistent results. All sequences showed very high similarity with *Sicyopterus* species in GenBank, with query coverage between 98–100% and similarity values above 99%. The matches were highly significant, as reflected by e-values of 0. Among the samples, four were identified as Sicyopterus squamosissimus (MKS_1_BMR, MKS_3_NKN, MKS_4_MRS, MKS_7_NPS and MKS_12_BMR), four samples are similar to Sicyopterus cynocephalus (MKS_2_LBY, MKS_6_KDR, MKS_8_NPS, and MKS_11__PGC), and three samples were identified as Sicyopterus lagocephalus (MKS_5_KDR, MKS_9_KNL, and MKS_10_KNL) (Figure 2). The sequence lengths, ranging from 652 to 667 bp, confirm that the

Table 2. The best match of the BLAST result from NCBI Genbank for the fish sequences

Sample name	Sample accession number	Query cover (%)	Similarity (%)	e-value	Accession length	Reference species from GenBank	Reference Accession Number
MKS_1_BMR	PX091362.1	98	99.84	0	652	*Sicyopterus squamosissimus*	PP132378.1
MKS_2_LBY	PX091156.1	98	100.00	0	667	*Sicyopterus cynocephalus*	MT706640.1
MKS_3_NKN	PX091157.1	98	100.00	0	652	*Sicyopterus squamosissimus*	PP132683.1
MKS_4_MRS	PX091363.1	98	99.84	0	652	*Sicyopterus squamosissimus*	PP132378.1
MKS_5_KDR	PX091364.1	99	100.00	0	625	*Sicyopterus lagocephalus*	PP132654.1
MKS_6_KDR	PX091365.1	100	99.80	0	667	*Sicyopterus cynocephalus*	MT706640.1
MKS_7_NPS	PX091158.1	99	99.39	0	658	*Sicyopterus squamosissimus*	PP132403.1
MKS_8_NPS	PX091159.1	98	100.00	0	652	*Sicyopterus cynocephalus*	PP132726.1
MKS_9_KNL	PX091160.1	98	100.00	0	652	*Sicyopterus lagocephalus*	PP132505.1
MKS_10_KNL	PX091161.1	100	99.85	0	679	*Sicyopterus lagocephalus*	HQ639041.1
MKS_11_PGC	PX091162.1	100	99.84	0	667	*Sicyopterus cynocephalus*	MT706640.1
MKS_12_PGC	PX091163.1	98	99.84	0	652	*Sicyopterus squamosissimus*	PP132378.1

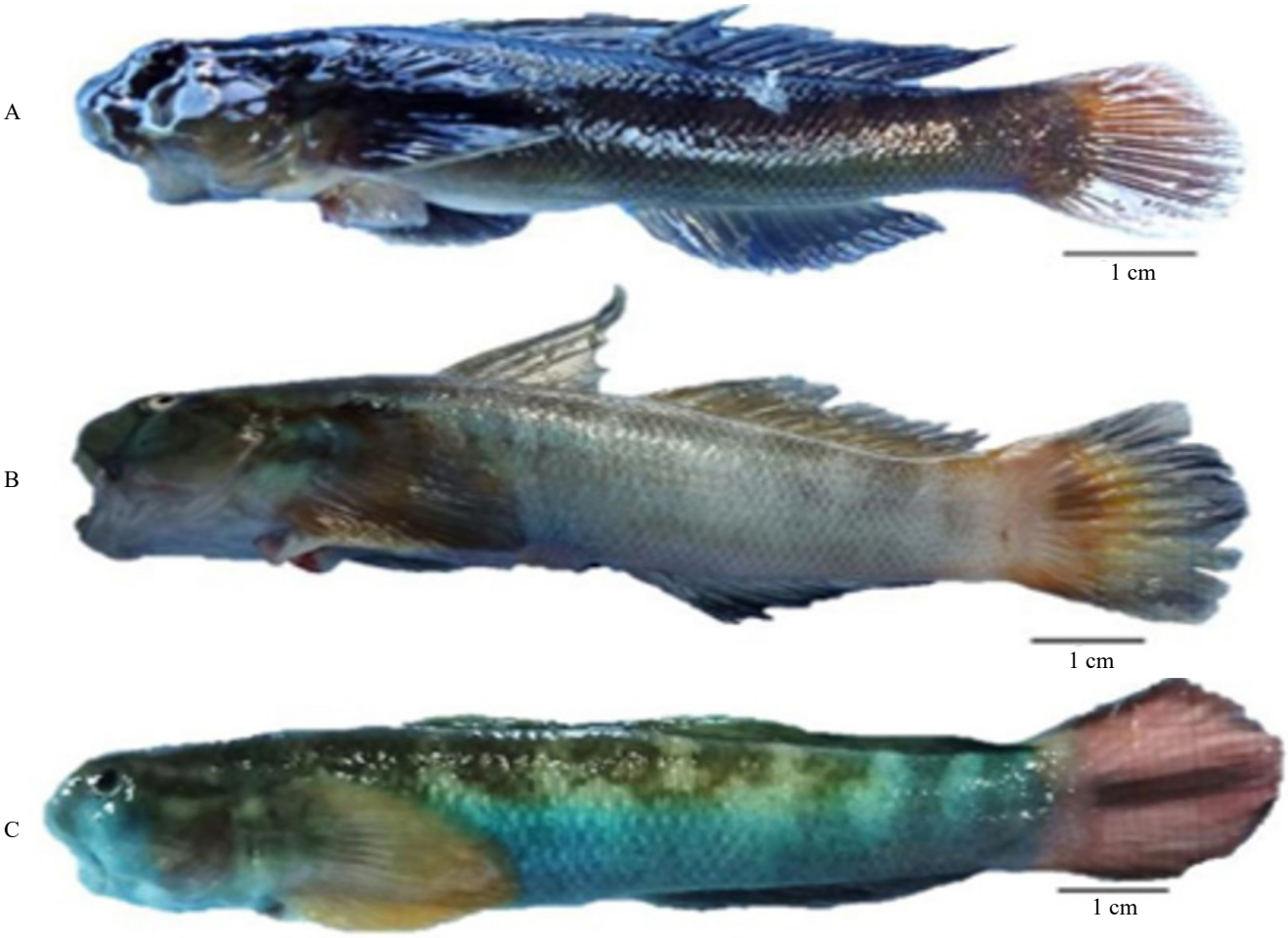

Figure 2. Sample photo of mungkus fish collected from the rivers in Bengkulu, (A) *S. squamosissimus*, (B). *S. cynocephalus*, and (C) *S. lagocephalus*

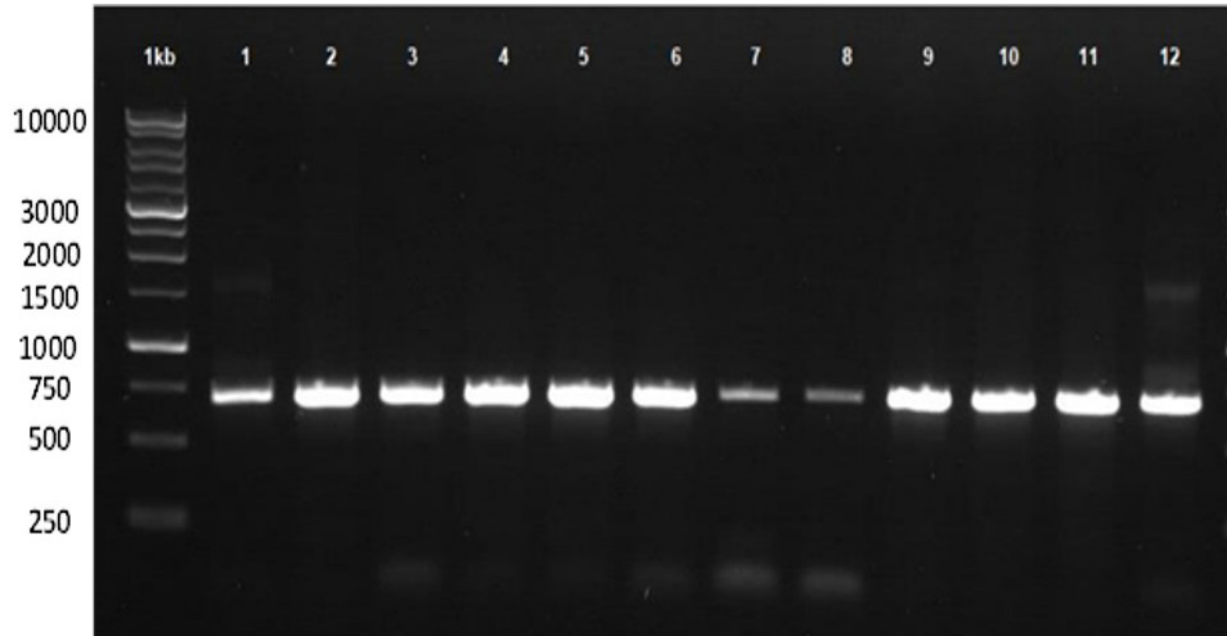

Figure 3. PCR amplification of the cytochrome oxidase subunit I (COI) gene (~650 bp) from Mungkus fish samples using the primers FishBCL and FishBCH under UV light

COI region was successfully amplified and sequenced. Taken together, these results show that DNA barcoding with COI is a reliable tool for distinguishing closely related *Sicyopterus* species, and highlight the presence of multiple species across the rivers in Bengkulu.

3.2. Genetic Distance and Species Delimitation

Genetic distance analysis was performed using pairwise distance in the MEGA 11 program. The genetic distance data among samples are shown in Table 3. The mean intraspecific genetic distances (K2P) were consistently low across all examined species, ranging from 0.003 in *S. cynocephalus* (n = 4) to 0.0093 in *S. lagocephalus* (n = 3), with *S. squamosissimus* showing an intermediate value of 0.0062 (n = 5). In contrast, interspecific distances were markedly higher, with *S. cynocephalus* vs. *S. lagocephalus* averaging 0.0466, *S. cynocephalus* vs. *S. squamosissimus* averaging 0.0975, and *S. lagocephalus* vs. *S. squamosissimus* reaching 0.1036.

Figure 4 illustrates that the intraspecific genetic distance of the three species is below 2%. It means that the samples belong to the three species with high confidence. The distance among the three species was more than 4 %. These higher values indicate clear genetic divergence among species. Overall, the results demonstrate a distinct separation between intraspecific and interspecific genetic distances, revealing a clear "barcode gap". It confirms that species delimitation based on the K2P 2% rule aligns with the BLAST result.

Table 3. Pairwise genetic distance of the COI sequence of the mungkus fish

Sample code	1	2	3	4	5	6	7	8	9	10	11	12
MKS_1_BMR												
MKS_2_LBY	0.093											
MKS_3_NKN	0.008	0.098										
MKS_4_MRS	0.008	0.102	0.004									
MKS_5_KDR	0.102	0.043	0.107	0.102								
MKS_6_KDR	0.096	0.002	0.100	0.105	0.045							
MKS_7_NPS	0.008	0.093	0.012	0.012	0.107	0.096						
MKS_8_NPS	0.095	0.002	0.100	0.104	0.045	0.004	0.095					
MKS_9_KNL	0.100	0.049	0.100	0.096	0.006	0.051	0.105	0.051				
MKS_10_KNL	0.102	0.051	0.103	0.098	0.012	0.053	0.107	0.053	0.010			
MKS_11_PGC	0.096	0.002	0.100	0.105	0.045	0.004	0.096	0.004	0.051	0.053		
MKS_12_PGC	0.002	0.096	0.006	0.006	0.105	0.098	0.006	0.098	0.102	0.105	0.098	

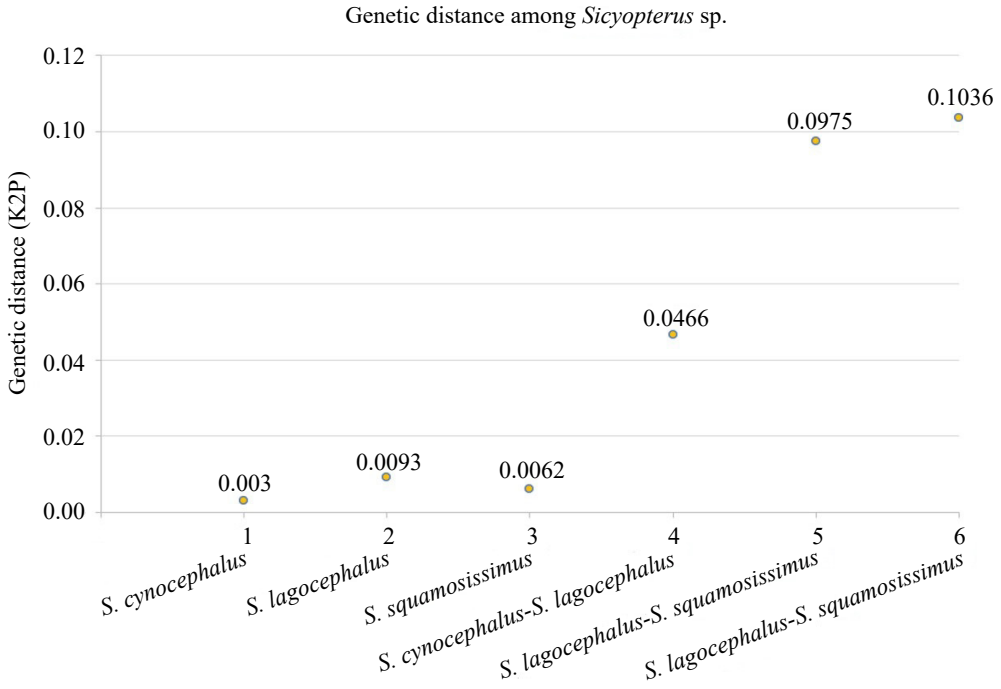

Figure 4. Genetic distance of three mungkus fish species (*S. cynocephalus, S. squamosissimus,* and *S. cynocephalus)*

ASAP species delimitation based on COI sequences supported a clear separation of the fish samples into three primary molecular operational taxonomic units (MOTUs) that were congruent with reference sequences and the phylogenetic clustering pattern. Specifically, the sequences grouped with reference lineages of *S. cynocephalus, S. lagochephalus,* and *S. squamosissimus*, while the outgroup (*Channa punctata*) formed a distinct, well-separated cluster (Supplementary Figure 1, 2, and Supplementary Table 1). Across the highest-ranked ASAP partitions, the three clades remained stable, indicating a pronounced barcode gap among these lineages and supporting their recognition as distinct species-level entities within the dataset.

3.3. Phylogenetic Tree Analysis

The Bayesian phylogenetic tree based on COI sequences clearly resolved the relationships among the samples and their closest reference taxa from GenBank (Figure 5). The sequences clustered consistently with their reference species. *Channa punctata* was used as the outgroup, providing a robust rooting for the phylogeny. Three samples (MKS_5_KDR, MKS_9_KNL, and MKS_10_KNL) were grouped with *Sicyopterus lagocephalus* at high posterior probability (PP), indicating strong evidence of conspecificity. Similarly, four samples (MKS_2_LBY, MKS_6_KDR, MKS_8_NPS, and MKS_11_PGC) clustered within the *S. cynocephalus* clade, supported by both high

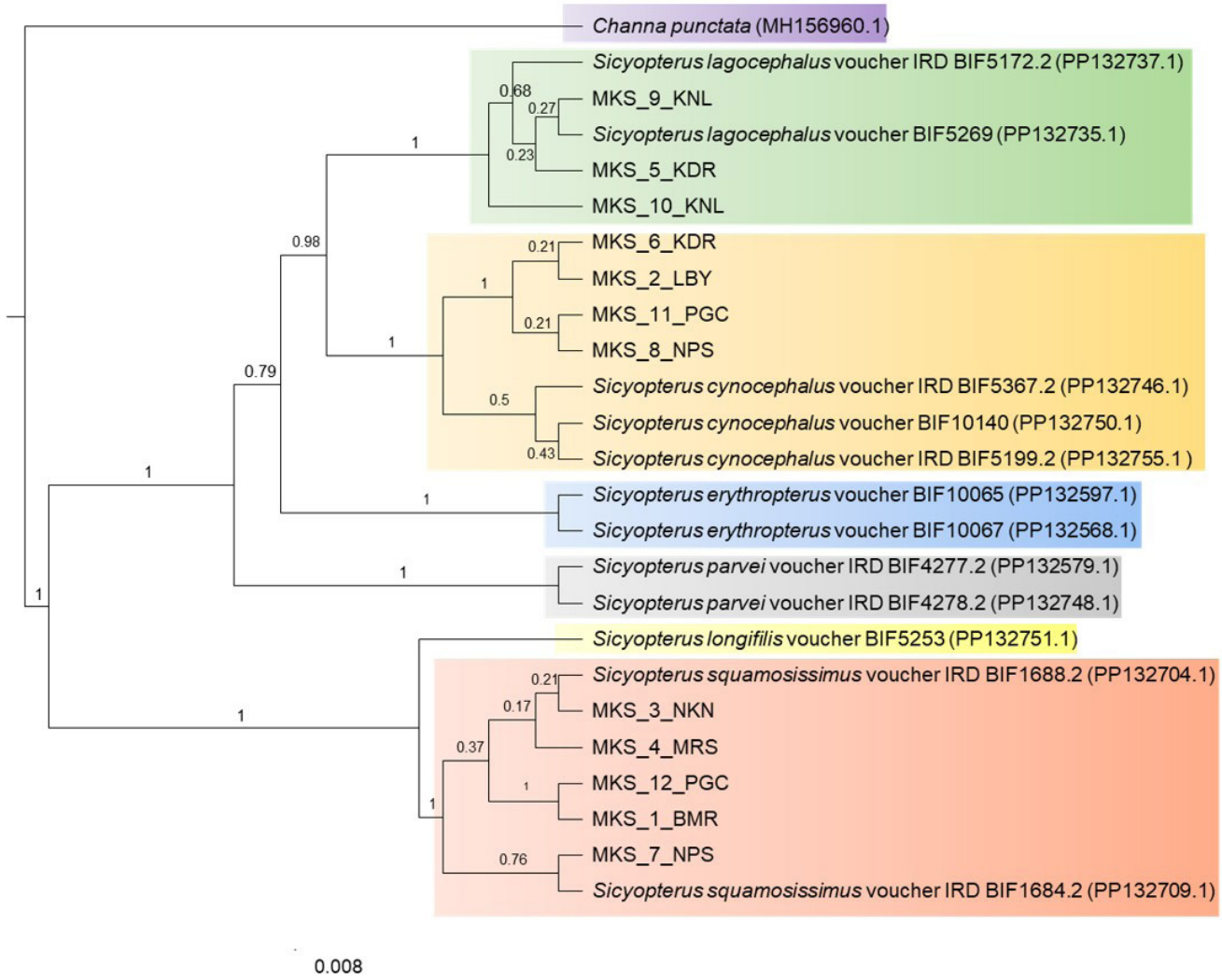

Figure 5. Bayesian inference phylogenetic tree of *Sicyopterus* species based on mitochondrial COI sequences, constructed in BEAST v1.10.4 and visualized in FigTree v1.4.4. Posterior probability values are shown at each node. *Channa punctata* (MH156960.1) was used as the outgroup. Colored clades indicate distinct species groups, each supported by ASAP analysis. Branch lengths are proportional to the number of substitutions per site

sequence similarity and robust nodal support values. Five additional samples (MKS_1_BMR, MKS_3_NKN, MKS_4_MRS, MKS_7_NPS, and MKS_12_PGC) were positioned within the *S. squamosissimus* clade. The clustering pattern across all clades showed congruence between the molecular results of this study and the reference sequences from GenBank.

Interestingly, the phylogeny also resolved additional *Sicyopterus* species from GenBank, including *S. erythropterus, S. parvei,* and *S. longifilis,* as distinct lineages separate from the samples. This reinforces the use of COI barcoding for distinguishing closely related species within the genus. The genetic distinctiveness among clades was consistent with the observed barcode gap (Figure 3), in which interspecific divergence (4.6–10.3%) was much higher than intraspecific variation (<1%). In short, these results validate the COI marker as an effective tool for species delimitation within the genus *Sicyopterus*.

3.4. Haplotype Characteristic

The haplotype characteristics of *Sicyopterus* fish species in Bengkulu showed divergence, with 4 haplotypes in *S. cynocephalus*, 3 in *S. lagocephalus*, and 4 in *S. squamosissimus* (Table 4). Involving the COI sequence references from the GenBank database, a total of 6 haplotypes of *S. cynocephalus*, 12 haplotypes of *S. lagocephalus*, and 6 haplotypes of *S. squamosissimus* were obtained in the present study. Compared to the COI sequence references, *S. lagocephalus* from Bengkulu, Maluku, and the Philippines had a close genetic relationship and were classified as haplotype 1 (H1). *S. lagocephalus* from Bengkulu and Maluku are also classified in the H3 group. *S. squamosissimus* from Bengkulu with H1 type was close to the same fish species in West Sumatra. Nonetheless, no close genetic relationship between *S. cynocephalus* at Bengkulu and Gorontalo is indicated by COI sequence diversity. However, the haplotype distribution map revealed that *S. lagocephalus* from Sulawesi Island and China had a close genetic relationship based on COI sequence diversity (Figure 6).

4. Discussion

DNA Barcoding using the COI gene successfully identified 12 samples from eight main rivers in Bengkulu Province, *S. lagocephalus, S. cynocephalus,* and *S. Squamosissimus*. Mungkus fish are widely distributed across Bengkulu Province, from the north to the south.

Table 4. Haplotype characteristics of *Sicyopterus* spp. in Indonesia and the Asia Pacific region

Species/Haplotype (H)	N	Location
S. cynocephalus		
H1	1	Bengkulu* (PX091156)
H2	1	Bengkulu* (PX091159)
H3	1	Bengkulu* (PX091162)
H4	1	Bengkulu* (PX091365)
H5	1	Gorontalo (MT706640)
H6	1	Gorontalo (MT706721)
S. lagocephalus		
H1	3	Bengkulu* (PX091160); Maluku (PP132505); Philippines (PP430689)
H2	1	Bengkulu* (PX091161)
H3	2	Bengkulu* (PX091364); Maluku (PP132654)
H4	3	Central Sulawesi (OR841563); Gorontalo (OQ137176); China (KX528228)
H5	1	Gorontalo (MT706641)
H6	1	Philippines (PP430687)
H7	1	Philippines (PP430688)
H8	1	Philippines (PP430690)
H9	1	Taiwan (PV856566)
H10	1	Taiwan (PV856570)
H11	1	Vanuatu (HQ639041)
H12	1	West Java (PP352554)
S. squamosissimus		
H1	2	Bengkulu* (PX091157); West Sumatra (PP132683)
H2	1	Bengkulu* (PX091158)
H3	2	Bengkulu* (PX091163); Bengkulu* (PX091362)
H4	1	Bengkulu* (PX091363)
H5	1	West Sumatra (PP132378)
H6	1	West Sumatra (PP132403)

N: number of observations, *present study

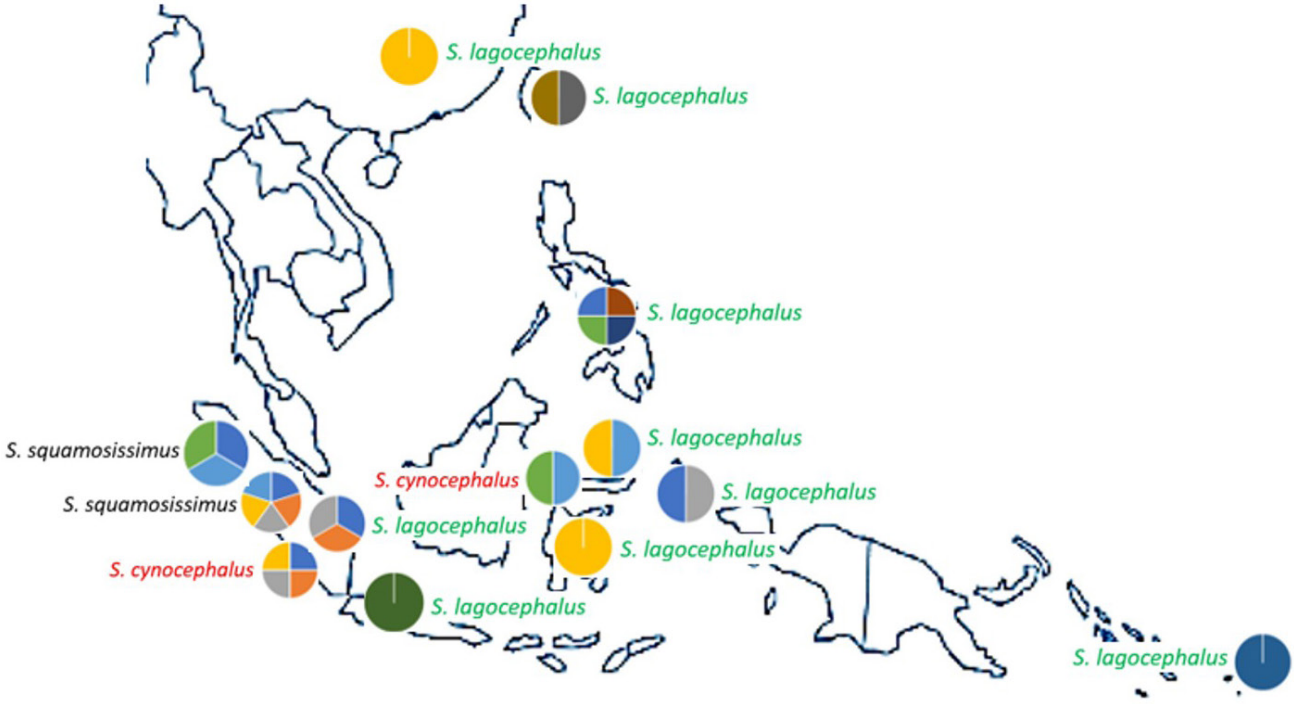

Figure 6. Haplotype distribution of *Sicyopterus* spp. in the Indo-Pacific based on the COI region. The various colors in the circle represent haplotype variation

In northern Bengkulu, *S. squamosissimus* was found in the Nokan River and Lubuk Muar, while *S. cynocephalus* was found only in Lubuk Banyau. *S. lagocephalus* was absent in northern Bengkulu. In the central region, the Maras River hosted *S. squamosissimus*. In southern Bengkulu, *S. squamosissimus* was found in the Nipis and Padang Guci rivers, while *S. cynocephalus* occurred in the Kedurang and Padang Guci rivers. Interestingly, *S. lagocephalus* was restricted to the Kinal River. The distribution of the Mungkus fish species is unique, as *S. lagocephalus* was found only in the southern part. The distribution of the Mungkus fish species is unique, as *S. lagocephalus* was recorded exclusively in the southern part of the river system. Such spatial segregation may reflect long-term ecological specialization and historical isolation within distinct riverine habitats. Freshwater fishes are strongly influenced by environmental filtering, including variation in flow regimes, substrate composition, salinity gradients, temperature, and nutrient availability, which can restrict species' occurrence to particular microhabitats (Flecker and Matthews 1999; Jackson *et al.* 2001). Over evolutionary timescales, geological processes such as river fragmentation, tectonic shifts, and climatic fluctuations may further promote population isolation and genetic divergence, leading to localized adaptation and restricted distributions (Albert and Reis 2011). Therefore, the confinement of *S. lagocephalus* to the southern region may reflect historical ecological adaptation that occurred thousands or even millions of years ago (Pasisingi *et al.* 2024).

The genus *Sicyopterus*, a group of freshwater gobies, exhibits diverse distributions across Indonesia, reflecting its ecological adaptations and biogeographical history. In Sulawesi. *S. lagocephalus, S. longifilis, S. cynocephalus,* and *S. pugnans* were commonly found in the fast-flowing rivers (Nurjirana *et al.* 2019; Olii *et al.* 2019; Muthiadin *et al.* 2020; Pangemanan *et al.* 2020). In Papua Island, the discovery of five new species in 2011 added *S. stiphodonoides, S. ocellaris, S. lengguru, S. erythropterus,* and *S. calliochromus*, found in the freshwater ecosystems (Keith *et al.* 2011). Across Indonesia, *Sicyopterus* species are influenced by habitat type, water quality, and geological history. In Sumatra, *S. microcephalus* thrives in fast-flowing streams, while *S. japonicus* is widespread across Java and Bali, adapting to both flowing and standing waters (Kottelat 2013).

The use of COI as a genetic marker is well established in ichthyology, as it is part of mitochondrial DNA that evolves relatively rapidly, making it a suitable target for studying species-level differentiation and population genetics (Hebert *et al.* 2003). Laskar *et al.* (2017) explored the phylogenetic relationships of gobiid fish in eastern

and north-eastern India using COI sequences. Their study demonstrated that COI was effective in distinguishing between closely related gobiid species and assessing evolutionary relationships. The findings from this study support the use of COI in identifying and separating Mungkus fish species. Sadurudeen *et al.* (2017) also highlighted the role of COI as a DNA barcode for fish species in marine ecosystems. Their research confirmed that COI is a robust marker for species identification and biodiversity assessment (Nurjirana *et al.* 2021; Sajjad *et al.* 2023).

The low mean intraspecific K2P distances observed in sample sequences reflected minimal genetic divergence within species, consistent with patterns expected for conspecific populations (Viswambharan *et al.* 2015). Conversely, interspecific distances were substantially higher, exceeding 4% in all pairwise comparisons, with the highest divergence observed between *S. lagocephalus* and *S. squamosissimus* (0.1036). The magnitude of the barcode gap strongly supports the distinctiveness of each *Sicyopterus* species examined and aligns with previous DNA barcoding studies in gobiid fishes, which have reported similar interspecific divergence levels (Lord *et al.* 2012a.).

Several studies have explored genetic diversity within *Sicyopterus* and other gobiid species, providing context for this research's findings. A comprehensive study of gobiid species across the Indo-Pacific region was conducted in 2010, highlighting the role of geographic barriers in shaping genetic diversity (Lord *et al.* 2010). In Sulawesi, studies have focused on the genetic diversity and evolutionary history of *Sicyopterus* species, particularly in relation to the island's complex geography and river system (Nurjirana *et al.* 2019). Papua also contributed to the understanding of *Sicyopterus* diversity in Indonesia (Keith *et al.* 2011). Their study identified several new species of *Sicyopterus*, highlighting the region's rich biodiversity. The genetic analyses conducted in Papua showed patterns of genetic variation similar to those observed in other parts of Indonesia, including Bengkulu. The discovery of new species in Papua underscores the importance of sustainable exploration and molecular analysis to document the biodiversity of the genus Sicyopterus in Indonesia fully.

The genetic diversity of the COI sequence in *Sicyopterus* sp. was high, indicating that the population is in genetic equilibrium without inbreeding pressure. The genetic diversity is controlled by various processes, including mutation, recombination, marker ascertainment, and demography (Stumpf 2004). In *S. japonicus*, a total of 74 haplotypes (Watanabe *et al.* 2006) and 102 haplotypes (Ju *et al.* 2013) of mitochondrial D-loop sequence variation have been reported from populations in Japan and Taiwan, respectively. Furthermore, the mitochondrial Cytochrome-b (Cyt-b) region can also discriminate the *Sicyopterus* sp. from French Polynesia and Mascarene-Comoros populations (Keith *et al.* 2005), West Sumatra (Dinata *et al.* 2025), and the Indo-Pacific region (Lord *et al.* 2012b). Therefore, Lord *et al.* (2012b) stated that the Western Pacific population of *S. lagocephalus* was the oldest, dating to the late Cenozoic (2.53-5.58 Myr). During that period, the Western Pacific region has become an important center of species diversification for many marine and terrestrial taxa due to the emergence of new islands and palaeo-oceanographic rearrangements (Planes and Galzin 1997).

In conclusion, this study confirms that DNA barcoding using the mitochondrial COI gene successfully identified three Sicyopterus species (*S. lagocephalus, S. cynocephalus,* and *S. squamosissimus*) across eight major rivers in Bengkulu Province, which demonstrates a clear barcode gap with low intraspecific and high interspecific genetic distances. *S. squamosissimus* and *S. cynocephalus* were broadly distributed, while *S. lagocephalus* was restricted to the southern Kinal River. The observed genetic diversity supports previous findings that the Western Pacific region plays an important role in the diversification of *Sicyopterus*. Overall, these results highlight the value of molecular approaches for resolving species identity, understanding distribution patterns, and supporting conservation of amphidromous freshwater gobies in Indonesia. This study has limitations, including a relatively small sample size and the lack of ecological covariates that may influence population structure and distribution. Future work should incorporate larger sample sizes, integrate environmental data, and apply high-throughput DNA approaches or multi-locus markers to improve resolution. Further ecological and reproductive studies are also necessary to support domestication and community-based aquaculture, alongside local policies for river protection and post-larval harvest regulation to sustain wild populations.

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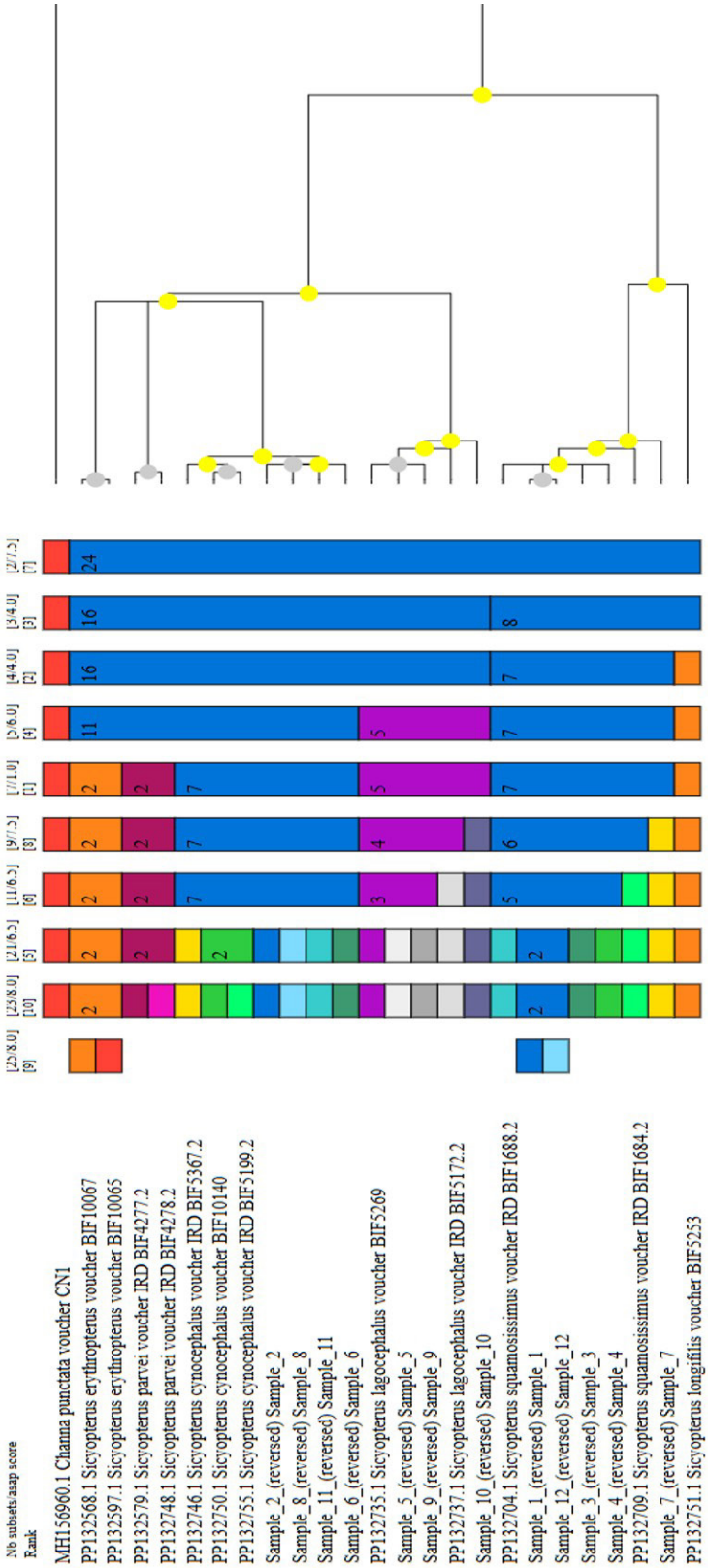
Supplementary Materials



Supplementary Figure 1. ASAP analysis result

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ModelFinder
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Best-fit model according to BIC: HKY+F+G4

List of models sorted by BIC scores:

Model              LogL         AIC      w-AIC        AICc     w-AICc         BIC      w-BIC
HKY+F+G4       -1979.188    4062.375 -   0.0165    4072.289 -   0.0277    4291.790 +    0.389
TPM3u+F+G4     -1976.341    4058.681 +    0.105    4068.995 +    0.144    4292.508 +    0.272
K3Pu+F+G4      -1977.164    4060.329 -    0.046    4070.642 +    0.063    4294.155 +    0.119
TPM2u+F+G4     -1977.936    4061.873 -   0.0213    4072.186 -   0.0291    4295.699 +   0.0552
HKY+F+R2       -1978.691    4063.383 -     0.01    4073.696 -   0.0137    4297.209 -   0.0259
HKY+F+I+G4     -1978.733    4063.466 -  0.00959    4073.779 -   0.0131    4297.292 -   0.0249
TN+F+G4        -1978.946    4063.892 -  0.00775    4074.205 -   0.0106    4297.718 -   0.0201
TIM3+F+G4      -1976.057    4060.114 +   0.0513    4070.836 +   0.0572    4298.352 -   0.0146
```

Supplementary Figure 2. Iqtree analysis as model finder

Supplementary Table 1. List of COI sequence references in *S. cynocephlus, S. lagocephalus,* and *S. squamosissimus*

Species	GenBank	Origin
S. cynocephalus	MT706640	Gorontalo
S. cynocephalus	MT706721	Gorontalo
S. lagocephalus	OR841643	Central Sulawesi
S. lagocephalus	KX528228	China
S. lagocephalus	MT706641	Gorontalo
S. lagocephalus	OQ137176	Gorontalo
S. lagocephalus	PP132502	Maluku
S. lagocephalus	PP132654	Maluku
S. lagocephalus	PP430687	Philippines
S. lagocephalus	PP430688	Philippines
S. lagocephalus	PP430689	Philippines
S. lagocephalus	PP430690	Philiipines
S. lagocephalus	PV856566	Taiwan
S. lagocephalus	PV856570	Taiwan
S. lagocephalus	HQ639041	Vanuatu
S. lagocephalus	PP352554	West Java
S. squamosissimus	PP132378	West Sumatra
S. squamosissimus	PP132403	West Sumatra
S. squamosissimus	PP132683	West Sumatra