

Molecular Analysis of Freshwater Goby (*Sicyopterus* sp.) from Bengkulu, Indonesia Based on Cytochrome b Mitochondrial Gene

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Abstract. *Sicyopterus* (locally known as mungkus in Bengkulu) is a genus of amphidromous goby inhabiting rocky stream ecosystems and commonly utilized as a local food resource in Indonesia. Accurate species identification within this genus is important for biodiversity assessment and conservation because many *Sicyopterus* species exhibit high morphological polymorphism and interspecific phenotypic similarity, which often complicate morphology-based identification. This study aimed to identify *Sicyopterus* species from Bengkulu, Indonesia, using combined morphological and molecular approaches and to evaluate their phylogenetic relationships. A total of 30 specimens were collected from Bengkulu Utara, Bengkulu Selatan, and Kaur Regency, of which 14 representative coded samples were selected for molecular analysis. Genomic DNA was extracted using the gSYNC™ DNA Extraction Kit, followed by PCR amplification of the mitochondrial cytochrome b gene using two DNA primers, Cytb F217 and Cytb R1043. Sequence analyses were conducted in MEGA II using the Neighbor-Joining and Maximum Likelihood methods based on an 817 bp fragment. Phylogenetic and genetic distance analyses identified lineages associated with *S. lagocephalus*, *S. squamosissimus*, *S. cynocephalus*, *S. parvei*, and a putative *S. aiensis* lineage. Sample PG1 from the Padang Guci River (Kaur Regency) showed close affinity with *S. parvei*, whereas sample KD3 from the Kedurang River showed tentative affinity with *S. aiensis*. In addition, sample KD7, associated with *S. squamosissimus*, showed the highest genetic divergence (2.22% p-distance), suggesting substantial intraspecific variation or a distinct lineage. These findings expand current knowledge of *Sicyopterus* distribution in western Indonesia and highlight the importance of integrating molecular and morphological approaches to resolve taxonomic uncertainty in amphidromous gobies. Nevertheless, additional genes and morphometric analyses are still needed, particularly for the *S. aiensis* - *S. cynocephalus* lineage, because these taxa showed relatively low mitochondrial divergence and limited phylogenetic support in the present study.

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INTRODUCTION

Amphidromous goby *Sicyopterus* (Family Gobiidae) exhibits unique morphological adaptations for survival in fast-flowing rocky riverine habitats. These adaptations comprise a suction disc formed by fused pelvic fins, thereby enabling individuals to adhere to rock surfaces and resist strong water currents while also providing support for locomotion and reproductive activities in lotic environments (Lord et al., 2019; Muthiadin et al., 2020; Maie, 2022). During

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the breeding season, adult *Sicyopterus* migrates from marine environments to rivers to spawn. The hatched larvae then migrate back to the sea, a life-history strategy known as amphidromous (Iida et al., 2017; Nurjirana et al., 2022; Ollie et al., 2017; Tran et al., 2018). The distribution of *Sicyopterus* is primarily restricted to tropical and subtropical regions of the Indo-Pacific, due to ecological and morphological specializations. Presently, approximately 33 species of *Sicyopterus* have been recognized worldwide, with at least 14 species reported from Indonesian waters. These are *Sicyopterus lagocephalus*, *S. squamosissimus*, *S. cynocephalus*, *S. lengguru*, *S. longifilis*, *S. microcephalus*, *S. parvei*, *S. calliochromus*, *S. erythropterus*, *S. hageni*, *S. micrurus*, *S. ouwensi*, *S. wichmanni*, and *S. macrostetholepis*, solidifying Indonesia's status as a global hotspot for gobiid fish biodiversity (Dinata et al., 2025; Froese & Pauly, 2025; Hadiaty et al., 2018; Salindeho, 2021).

In Indonesia, freshwater ecosystems supporting *Sicyopterus* populations are increasingly threatened by anthropogenic pressures, including overexploitation, habitat degradation from sand mining and dam construction, water pollution from agricultural and urban runoff, and ecological disruption by invasive species (Baihaqi et al., 2025; Dinata et al., 2025). These pressures are reflected in the conservation status of several *Sicyopterus* species. Currently, *S. eudentatus*, *S. sarasini*, and *S. rapa* are categorized as Endangered (EN) due to population declines over the past decade, while *S. ocellaris* is designated Vulnerable (VU) owing to its restricted range and fragmented habitats. An additional four species (*S. erythropterus*, *S. aiensis*, *S. calliochromus*, and *S. stimsoni*) are listed as Near Threatened (NT), indicating that their populations may continue to decline if environmental pressures persist (IUCN, 2025). Their life cycle depends on the connectivity between freshwater and marine ecosystems. Habitat fragmentation, declining water quality, river modification, and disruption of migratory pathways may directly affect larval dispersal, recruitment success, and long-term population persistence (Lord et al., 2019; Nurjirana et al., 2022). Consequently, accurate taxonomic identification and distributional data are essential for supporting conservation planning and evaluating the conservation status of *Sicyopterus* populations in Indonesia.

Bengkulu Province is one of the regions where postlarval *Sicyopterus* (locally known as "ikan mungkus") are commonly harvested for food and local trade, often using non-selective fishing methods. However, information regarding species diversity, spatial distribution, spawning habitats, and genetic diversity of *Sicyopterus* in Bengkulu remains limited. This is significant because amphidromous gobies rely on the connectivity between freshwater and marine habitats during their reproductive cycle and larval migration. Identification of spawning habitats and migratory routes may therefore support more effective management strategies, including habitat protection and seasonal harvest regulation. In addition, the limited genetic data may complicate species identification and diminish our comprehension of population structure and the potential for cryptic diversity within *Sicyopterus* populations in Bengkulu. Therefore, molecular-based studies are necessary to provide a baseline for biodiversity assessment and future conservation planning of amphidromous gobies in western Indonesia.

Lestari et al. (2021) initiated efforts to address the limited information regarding *Sicyopterus* diversity and distribution in Bengkulu Province through field surveys, revealing the presence of three *Sicyopterus* species in the region: *S. lagocephalus*, which was exclusively found in South Bengkulu Regency, and *S. cynocephalus* and *S. squamosissimus*, both distributed across the northern and southern areas of Bengkulu Province. However, accurately uncovering the true species diversity of *Sicyopterus* fish in Bengkulu Province presents a significant challenge due to pronounced interspecific similarities and intraspecific polymorphism within the genus. This study was undertaken to enhance the precision of species identification and to bring current information concerning the diversity and distribution of *Sicyopterus* in Bengkulu Province up to date. This study combined morphological observations with molecular analysis.

To achieve molecular identification, the mitochondrial Cytochrome b gene was selected as a marker due to its extensive utilization in phylogenetic and taxonomic studies of gobiid fishes, including *Sicyopterus* (Čekovská et al., 2023; Dinata et al., 2025; Lord et al., 2019; Maie, 2022; Roesma et al., 2020). Compared to other mitochondrial markers, Cytochrome b has shown good resolution for distinguishing closely related fish species due to its relatively conserved regions within species and sufficient variation among species (Lord et al., 2019; Meyer, 1994; Perdices & Doadrio, 2001;). Furthermore, Cytochrome b sequence data for numerous *Sicyopterus* species are presently accessible via GenBank, enabling more reliable comparative analyses and phylogenetic reconstruction.

The objective of this study was to determine the diversity of *Sicyopterus* species in Bengkulu Province through the combination of morphological and molecular approaches, and to document their distribution patterns across the region. The findings are expected to provide baseline information for future biodiversity assessments and conservation planning. Particularly, considering the ecological and socio-economic importance of *Sicyopterus* in Bengkulu as a freshwater fish resource that is commonly utilized by local communities. By improving species identification and clarifying taxonomic uncertainty within the genus, this study may also contribute to future management and conservation efforts for amphidromous gobies in western Indonesia.

MATERIALS AND METHODS

Sample Collection

The research was conducted from July to September of 2024. A total of 30 *Sicyopterus* specimens were collected from four river systems in Bengkulu Province, Indonesia, consisting of the Air Lais River (n = 14), the Air Besi River (n = 5), the Kedurang River (n = 7), and the Padang Guci River (n = 4). The fish specimens were obtained from local fishermen operating directly in the river systems and from nearby traditional markets, located close to the sampling areas. Information regarding the provenance of each specimen was confirmed through direct interviews with fishermen and fish sellers immediately after collection, including the fishing location and capture area. Only specimens reported to originate from the targeted rivers were included in this study. However, because some specimens were obtained from traditional markets, the possibility of minor uncertainty in locality information cannot be completely excluded and is acknowledged as a limitation of this study.

All *Sicyopterus* specimens were identified based on physical traits, using taxonomic references from Keith et al. (2004, 2015), Froese & Pauly (2024), and Lestari et al. (2021) as primary sources. Each specimen was measured, photographed, and cataloged for documentation purposes. To avoid the repeated sequencing of morphologically similar samples from the same area, representative individuals with differing morphology and sampling localities were selected for molecular analysis from the complete collection. Muscle tissue samples from selected individuals were preserved in Falcon tubes containing 95% ethanol and stored at -20°C for further molecular analysis. Intact specimens were conserved in 70% ethanol and stored at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Bengkulu, for future taxonomic research.

DNA Extraction

The genomic DNA extraction was conducted using the gSYNC™ DNA Extraction Kit, following the manufacturer's protocol for animal tissue samples. Generally, DNA extraction comprises four steps: cell lysis, DNA binding, DNA washing, and DNA elution. Approximately 20-25 mg of muscle tissue was dissected from each selected specimen and transferred into a 1.5 mL microcentrifuge tube. Tissue samples were treated with proteinase K, and the lysis solution was

incubated at 60°C until full tissue digestion was accomplished. After that, the lysate was transferred into a GS column so that DNA could attach to the silica membrane. To remove proteins and other impurities, the attached DNA was cleaned using the supplied wash buffers. Lastly, 100 µL of elution buffer was used to elute the pure genomic DNA. Before Polymerase Chain Reaction (PCR) amplification, DNA content and purity were assessed using NanoDrop spectrophotometry based on A260/A280 absorbance ratios.

Amplification and DNA Sequencing

PCR amplification of the mitochondrial Cytochrome b gene was performed using forward primer Cyt b F217 (TCG AAA YAT ACA TGC CAA TGG), and reverse primer Cyt b R1043 (GAA GTA YAG GAA GGA YGC AAT TT), following Lord et al. (2012). The amplicon size was predicted to be around 812 bp. A total of 25 µL was used for the PCR reactions, which included 12.5 µL of GoTaq® G2 Green Master Mix, 1 µL of forward primer, 1 µL of reverse primer, 7.5 µL of nuclease-free water, and 3 µL of DNA template. PCR amplification involved 2 minutes of initial denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 52.6°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72 °C for 5 minutes. The amplified PCR products were visualized with 0.1 mg/mL ethidium bromide staining and separated on a 1.2% agarose gel at 50 volts for 45 minutes. An Axygen® gel documentation system was used to record DNA bands under UV transillumination ($\lambda = 302$ nm). The existence of distinct DNA bands at the anticipated fragment size indicated successful amplification. Successfully amplified PCR products were sequenced bidirectionally by PT Genetika Science Indonesia. Before molecular analysis, sequence chromatograms were edited, clipped, and aligned.

Data Analysis

ChromasPro software (Technelysium, 2022) was used to edit and assemble the acquired cytochrome b sequences. The Basic Local Alignment Search Tool-Nucleotide (BLASTn) was used to identify sequences, compared to reference sequences found in GenBank (<https://www.ncbi.nlm.nih.gov/>). Before phylogenetic analysis, all nucleotide sequences were converted into amino acid sequences in MEGA 11 to ensure that stop codons were absent. ClustalW implementation was used to do multiple sequence alignment (Tamura et al., 2021). The Tamura-Nei model was chosen for phylogenetic reconstruction based on the best-fit nucleotide substitution model identified in MEGA 11 using the Bayesian Information Criterion (BIC). Neighbor-Joining (NJ) and Maximum Likelihood (ML) phylogenetic analyses were conducted with 1000 bootstrap replicates to assess the consistency of tree topology. Pairwise genetic distances among sequences were calculated using uncorrected p-distance values (Kimura, 1980).

RESULTS AND DISCUSSION

A total of 30 *Sicyopterus* individuals were collected from four river systems in Bengkulu Province, consisting of Air Lais River (n = 14), Air Besi River (n = 5), Kedurang River (n = 7), and Padang Guci River (n = 4). Based on preliminary morphological identification following Lestari et al. (2021), the collected specimens were initially grouped into morphologically similar categories representing *S. squamosissimus*, *S. lagocephalus*, and *S. cynocephalus*. From all these specimens, 14 representative individuals were selected for molecular analysis, consisting of HL9 and HL11 (Air Lais River); HB2, HB3, and HB4 (Air Besi River); KD1 - KD7 (Kedurang River); and PG1 and PG4 (Padang Guci River) (Figure 1).

Table 1. List of *Sicyopterus* specimens collected from Bengkulu Province, Indonesia

Sample Code	Locality	Characteristics
HL9	Air Lais River, North Bengkulu	Total length approximately 10 cm; body elongated and laterally compressed; body blackish with yellowish dorsal region; caudal fin orange-reddish; dorsal region exhibiting yellow–black banding pattern; upper lip possessing paired wrinkled lateral clefts; first dorsal fin VI spines, filamentous; second dorsal fin I,10 soft rays.
HL11	Air Lais River, North Bengkulu	Total length approximately 9 cm; body slender and laterally compressed; body grayish-white with black patternation; caudal fin reddish; first dorsal fin VI spines, filamentous; second dorsal fin I,10 soft rays.
HB2	Air Besi River, North Bengkulu	Total length approximately 10 cm; body uniformly black without distinct markings; dorsal and caudal fins dark black to faded black; caudal fin rounded; eyes blackish; first dorsal fin VI spines, elongated; second dorsal fin I,10 soft rays.
HB3	Air Besi River, North Bengkulu	Total length approximately 14 cm; body uniformly deep black without distinct patterns; caudal fin orange; eyes blackish; first dorsal fin VI spines, filamentous; second dorsal fin I,10 soft rays.
HB4	Air Besi River, North Bengkulu	Total length approximately 10 cm; body blackish with yellowish dorsal region; caudal fin reddish-orange; dorsal region exhibiting yellow–black banding pattern; upper lip possessing paired lateral clefts; first dorsal fin VI spines, filamentous; second dorsal fin I,10 soft rays.
KD1	Kedurang River, South Bengkulu	Total length approximately 9 cm; body bluish in coloration with robust body form; caudal fin reddish-orange with black median stripe; eyes blackish; first dorsal fin VI spines, elongated; second dorsal fin I,10 soft rays.
KD2	Kedurang River, South Bengkulu	Total length approximately 12 cm; body whitish-brown; dark longitudinal stripes below eyes; iris reddish; pectoral fins striped; first dorsal fin VI spines, elongated; second dorsal fin I,10 soft rays.
KD3	Kedurang River, South Bengkulu	Total length approximately 12 cm; body bluish-white; body lacking distinct markings except on dorsal region; caudal region pale; first dorsal fin VI spines; second dorsal fin I,10 soft rays.
KD4	Kedurang River, South Bengkulu	Total length approximately 9 cm; body brownish; dorsal region exhibiting tiger-like banding pattern; caudal fin with central black marking; eyes blackish; first dorsal fin VI spines, filamentous; second dorsal fin I,10 soft rays.
KD5	Kedurang River, South Bengkulu	Total length approximately 8 cm; body bluish-white with body-wide patternation; caudal fin yellowish; morphologically similar to KD1 but differing in eye coloration and absence of median caudal stripe; first dorsal fin VI spines, filamentous; second dorsal fin I,10 soft rays.
KD6	Kedurang River, South Bengkulu	Total length approximately 9 cm; body brownish with black patternation; dorsal surface whitish without distinct markings; eyes blackish; first dorsal fin VI spines; second dorsal fin I,10 soft rays.
KD7	Kedurang River, South Bengkulu	Total length approximately 9 cm; body blackish with whitish ventral region; caudal fin orange; body lacking distinct patterns; first dorsal fin VI spines, filamentous; second dorsal fin I,10 soft rays.
PG1	Padang Guci River, Kaur Regency	Total length approximately 11 cm; body dark brown to blackish; caudal fin exhibiting black–yellow patternation; dorsal region lacking tiger-like markings; eyes blackish; first dorsal fin VI spines, elongated; second dorsal fin I,10 soft rays.
PG4	Padang Guci River, Kaur Regency	Total length approximately 9 cm; body grayish; black patternation extending from body to caudal region; caudal peduncle slender; first dorsal fin VI spines; second dorsal fin I,10 soft rays.

Distinct morphological characters and observed intraspecific variation among samples, including body coloration patterns, head shape, fin morphology, body size proportions, and meristic characters such as fin ray counts. These variations were compared among sampling locations to represent the morphological diversity of *Sicyopterus* in Bengkulu (Figure 2). Specimens showing identical or highly similar morphological characteristics from the same locality were not included in molecular analysis to avoid redundant sequencing. Therefore, the selected samples were considered representative of the morphological variation observed among the collected specimens. A complete list of all collected specimens and their preliminary morphological identification is provided in Table 1. The partial mitochondrial cytochrome b gene was successfully amplified from all selected samples. BLASTn analysis showed that samples HL9, HL11, HB2, HB3, HB4, KD4, KD6, and KD7 shared high genetic similarity with *S. squamosissimus* from Sumatra, Indonesia (NC_044151), with similarity values ranging from 97.89% to 99.89%. Meanwhile, samples KD1, KD5, and PG4 showed 99.14–99.75% similarity with *S. lagocephalus* from Europe and Taiwan. Samples KD2 and KD3 showed high similarity (99.26–99.75%) with *Sicyopterus cynocephalus* (MK496936) from the Solomon Islands. In addition, sample PG1 showed 99.75% similarity with *S. parvei* (NC044145) from Java, Indonesia. Query coverage values for all analyzed samples reached 100% (Table 2).

We employed the Megablast database to identify high-confidence species matches (Identity >99%, Query Cover = 100%) using cytochrome b mitochondrial gene sequences (Schlaberg et al., 2012; Pearson, 2013). Megablast analysis revealed that the samples studied related to four *Sicyopterus* taxa found in Bengkulu waters: *S. squamosissimus*, *S. lagocephalus*, *S. cynocephalus*, and *S. parvei* (Table 2). *S. parvei* has the potential to set a new distribution record for Bengkulu Province when compared to previous findings by Lestari et al. (2021). Most samples revealed high similarity (>99%) to GenBank reference sequences. However, sample KD7 showed lower similarity (97.89%) with *S. squamosissimus* from Sumatra, indicating greater genetic divergence than the other samples studied.

High sequence similarity was found between samples KD2 and KD3 and *S. cynocephalus* (Solomon Islands), *S. aiensis* (Vanuatu), and *S. lengguru* (Papua, Indonesia). While sample KD3 had a very equal affinity to both *S. cynocephalus* (99.26%) and *S. aiensis* (99.02%), sample KD2 exhibited the most similarity with *S. cynocephalus* (99.75%). Additional analysis of genetic distance and phylogenetic reconstruction consistently placed KD2 closer to *S. cynocephalus*, despite the relatively near similarity values between these taxa. Despite the very poor phylogenetic support for this lineage, KD3 showed a slightly closer relationship with *S. aiensis* (Table 2). Moreover, a phylogenetic tree was constructed to visualize the relationship between individuals being compared (samples and individuals whose sequence data were downloaded from GenBank (Figure 1). According to the findings of Lord et al. (2019), the minimum interspecific genetic distance among the *Sicyopterus* group is 1.17%, and the maximum divergence is 11.36%. However, until further investigations employing complementary molecular markers and more thorough morphometric comparisons are conducted, the identification of KD3 as *S. aiensis* should be regarded as dubious.

Sample KD5 was highly comparable to both *S. lagocephalus* (99.51%) and *S. marquesensis* (98.89%). However, *S. lagocephalus* has a wide Indo-Pacific distribution, including Southeast Asian waters, whereas *S. marquesensis* has been reported only from the Marquesas Islands in French Polynesia. Given the somewhat greater similarity score, phylogenetic location, and geographic proximity of previously described *S. lagocephalus* populations in western Indonesia and Taiwan, KD5 was tentatively classified as *S. lagocephalus*. The unusually high similarity between these taxa may represent close evolutionary ties across multiple Indo-Pacific *Sicyopterus* species, as well as the migratory capacity of amphidromous gobies, which can disperse larvae over great distances via marine currents (Lord et al., 2019).

Table 2. The result of species identification for *Sicyopterus* samples captured in Bengkulu based on the cytochrome b gene (BLASTn)

Voucher	Species	Accession	BLASTn			Origin Country
			Query Cover (%)	E-value	Percent Identity (%)	
HL9	<i>S. squamosissimus</i>	NC_044151	100	0.0	99.15	Sumatra (Indonesia)
	<i>S. stiphodonoides</i>	NC_044152	100	0.0	94.02	Solomon Island
	<i>S. longifilis</i>	MK496959	100	0.0	93.41	Indonesia
HL11	<i>S. squamosissimus</i>	NC_044151	100	0.0	99.75	Sumatra (Indonesia)
	<i>S. stiphodonoides</i>	NC_044152	100	0.0	94.04	Solomon Island
	<i>S. longifilis</i>	MK496959	100	0.0	93.80	Indonesia
HB2	<i>S. squamosissimus</i>	NC_044151	100	0.0	99.26	Sumatra (Indonesia)
	<i>S. stiphodonoides</i>	NC_044152	100	0.0	94.07	Solomon Island
	<i>S. longifilis</i>	MK496959	100	0.0	93.57	Indonesia
HB3	<i>S. squamosissimus</i>	NC_044151	100	0.0	99.39	Sumatra (Indonesia)
	<i>S. stiphodonoides</i>	NC_044152	100	0.0	94.51	Solomon Island
	<i>S. longifilis</i>	MK496959	100	0.0	93.65	Indonesia
HB4	<i>S. squamosissimus</i>	NC_044151	100	0.0	99.51	Sumatra (Indonesia)
	<i>S. stiphodonoides</i>	NC_044152	100	0.0	94.10	Solomon Island
	<i>S. longifilis</i>	MK496959	100	0.0	93.61	Indonesia
KD1	<i>S. lagocephalus</i>	KF415661	100	0.0	99.14	European
	<i>S. marquesensis</i>	MK496961	100	0.0	95.97	Marquesas Islands
	<i>S. lengguru</i>	NC_044140	100	0.0	94.13	Papua (Indonesia)
KD2	<i>S. cynocephalus</i>	MK496936	100	0.0	99.75	Solomon Islands
	<i>S. aiensis</i>	MK426281	100	0.0	98.77	Vanuatu
	<i>S. lengguru</i>	NC_044140	100	0.0	98.65	Papua (Indonesia)
KD3	<i>S. cynocephalus</i>	MK496936	100	0.0	99.26	Solomon Islands
	<i>S. aiensis</i>	MK426281	100	0.0	99.02	Vanuatu
	<i>S. lengguru</i>	NC_044140	100	0.0	98.65	Papua (Indonesia)
KD4	<i>S. squamosissimus</i>	NC_044151	100	0.0	99.26	Sumatra (Indonesia)
	<i>S. stiphodonoides</i>	MK496987	100	0.0	94.36	Solomon Island
	<i>S. longifilis</i>	MK496959	100	0.0	93.50	Indonesia
KD5	<i>S. lagocephalus</i>	NC_022838	100	0.0	99.51	Taiwan
	<i>S. marquesensis</i>	MK496961	100	0.0	98.89	Marquesas Islands
	<i>S. aiensis</i>	MK426281	100	0.0	94.21	Vanuatu
KD6	<i>S. squamosissimus</i>	NC_044151	100	0.0	99.38	Sumatra (Indonesia)
	<i>S. stiphodonoides</i>	MK496987	100	0.0	94.44	Solomon Island
	<i>S. longifilis</i>	MK496959	100	0.0	93.70	Indonesia
KD7	<i>S. squamosissimus</i>	NC_044151	100	0.0	97.89	Sumatra (Indonesia)
	<i>S. stiphodonoides</i>	MK496987	100	0.0	94.42	Solomon Island
	<i>S. pugnans</i>	MK496973	100	0.0	93.42	Society Islands
PG1	<i>S. parvei</i>	NC_044145	100	0.0	99.75	Jawa (Indonesia)
	<i>S. marquesensis</i>	MK496961	100	0.0	93.73	Marquesas Island
	<i>S. cynocephalus</i>	MK496936	100	0.0	93.52	Solomon Islands
PG4	<i>S. lagocephalus</i>	NC_022838	100	0.0	99.75	Taiwan
	<i>S. marquesensis</i>	MK496961	100	0.0	96.15	Marquesas Islands
	<i>S. aiensis</i>	MK426281	100	0.0	94.23	Vanuatu

Remark : The sequences of the cytochrome b gene of each non-sample species were downloaded from GenBank

After matching with reference sequences, an 817 bp cytochrome b gene fragment was obtained from 14 *Sicyopterus* samples. This fragment covers 71.6% of the known complete cytochrome b gene, 1140 bp in length (Chiang et al., 2013a; Chiang et al., 2013b). The fragment is located at nucleotide positions 247-1063 bp within the complete cytochrome b gene, encompassing the highly conserved 5'-end, variation sites within the coding region, and the 3'-end, which exhibit distinct mutations. Functionally, these regions correspond to the three mutational domains in vertebrate cytochrome b genes: the more conserved external domain, moderately variable interfacial domain, and the most variable internal domain, as described by Irwin et al. (1991). Therefore, the selection of this 817 bp fragment is highly strategic, as it includes all three functional domains, provides sufficient informative sites for phylogenetic analysis, and allows comparisons with previous studies that used similar fragments.

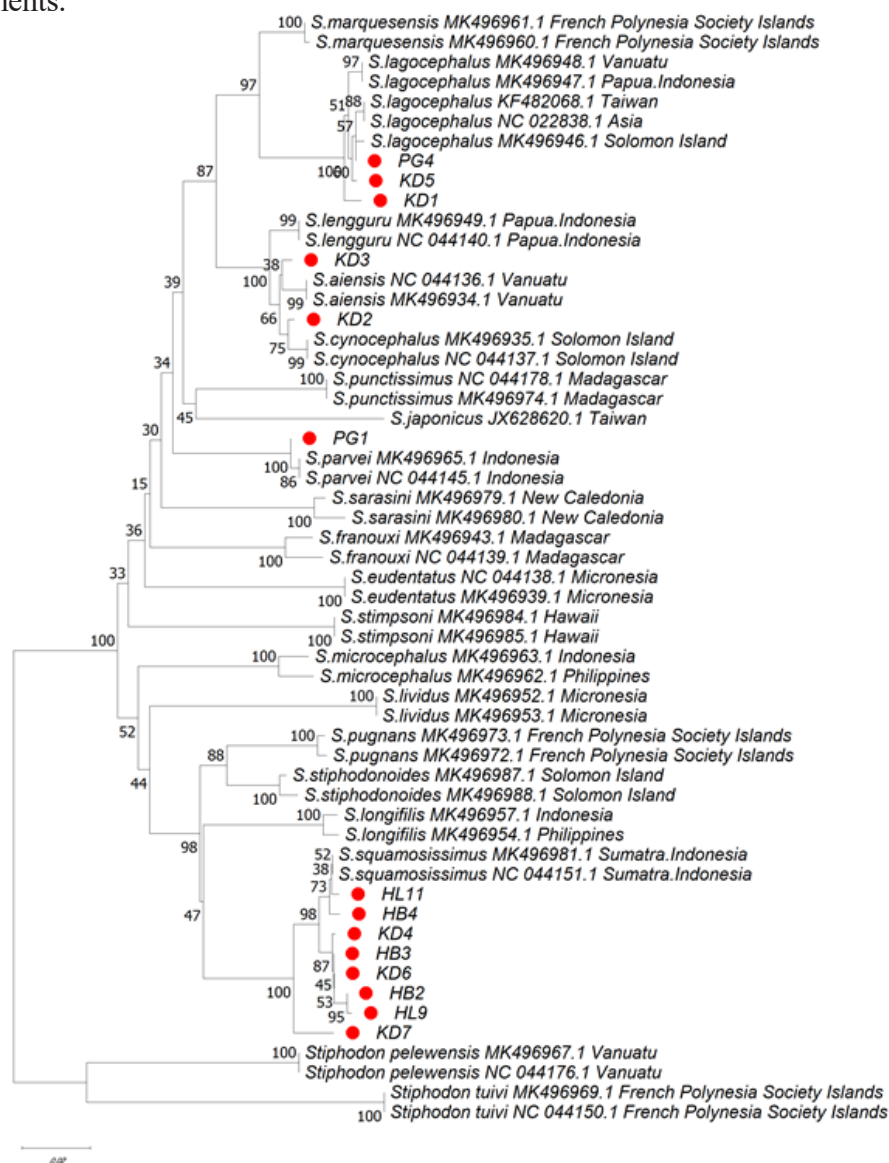


Figure 1: The phylogenetic relationships among 14 individuals of *Sicyopterus* from Bengkulu, Indonesia, 18 reference *Sicyopterus* taxa retrieved from GenBank, and 2 outgroup species from the *Stiphodon* genus. The phylogenetic tree was reconstructed using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods with the Tamura-Nei model, 1000 bootstrap replicates. The analysis was based on partial sequences of the Cytochrome b gene (817 bp length). Bootstrap values are shown at branch nodes and Bengkulu samples are indicated by the red circle marks (●)

Phylogenetic analysis using the Neighbor-Joining method (Figure 1) revealed that the *Sicyopterus* samples from Bengkulu were divided into five lineages, including *S. lagocephalus*, *S. squamosissimus*, *S. cynocephalus*, *S. aiensis*, and *S. parvei*. Samples PG4, KD5, and KD1 clustered with *S. lagocephalus*, while PG1 was closely related to *S. parvei*. In addition, samples HL9, HL11, HB2, HB3, HB4, KD4, KD6, and KD7 formed a cluster with *S. squamosissimus*. Samples KD2 and KD3 were placed near *S. cynocephalus* and *S. aiensis*, respectively. Most main clades have strong bootstrap support values, especially the *S. lagocephalus*, *S. squamosissimus*, and *S. parvei* lineages, indicating very stable evolutionary connections. However, the bootstrap support for the *S. aiensis* associated lineage was low (45%), while the *S. cynocephalus* branch had considerable support (75%). Low bootstrap values generally indicate weak support for branch stability and should be interpreted cautiously in phylogenetic reconstruction (Ecker et al., 2024; Lemoine & Gascuel, 2024; Tagliacollo et al., 2024). Therefore, the placement of KD3 within the *S. aiensis* associated lineage should be considered tentative pending additional analyses using complementary molecular markers and broader taxon sampling.

The presence of *S. parvei* and the potential *S. aiensis* branch in Bengkulu is biogeographically significant because previous research has primarily recorded these taxa from eastern Indonesia and the western Pacific areas. *S. parvei* was once found by Sahami et al. (2021) in Gorontalo, Sulawesi Island, Indonesia, but has never been recorded on the island of Sumatra. Keith et al. (2004) reported *S. aiensis* in Vanuatu in 2004, whereas there have been no reports of this species in Indonesia, especially in Sumatra. Bengkulu is located on Sumatra's western coast, isolated from Sulawesi and Papua by large sea and island systems. The approximate oceanic distance between western Sumatra and Sulawesi exceeds 2,000 kilometers, whereas the distance between Sumatra and Papua is much greater. The approximate marine distance between western Sumatra and Sulawesi exceeds 2,000 km, whereas the distance between Sumatra and Papua is considerably greater. Despite this separation, amphidromous gobies possess marine larval stages that enable dispersal through ocean currents during early development (Lord et al., 2019). This amphidromous life-history strategy may facilitate connectivity among river systems and islands across the Indo-Pacific region and may contribute to the relatively high genetic similarity observed among geographically distant *Sicyopterus* populations (Nurjirana et al., 2022, 2025; Ollie et al., 2017; Tran et al., 2018; Zarei et al., 2022;).

Previous research on amphidromous gobies has demonstrated that larval dispersal across maritime habitats can minimize genetic divergence between populations from various islands and coastal locations (Iida et al., 2017; Ollie et al., 2017; Tran et al., 2018; Zarei et al., 2022). This method could explain the close evolutionary relationships found across multiple *Sicyopterus* taxa in the current study, despite their widespread geographic range. Nonetheless, the relatively low bootstrap support for the *S. aiensis*-associated branch suggests that the current phylogenetic evidence is insufficient for final taxonomic confirmation. Additional analyses employing nuclear markers, increased geographic sampling, and extensive morphometric comparisons are still required to determine the taxonomic position of this lineage in Bengkulu waters.

Preliminary identification of *Sicyopterus* samples was performed based on external morphological characters, following Keith et al. (2004, 2015) and Lestari et al. (2021). Diagnostic characters used during sample grouping included body coloration pattern, head shape, fin coloration, and pigmentation pattern. These morphological observations were subsequently used to inform the inclusion and exclusion of representative samples selected for molecular analysis.

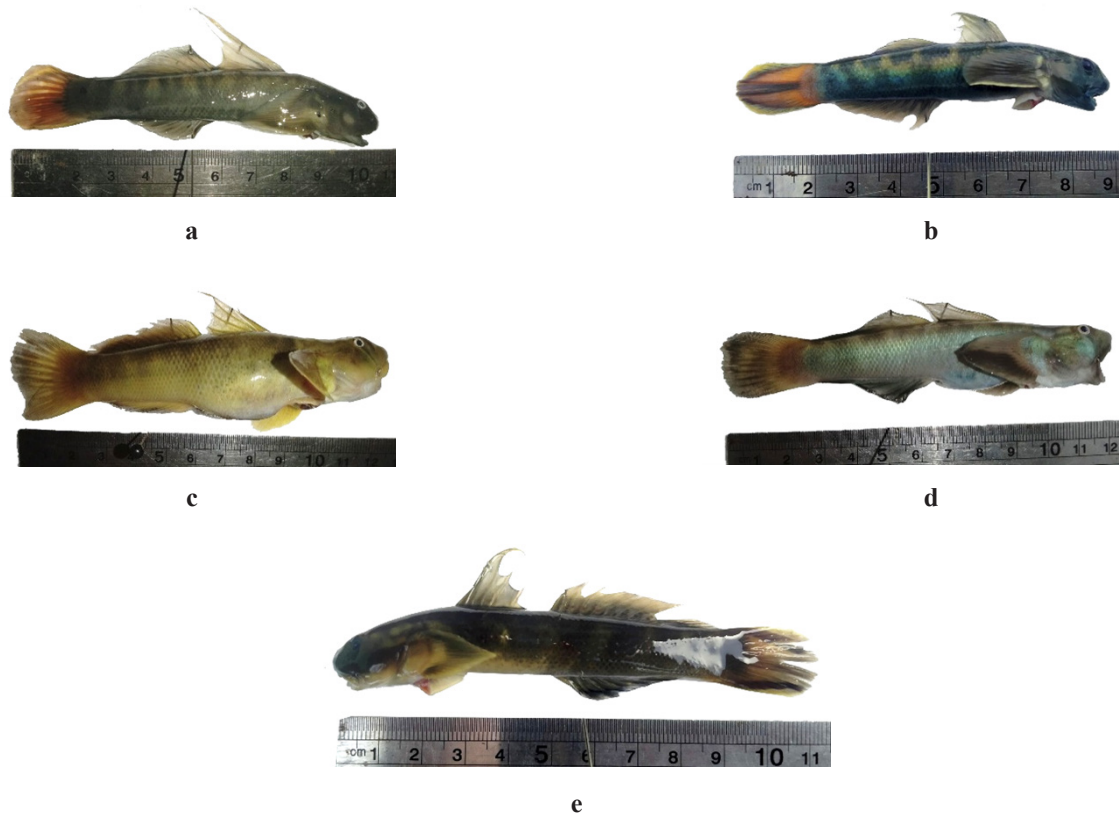


Figure 2: Representative *Sicyopterus* specimens collected from Bengkulu, Indonesia, based on preliminary morphological identification: (a) *S. squamosissimus*, (b) *S. lagocephalus*, (c) *S. cynocephalus*, (d) possible *S. aiensis*, and (e) *S. parvei*. Morphological observations, including body coloration patterns, fin pigmentation, and head morphology, served as the initial basis for sample grouping and selection before molecular analysis.

Morphological observations showed noticeable variation in *S. lagocephalus* and *S. squamosissimus*, particularly in body coloration, eye size, fin shape, and pigmentation patterns. Similar intraspecific variation within the genus *Sicyopterus* has also been reported in previous studies (Lord et al., 2019). Variability in body coloration, body size, eye morphology, and fin characters among amphidromous gobies has been documented in several Indo-Pacific populations (Ellien et al., 2016; Jensen et al., 2023; Moody et al., 2015; Pasingi et al., 2024; Thomas et al., 2018). In the present study, molecular analysis supported the occurrence of considerable morphological polymorphism within *S. lagocephalus* and *S. squamosissimus*. However, these morphological differences do not necessarily indicate distinct species, as substantial phenotypic variation may occur within the same species across different habitats and environmental conditions.

Amphidromous gobies' morphological variation may be influenced by local adaptation, habitat features, and environmental factors. Previous studies suggested that factors such as water temperature, elevation, habitat type, hydrological conditions, and ecological pressure may contribute to phenotypic variation among populations (Diamond et al., 2016, 2024; Ellien et al., 2016; Jensen et al., 2023; Moody et al., 2015, 2017; Shuai et al., 2018). Such variation may complicate species identification when based solely on external morphology, particularly among closely related or cryptic taxa. Therefore, combining morphological observations with molecular analysis can improve identification accuracy and reduce the risk of taxonomic misclassification within morphologically variable *Sicyopterus* populations.

Table 3. The summary of genetic distance percentage for the goby *Sicyopterus* from Bengkulu to closely related species based on an 817 bp cytochrome b gene sequence

Genetic Distance	Mean (%)
<i>Sicyopterus</i> sp. (KD3) vs <i>S. aiensis</i>	0,67
<i>Sicyopterus</i> sp. (KD3) vs <i>S. cynocephalus</i>	1,14
<i>Sicyopterus</i> sp. (KD2) vs <i>S. cynocephalus</i>	0,50
<i>Sicyopterus</i> sp. (KD1, KD5, PG4) vs <i>S. lagocephalus</i>	0,65
<i>Sicyopterus</i> sp. (PG1) vs <i>S. parvei</i>	0,17
<i>Sicyopterus</i> sp. (HB2, HB3, HB4, HL9, HL11, KD4, KD6, KD7) vs <i>S. squamosissimus</i>	0,83
<i>Sicyopterus</i> sp. (KD7) vs <i>S. squamosissimus</i>	2,22

Genetic distance analysis based on uncorrected p-distance supported the phylogenetic relationships observed in Figure 1 (Table 3). Previous studies reported that interspecific divergence among *Sicyopterus* species based on mitochondrial protein-coding genes ranged from approximately 1.17% to 11.36% (Lord et al., 2019). Particularly, Lord et al. (2019) highlighted that *S. aiensis*, *S. cynocephalus*, and *S. lengguru* showed relatively low genetic divergence of around 1%, despite forming distinct phylogenetic lineages with strong posterior probability support. These findings indicate that closely related *Sicyopterus* species may exhibit relatively limited mitochondrial divergence while still representing separate evolutionary lineages.

In the present study, most Bengkulu samples showed relatively low genetic distances to their corresponding reference sequences, supporting their placement within previously recognized *Sicyopterus* taxa. Sample PG1 showed the lowest genetic distance (0.17%) to *S. parvei*, indicating a very close genetic affinity between both sequences. Similarly, samples KD1, KD5, and PG4 showed low genetic distances to *S. lagocephalus* (0.65%), while KD2 showed the closest relationship to *S. cynocephalus* (0.50%). Sample KD3 showed relatively close genetic distances to both *S. aiensis* (0.67%) and *S. cynocephalus* (1.14%). This pattern is consistent with the phylogenetic relationships reported by Lord et al. (2019), in which closely related species within the *S. aiensis*, *S. cynocephalus*, and *S. lengguru* clade also exhibit relatively low mitochondrial divergence. Although KD3 showed a closer genetic affinity to *S. aiensis*, the bootstrap support for this lineage remained low. Therefore, the placement of KD3 within the *S. aiensis*-associated lineage should remain tentative pending additional analyses with nuclear markers and broader morphometric comparisons.

Among the analyzed samples, KD7 showed the greatest divergence from the reference *S. squamosissimus* sequence, with a genetic distance of 2.22%. This value was substantially higher than the average genetic distance observed among the other *S. squamosissimus*-associated samples (0.83%). Although the divergence observed in KD7 remained lower than most interspecific divergence values previously reported among recognized *Sicyopterus* species (Lord et al., 2019). The relatively elevated divergence may indicate substantial intraspecific variation, geographic population structuring, or a potentially distinct lineage within western Indonesian *Sicyopterus* populations. Morphologically, KD7 also showed slight differences in pigmentation and body coloration compared with the other *S. squamosissimus* samples, although these differences were not sufficiently distinct for definitive taxonomic separation.

Similar cases of elevated mitochondrial divergence within morphologically similar gobiid populations have been associated with cryptic diversity and geographically structured populations in amphidromous fishes (Čekovská et al., 2023; Pasisingi et al., 2024). Therefore, KD7 represents an important lineage that warrants further investigation using additional molecular markers, expanded geographic sampling, and detailed morphometric analyses. Complete mitochondrial genome data and nuclear gene markers may provide better resolution for evaluating whether KD7 represents intraspecific variation or a distinct cryptic lineage within *S. squamosissimus*.

The detection of five *Sicyopterus* lineages in Bengkulu, including *S. parvei* and the putative *S. aiensis* lineage, expands current knowledge regarding the distribution of amphidromous gobies in western Indonesia. These findings also highlight the importance of conserving river ecosystems in Bengkulu, as amphidromous gobies depend on connectivity between freshwater and marine environments throughout their life cycles (Lord et al., 2019; Zarei et al., 2022; Nurjirana et al., 2025). Additional field surveys and molecular studies are still needed to better understand the diversity, population structure, and biogeographic patterns of *Sicyopterus* in Sumatra and the surrounding Indo-Pacific regions. In addition to their taxonomic importance, these findings also have ecological and conservation implications. Amphidromous *Sicyopterus* species depend on the connectivity between upstream freshwater habitats and marine environments to complete their life cycle. Therefore, habitat fragmentation, river modification, declining water quality, and disruption of migratory pathways may directly affect population persistence and dispersal processes (Lord et al., 2019; Nurjirana et al., 2025; Zarei et al., 2022). The occurrence of multiple *Sicyopterus* lineages in Bengkulu further highlights the importance of maintaining river ecosystem integrity and connectivity in western Sumatra.

Several limitations should be considered in interpreting the present findings. This study relied on a single mitochondrial marker (Cytochrome b) and relatively limited sample representation from several river systems in Bengkulu. Although mitochondrial DNA is widely used for species identification and phylogenetic reconstruction, analyses based solely on mitochondrial markers may not fully resolve closely related taxa or distinguish recent divergence from intraspecific variation. In addition, some phylogenetic relationships, particularly within the *S. aiensis*-*S. cynocephalus* lineage, showed relatively low bootstrap support, suggesting that further confirmation is still required.

Future studies should therefore incorporate approaches using additional mitochondrial and nuclear markers, broader geographic sampling across western Indonesia, and more comprehensive morphometric and meristic analyses. Population-level studies integrating ecological and biogeographic data would also be valuable for understanding dispersal dynamics, population connectivity, and potential cryptic diversity within Indonesian *Sicyopterus*. Such approaches may provide a more robust framework for taxonomic revision and conservation planning of amphidromous gobies in the Indo-Pacific region.

CONCLUSION

This study identified five *Sicyopterus* lineages from Bengkulu Province, Indonesia (Air Lais, Air Besi, Kedurang, and Padang Guci Rivers) based on combined morphological observations and mitochondrial cytochrome b gene analysis (817 bp). Phylogenetic reconstruction and genetic distance analysis supported the presence of *S. lagocephalus*, *S. squamosissimus*, *S. cynocephalus*, and *S. parvei* in Bengkulu waters, whereas the lineage associated with *S. aiensis* should still be considered tentative, given its relatively low bootstrap support. The detection of *S. parvei* and the putative *S. aiensis* lineage extends the known distribution of *Sicyopterus* in western Indonesia,

particularly Sumatra, beyond its previously documented occurrence in Java, Sulawesi, and other Indo-Pacific regions. Sample KD7 showed the highest genetic divergence (2.22% p-distance) relative to *S. squamosissimus*, suggesting substantial intraspecific variation, geographic population structuring, or a distinct cryptic lineage. These findings highlight the importance of integrating molecular and morphological approaches to improve taxonomic resolution in morphologically variable amphidromous gobies. Nevertheless, this study relied on a single mitochondrial marker and had relatively limited geographic sampling. Therefore, additional studies using other gene approaches, expanded sampling coverage, and more comprehensive morphometric and ecological analyses are still needed to clarify species boundaries, population structure, and evolutionary relationships within *Sicyopterus* populations in Indonesia. Such information is important not only for taxonomic revision but also for supporting conservation strategies, particularly because amphidromous gobies are highly dependent on river connectivity and sensitive to environmental disturbance.

AUTHOR CONTRIBUTION

D.F.L. designed the study and conducted the research, **H.T.** collected field data samples, **S.N.K.** conducted the research and drafted the manuscript, **S.** supervised the research process, **M.R.A.H.** assisted in writing and reviewed the final manuscript, and **N.M.** contributed to manuscript writing.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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