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Unveiling the occurrence of vulnerable Sisorid catfish (Teleostei: Sisoridae) in Bangka based on morphological and molecular evidence

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Abstract. *Valen FS, Mamat NB, South J, Ottoni FP, Vieira LO, Kamarudin AS, Afandi AY, Hasan V. 2024. Unveiling the occurrence of vulnerable Sisorid catfish (Teleostei: Sisoridae) in Bangka based on morphological and molecular evidence. Biodiversitas 25: 4543-4550. Glyptothorax robustus* Boeseman 1966 is a vulnerable catfish species that is known to be distributed across Java and Sumatra. The objective of this research is to document, for the first time, the presence of *G. robustus* in Bangka, Indonesia, and to update the species' geographic distribution. This discovery of a freshwater fish in a new location significantly contributes to the understanding of its biogeography. A purpose-sampling approach was employed to collect the specimens. The species were identified morphologically and molecularly through DNA barcoding. On 20 January 2023, five *G. robustus* specimens were collected from the Bumang River in Bangka Island using fish traps. The occurrence of *G. robustus* at this location represents the most northerly record of this species and extends its known geographic range. Additionally, this new site is approximately 200 km north of the nearest known location on Sumatra Island and 300 km north of the nearest known location in Java. This record expands the documented distribution range of *G. robustus* and enhances our understanding of this species. Furthermore, an updated record of the *G. robustus* DNA sequence from the cytochrome c oxidase subunit I (COI) gene is presented. This sequence constitutes the first DNA barcode for Indonesia and has the potential to support future studies in biogeographical and ichthyological research. The DNA sequence was registered in NCBI GenBank under the accession code OR144414. This DNA barcode can serve as a standard for identifying *G. robustus* and will support future DNA and biotechnology-based studies.

Keywords: Aquatic environment, checklist, fisheries, life below water

INTRODUCTION

Western Indonesia is one of the world's hotspots for freshwater fish (Kottelat et al. 1993; von Rintelen et al. 2017; Roesma et al. 2024). Partly explained by the west area of the Indonesian archipelago being connected to Sunda Shelf. Despite huge biodiversity, Indonesian freshwater fish distribution patterns are somewhat unexplored (Kolanowska et al. 2016; Kurniawan et al. 2021; Kurniawan et al. 2022). There are more than 1200 native freshwater fish species in Indonesia, including 134 endemic species (Hubert et al. 2015). Within the three main islands (Borneo, Sumatra, and Java), there are several rivers with varied topographies due to the paleogeology of the region (Kottelat 2013; Hasan and Widodo 2020; Haryono and Wahyudewantoro 2020). The catfish genus *Glyptothorax* (family Sisoridae) is one of the most diverse fish genera from western Indonesia, comprising approximately 80 valid species, and is widely distributed in the Asian hillstream (Kottelat et al. 1993; Kottelat 2013; Ng and Kottelat 2016; Jiang et al. 2023). This genus spans the entire range of the family, from the Euphrates River drainage in eastern Turkey southward to the Indian subcontinent and the Greater Sunda Islands and eastward to the Yangtze River drainage (Rameshori and Vishwanath 2014; Jiang et al. 2023). One of the species found in this region is *Glyptothorax robustus* Boeseman 1966, which is limited to the western tip of Java and the southeastern tip of Sumatra (Kettela 2013; Ng and Kottelat 2016; Ng 2020). New records of *G. robustus* are critical to accurately resolve evolutionary diversification patterns within the region.

The existence of *G. robustus* in Java and Sumatra is recorded as Vulnerable (VU) based on the International Union for Conservation of Nature (hereafter IUCN) Red List of Threatened Species (Ng 2020). The populations of *G. robustus* in Sumatra and Java have dramatically declined due to pollution, habitat fragmentation, and the presence of invasive fish, which causes competition for resources, predation, and disease transmission (Margono et al. 2014; Hasan et al. 2020a; Rahmi et al. 2023). Despite its

vulnerable status, no conservation measures have been implemented for this species. Therefore, to manage a conservation plan, it is essential to study its life history, population dynamics, associations and potential threats.

DNA barcoding can facilitate understanding of species evolution and speciation alongside acting as reference tool for species distribution studies (Imtiaz et al. 2017; Sektiana et al. 2022; Syarif et al. 2023). DNA-barcoding serves as a tool for the rapid identification of species using one or several standardized mitochondrial DNA gene regions (Panprommin et al. 2019; Andriyono et al. 2022; Priyono et al. 2022). The cytochrome c oxidase subunit I (COI) gene is one of the standard fish species identification genes (Zou et al. 2020). The COI gene has been globally adopted as a standardized tool for the identification and molecular taxonomy, providing a reliable basis for differentiating between species (Dwifajri et al. 2022; Irmawati et al. 2022; Nurjirana et al. 2023). It has been successfully used as a species identification tool for freshwater fish in Indonesia (Widodo et al. 2020; Sajjad et al. 2023; Robin et al. 2023).

In this study, we discovered a new distribution location for *G. robustus* on Bangka and conducted the first DNA barcoding of this species based on the COI gene. The DNA-barcoding data were uploaded to the GenBank NCBI database as a standard for identifying *G. robustus* based on the COI gene. This research will enhance rapid species identification, contribute to understanding biodiversity and increases in species genetic diversity, and provide insights into the species' life history. In the long term, this work will support conservation actions and development.

MATERIALS AND METHODS

Sampling site

The study was conducted in the Swamp Forest Stream of Bumang River (2°04'56.0"S, 105°57'39.0"E) (Figure 1), Kemujang Village, Mendo Barat Sub-district, Bangka District, Bangka Island, Indonesia, from 8 to 14 July 2024. Samples were collected using environmentally sustainable fishing equipment, specifically scoop nets. The *G. robustus* specimens were captured throughout the sampling process. All the captured specimens were collected and delivered to the laboratory for the next stage of the study.

Preserve fish and morphological analysis

Five *G. robustus* specimens were captured using a scoop net over 1 week of trapping. The remaining 12 specimens were retained as livestock for breeding and domestication at the Fish Reproduction Laboratory, Aquaculture Department, Bangka Belitung University, Indonesia. The pectoral fin on the right side of one fish was surgically removed to collect the tissue sample. The fins were stored in a solution of 70% alcohol for subsequent molecular analysis. The left side was used to gather morphometric data. It was then stored in a 10% formalin solution (Kusumah et al. 2023) and then deposited to the Ichthyological Collection of the Aquaculture Laboratory at Universitas Bangka Belitung, Indonesia. The specimens were examined for diagnostic morphological characteristics according to Ng and Kottelat (2016).

DNA extraction and amplification processes

DNA was extracted using the 10% Chelex protocol. After the extraction process, the partial fragment of the mitochondrial CO1 was amplified using the BIONESIA method with FISH-F1 (5′- `TCA ACC AAC CAC AAA GAC ATT GGC AC` -3′) and FISH-R1 (5′- `TAG ACT TCT GGG TGG CCA AAG AAT CA` -3′) primers (Ward et al. 2005). The total PCR reaction volume was 25 µL, comprising 2 µL of extracted DNA template, 1.25 µL of each primer at a concentration of 10 mM, 4.5 µL of ddH_2O, 1.5 µL of 10× PCR buffer, 2.5 µL of dNTPs, 2.0 µL of $MgCl_2$, and 0.125 µL of PE Amplitaq. The reaction mixture was amplified using a 2720 Thermal Cycler (Applied Biosystems).

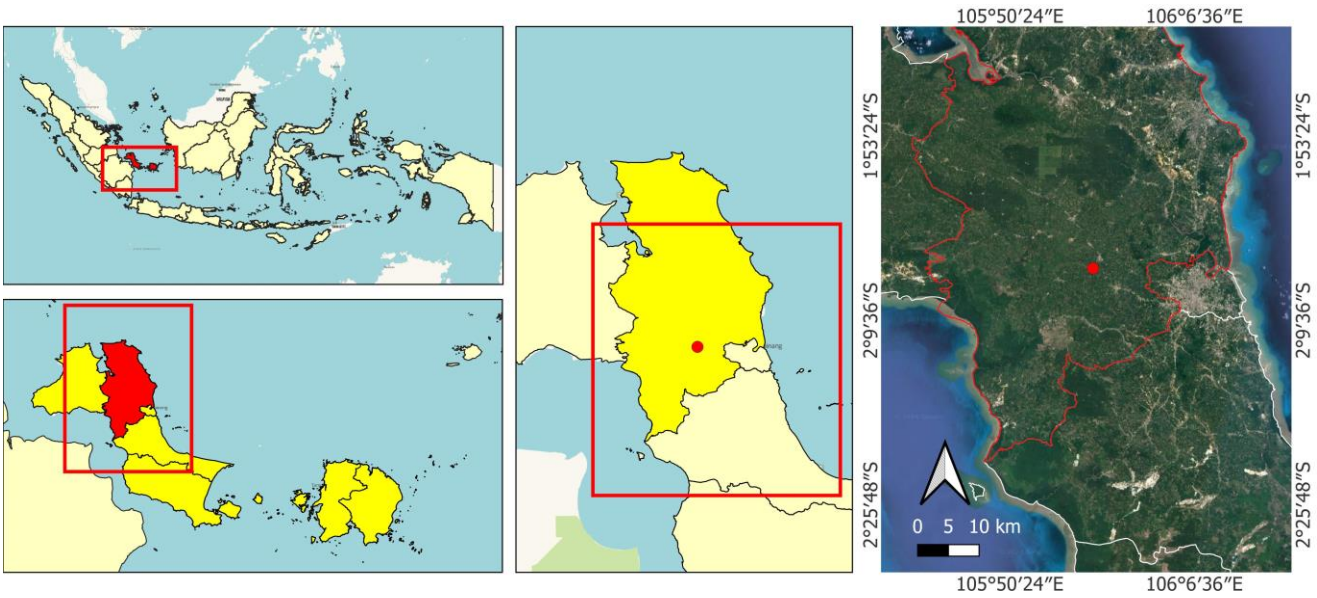

Figure 1. Collecting sites of Bumang River, Bangka, Kepulauan Bangka Belitung, Indonesia

PCR cycling parameters consisted of an initial denaturation phase at 94°C for 3 min, followed by denaturation at 94°C for 30 s, annealing at 48°C for 30 s, and extension at 72°C for 45 s, repeated for 38 cycles. Subsequently, the PCR results were visualized on a 1% agarose gel through electrophoresis, stained with Nucleic Acid Gel Stain (GelRed®) (Valen et al. 2024a). A positive sample, identified by DNA bands on an agarose gel, was subjected to DNA sequencing using the Sanger dideoxy method (Sanger et al. 1977; Al-Shuhaib and Hashim 2023), generating a partial COI sequence with 636 base pairs.

Data analysis

Species identification was conducted using BLASTn (Basic Local Alignment Search Tool nucleotide) in the National Center for Biotechnology Information (NCBI)-GenBank (https://blast.ncbi.nlm.nih.gov). Previously, the quality of the DNA sequences of the species was visually assessed using Sequence Scanner software, which displays DNA fragment nucleotide chromatograms. Graphs with well-separated high-peak sequencing results are of superior quality. Graphs with peaks that slope or are not separated from each other indicate low-quality sequencing findings. To create a base arrangement whose outcomes were reliable, low-quality portions of the sequence were removed. The superior-quality sequences were aligned using the muscle algorithm. The evolutionary history was inferred using the neighbor-joining method using MEGA X (Kumar et al. 2018). Bootstrap analysis (Felsenstein 1985) with 1,000 replicates was performed to assess the robustness of the tree topology using MEGA X (Kumar et al. 2018). Evolutionary distances were computed and expressed as the number of base substitutions per site by the Maximum Composite Likelihood method. These evolutionary analyses, along with assessments of nucleotide composition and polymorphic sites, were conducted using MEGA X (Kumar et al. 2018).

RESULTS AND DISCUSSION

Identification of morphological features

We identified *G. robustus* (Figure 2) based on the characteristics proposed by Ng and Kottelat (2016). This species is distinguished from its congeners by the combination of the following characteristics: *G. robustus* is distinguished from all Sundaic congeners in absent (vs. having) a medial pit on the thoracic adhesive apparatus and having (vs. lacking) prominent dark longitudinal stripes running through each caudal-fin lobe; premaxillary tooth band approximately half exposed when mouth is closed; anteromedial striae in thoracic adhesive apparatus absent; smooth posterior margin of dorsal-fin spine; margin of dorsal fin concave; straight edge of dorsoposterior adipose fin; the body is nearly uniform with scattered pale patches and absent (vs. having) dark vertical bars at the adipose-fin base and the base of the caudal fin. Other specific morphological characteristics were as follows: depressed head and sub-cylindrical body. The dorsal profile rises evenly from the tip of the snout to the base of the dorsal fin, sloping gently ventrally from the base of the dorsal fin to the tip of the caudal peduncle. The ventral profile is straight to the base of the anal fin, then slopes dorsally from the base of the anal fin to the tip of the caudal peduncle. The anal and urogenital openings were located vertically through the middle of the compressed pelvic fins, tuberculous skin with even-sized tubercles on the sides of the body. Complete lateral and mid-lateral lines.

Distribution range

The occurrence of *G. robustus* in Bumang River, Kemujang Village, Mendo Barat Sub-District, Bangka District, Bangka Island, Indonesia, is the northernmost record for this species, expanding its geographic distribution. In addition, the new record site is about 200 km northeast of the nearest locality in Sumatra and about 300 km north of the nearest locality in Java (Figure 3). New records of freshwater fishes are essential contributions to the natural sciences (Saptadjaja 2020; Hasan et al. 2024). They play a crucial role in supporting appropriate conservation-related decisions and environmental impact assessments (Ihwan et al. 2020; Valen et al. 2020; Hasan et al. 2022; Ndobe et al. 2023). Hence, this new record fills an important gap in the species' geographic distribution and is registered as an additional island for the species (Figure 1).

Figure 2. *Glyptothorax robustus* collected from Bumang River, Bangka, Indonesia (63.3 mm SL)

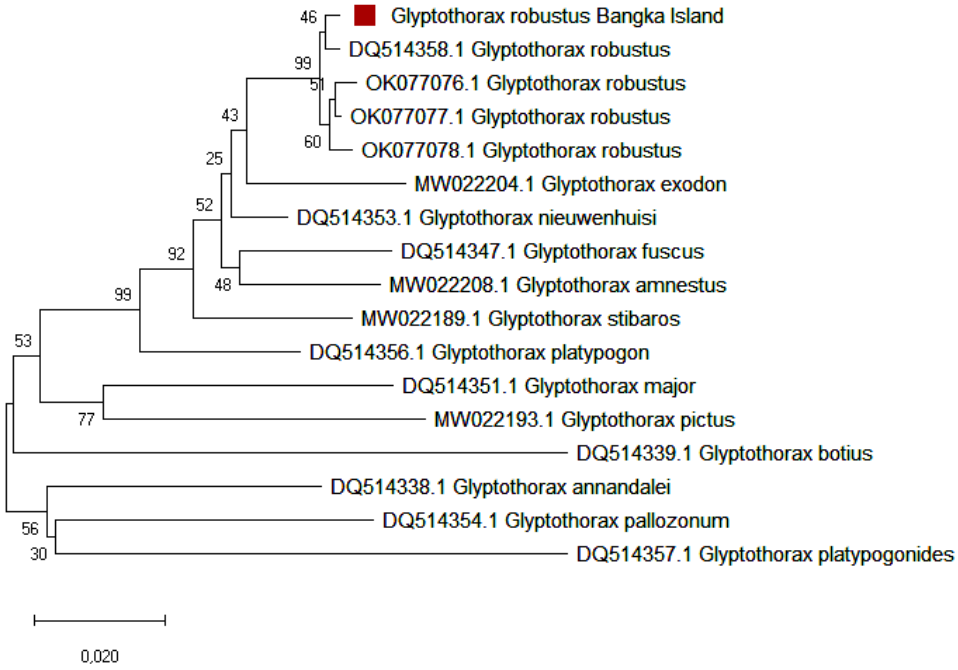

Figure 3. Neighbor-joining phylogeny of the *Glyptothorax* of Sundaland based on COI genes. The number above branches are bootstrap values

Conservation status

Glyptothprax robustus is categorized as Vulnerable (VU) by the IUCN because their habitats are located near human settlements (Ng 2020). The consequences of human actions on freshwater ecosystems have become apparent with the profound, recent global declines in fish populations, including *G. robutus*. However, despite their ecological significance, populations of many species of Sosorid catfish are declining on a global scale, mainly due to habitat fragmentation and pollution (Ng and Kottelat 2016; Ng 2020). In addition, the entry of invasive species can cause the loss of local fish populations in Indonesia (Hasan et al. 2020b; Serdiati et al. 2020; Serdiati et al. 2021). In the future, more complete data collection is needed to assess the occurrence of *G. robustus* and to evaluate the importance of Bangka waters as the natural habitat of *G. robustus*.

DNA barcoding of *G. robustus*

The DNA barcode of *G. robustus* from Bangka Island was accurately determined by sequencing the COI gene using Fish_F2 and Fish_R2 primers, resulting in a length of 686 bp. Hebert et al. (2003) found that COI genes with lengths greater than 658 bp can serve as reliable criteria for identifying various animal species.

DNA barcoding of *G. robustus* from Bumang River, Bangka, Indonesia:

TTTGGTGCTTGGGCCGGAATAGTGGGCACAGCCCTTAGT
CTTCTGATCCGGGCGGAGCTAGCCCAACCCGGAGCTCTA
CTAGGCGATGATCAAATCTATAATGTCATCGTTACTGCA
CACGCCTTTGTTATAATCTTCTTTATAGTAATACCAATC
ATGATTGGGGGGTTCGGCAACTGACTTGTCCCCCTAATG
ATTGGAGCACCAGACATAGCATTCCCTCGAATAAACAAC
ATAAGCTTCTGACTACTGCCCCCATCCTTTCTCCTACTT
CTCGCCTCTTCAGGTGTTGAAGCCGGAGCTGGGACAGGG
TGGACAGTATACCCCCCGCTTGCCGGAAACCTAGCACAT
GCTGGAGCCTCCGTAGACTTAACAATCTTCTCACTGCAT
CTTGCAGGGGTGTCATCAATTCTAGGGGCCATCAACTTC
ATCACAACGATTATTAATATGAAACCCCCAGCAATTTCA
CAATATCAAACACCTTTATTTGTATGAGCTGTCCTAATT
ACAGCAGTACTCCTATTACTGTCACTACCAGTACTTGCT
GCAGGCATTACAATACTGCTAACAGACCGAAATCTAAAT
ACAACTTTCTTTGACCCAGCAGGAGGAGGTGACCCCATT
CTATACCAACA

DNA barcoding is a taxonomic tool that uses short genetic markers in the DNA of an organism for identification at the species level. For the identification of fish species, the COI gene is a standardized genetic target; this method was well-suited because it displays a high level of divergence between species and a low divergence within species (Bingpeng et al. 2018). The COI gene in the DNA can function as a universal barcode for all animal species. Furthermore, the DNA barcoding of *G. robustus* Bangka was evaluated using Sequence Scanner software, which presents nucleotide chromatograms of the DNA fragments. Graphs displaying distinct, prominent sequencing results indicate higher quality (Liu et al. 2020). Graphs exhibiting merged or overlapping peaks indicate poor sequencing results. To provide a reliable foundation, we eliminated low-quality sequences. The DNA barcoding shows 642 high-quality DNA fragments from the COI gene of *G. robustus* from Bangka Island, with each fragment consisting of a base pair.

DNA barcoding is an essential tool for the development of scientific knowledge, particularly in biotechnology and taxonomy (Valen et al. 2023; Lamadi et al. 2023; Modeel

et al. 2024). Additionally, the DNA barcode of *G. robustus* from Bangka Island was the first barcode reported in GenBank and the primary report originating from Indonesia. This sequence contributed to the Barcode of Life (BOL) project by recording the diversity of eukaryotic organisms. Moreover, genetic samples of species are important for comprehending fish population structures, especially if considered for captive breeding purposes. Declining freshwater fish biodiversity, as a result of habitat destruction, non-native invasive species and climate change, mean that assessing natural diversity is a priority to create conservation measures (Ottoni et al. 2023).

Species identification based on COI gene

The COI gene of the Bangka Island species was examined and compared with the NCBI GenBank using the Basic Local Alignment Search Tool-nucleotide method (https://blast.ncbi.nlm.nih.gov) (Table 1).

Species identification was accomplished using sequences extracted from the COI gene found in mitochondrial DNA. Utilization of the COI gene has been proven to be informative for the identification of multiple freshwater species (Robin et al. 2023; Syarif et al. 2023; Valen et al. 2024a). The COI gene of the species from Bangka Island was examined and compared with the NCBI GenBank using the Basic Local Alignment Search Tool-nucleotide (BLASTn) method (https://blast.ncbi.nlm.nih.gov), which was used to identify regions with local similarity between protein and nucleotide sequences. This program compares protein or nucleotide sequences to sequences from a database and calculates the matched statistical significance.

Based on the BLAST analysis, the genetic sequence of *G. robustus* from Bangka Island had similarity thresholds of 99% to other *G. robustus* from GenBank. According to Hebert et al. (2003), sequences that have a similarity range of 97%-100% are typically considered identical, whereas organisms with differences of 3% or more are considered distinct species. However, the DNA barcode of *G. robustus* contained nucleotide bases (A, T, G, and C) with a nucleotide composition percentage of T (28.1%), C (29.2%), A (27.0%), and G (15.8%). The COI gene of this genus was classified as an A-T-rich group (A-T rich) because of the high average quantities of adenine and thymine found in the DNA barcode of *G. robustus* with 55.1%. There is a greater chance of species mutation because the A-T has two hydrogen bonds, whereas the G-C hydrogen bond has three (Insani et al. 2022).

Genetic distances and molecular phylogeny

Based on the genetic distance, the population of *G. robustus* from Bangka Island was close to that of other populations of *G. robustus* from the GenBank database, which was approximately 0.005 (Table 2).

Diversity is the foundation upon which breeding and evolution occur. Any population with genetic variation benefits from being able to guide diverse life forms toward genetic advancement and improved adaptation to changing environmental factors (Ha et al. 2017; Valen et al. 2024b). Genetic distance is correlated with geography. Based on the genetic distance, the population of *G. robustus* from the GenBank database was approximately 0.005 (Table 2) (Hasan et al. 2021). This indicates that among a collection of 1000 base pairs, their base pairs were different with *G. robustus* being isolated from Bangka Island.

After determining the genetic distance between the *Glyptothorax* species, we reconstructed the phylogenetic connection based on the mitochondrial COI gene to determine the evolutionary history of the Sundaland *Glyptothorax* (Figure 3). We constructed a phylogenetic tree with a bootstrap level of 1000 replications based on genetic distance. The evolutionary tree of the Sundaland *Glyptothorax* indicated a high level of accuracy and consistency in branching patterns. Branches of the phylogenetic tree with a support value greater than 70% were considered to have a high level of confidence with a 95% confidence interval. The phylogenetic tree consists of two branches and two clades.

Genetic polymorphisms

There were eight nucleotide bases out of a total of 642 that differed between *G. robustus* from Bangka Island and others *G. robustus* from the GenBank database (Table 3). Genetic polymorphisms are heritable alterations in DNA sequences (Prasertlux et al. 2024). Variation in the population is often caused by mutations (Martinez et al. 2018). This determines the diversity of the individuals. Polymorphic site analysis can be used to identify differences in the nucleotide sequences of a species. This study aimed to determine the locations of sites that exhibit differences within the same species; out of a total of 642 base pairs, eight nucleotides from *G. robustus* from Bangka Island differed from other *G. robustus* from the GenBank database.

Table 1. Species identification and similarity of the *Glyptothorax* from Bangka

Species outcome	Family	Genbank accession ID	Query coverage (%)	Percent identity (%)
Glyptothorax robustus Boeseman 1966	Sosoridae	DQ514358.1	100	99
Glyptothorax exodon Ng & Rachmatika 2005	Sosoridae	MW022204.1	100	96
Glyptothorax amnestus Ng & Kottelat, 2016	Sosoridae	MW022196.1	100	95
Glyptothorax platypogon Valenciennes 1840	Sosoridae	DQ514356.1	100	94
Glyptothorax fuscus Fowler 1934	Sosoridae	DQ514347.1	100	96
Glyptothorax schmidti Volz 1904)	Sosoridae	DQ514359.1	100	96
Glyptothorax nieuwenhuisi Vaillant 1902	Sosoridae	DQ514353.1	100	97

Table 2. Genetic distances of the *Glyptothorax* of Sundaland based on COI genes

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
a														
b	0.005													
c	0.104	0.106												
d	0.098	0.099	0.115											
e	0.138	0.139	0.135	0.141										
f	0.041	0.042	0.116	0.110	0.141									
g	0.024	0.026	0.098	0.097	0.126	0.035								
h	0.105	0.106	0.125	0.094	0.131	0.112	0.107							
i	0.059	0.059	0.087	0.097	0.130	0.076	0.047	0.110						
j	0.139	0.138	0.147	0.121	0.173	0.149	0.134	0.128	0.139					
k	0.045	0.051	0.096	0.100	0.143	0.056	0.037	0.120	0.057	0.143				
l	0.108	0.108	0.094	0.128	0.145	0.115	0.106	0.127	0.102	0.145	0.107			
m	0.039	0.040	0.100	0.104	0.133	0.061	0.037	0.106	0.061	0.145	0.056	0.104		
n	0.042	0.044	0.100	0.104	0.149	0.045	0.037	0.129	0.056	0.135	0.049	0.112	0.053	

Note: a: *G. robustus* Bangka; b: *G. robustus*; c: *G. major*; d: *G. annandalei*; e: *G. botius*; f: *G. fuscus*; g: *G. nieuwenhuisi*; h: *G. pallozonum*; i: *G. platypogon*; j: *G. platypogonides*; k: *G. stibaros*; l: *G. pictus*; m: *G. exodon*; n: *G. amnestus*

Table 3. Genetic polymorphisms/polymorphic sites between *G. robustus*

	58	67	124	184	217	260	265	334
Glyptothorax robustus Bangka	G	G	T	C	A	C	G	A
OK077076.1 *Glyptothorax robustus*	A	.	C	T	G	.	A	.
OK077077.1 *Glyptothorax robustus*	A	.	.	T	G	.	A	G
OK077078.1 *Glyptothorax robustus*	A	A	.	.	G	.	A	G
DQ514358.1 *Glyptothorax robustus*	.	.	.	.	G	T	A	.

The genetic mutations were primarily transition mutations occurrences of A↔G and C↔T, and there are no transversion mutations. Transition mutations included the replacement of purines (A, G) with fellow purines or the replacement of pyrimidines (C and T) with fellow pyrimidines. Transversion replaces a pyrimidine with a purine or vice versa. Transversions usually result in a greater probability of protein change than transitions because there is a more drastic change in the process of amino acid formation. There are four possible mutations in transition (A↔G, C↔T) and eight possible mutations in transversion (A↔C, A↔T, G↔C, G↔T) (Insani et al. 2022).

In conclusion, *G. robustus* is a vulnerable species whose distribution is not only in Java and Sumatra but has also recently been discovered in Bangka. This study's morphological features, such as color, body form, and the Cytochrome c oxidase subunit I (COI) gene, showed that the samples obtained were valid as *G. robustus*, a local type of Bangka. This discovery is important for the species' conservation status.

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