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Unveiling the Enigmatic Dwarf Horseface Loach *Acanthopsoides molobrion*: A Groundbreaking Discovery in Indonesia

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Abstract. *Acanthopsoides molobrion* is a freshwater previously known to be spread across Malaysia and Borneo. This study aimed to record for the first time the occurrence of the *A. molobrion* in Bangka Island, Indonesia, and update the geographic distribution of this species. This new record of freshwater fish provides an important contribution to the comprehension of the biogeography of the species. The purpose sampling approach was employed for this study to collect specimens. The species were then identified morphologically by the application of morphometric and meristic methods, and molecularly through the employ of DNA Barcoding. On 20th January 2023, two *A. molobrion* specimens were collected from the Bumang Kemuja River, Bangka Island using a fish trap. The new record of *A. molobrion* found here is the southernmost record for this species, expanding its geographic distribution. In addition, the latest record site is about 500 km south of the nearest locality in Peninsular Malaysia, and about 750 km southwest of the nearest locality in Borneo. The new record of *A. molobrion* has expanded the species' recorded distribution range, which has added to our understanding of this species. Furthermore, we present an updated record for the *A. molobrion* DNA sequence based on the COI gene. This sequence is the first DNA Barcode to Indonesia. Subsequently, the DNA sequence was registered into NCBI Genbank with the access code OR144414. This DNA barcode will be used as a standard for identifying *A. molobrion* and will aid in DNA and biotechnology-based studies in the future.

Keywords: COI gene, DNA barcoding, freshwater species, geographical distribution, new record

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INTRODUCTION

Indonesia boasts a remarkably high diversity of freshwater fish species. As of 2022, there are 1,266 species documented in Indonesian inland waters (Robin *et al.*, 2023a). The diversity of Indonesian freshwater fish comprises endemic, native, introduced, and reintroduced species (Hasan *et al.*, 2023a; Insani *et al.*, 2023; Valen *et al.*, 2023a). However, *Acanthopsoides molobrion* is native to Indonesia, this species belongs to the Cobitidae family and the class Actinopterygii. This species has an IUCN Red List (International Union for Conservation of Nature's Red List of Threatened Species) status of least concern (LC) (Daniels & Allen 2020), usually occurring in clear water river channels, lakes, or swamps and primarily feed on small animals found in the

sediments.

According to Kottelat (2020), *A. molobrion* has a distribution range from Malaysia (Malay Peninsula and parts of Sarawak state) to Borneo (Mahakam River Basin). In this study, we report the discovery of *A. molobrion* in Bangka Island, marking the second recorded presence of this species in Indonesia, following a previous sighting in the Mahakam River Basin in Borneo, with additional records in Malaysia. These findings contribute to our understanding of the geographical distribution and range of *A. molobrion* (Hasan *et al.,* 2024; Ndobe *et al.,* 2022; Valen *et al.,* 2022). This record represents the southernmost record for this species, extending its geographical distribution further south. Moreover, this study provides a detailed and updated map of the geographic distribution of *A. molobrion*.

Furthermore, this study also presents the first DNA barcode of *A. molobrion* based on the Cytochrome C Oxidase Subunit I (COI) gene, specifically for Bangka Island, Indonesia. This groundbreaking report represents the first sequence submitted to the Genbank database. The COI gene is widely used as a universal gene for species identification or DNA barcoding of fishes (Tsoupas *et al.,* 2022; Bingpeng *et al.,* 2018). Its utilization allows for rapid and accurate species identification by analyzing one or several gene segments of mitochondrial DNA (Fawzia *et al.,* 2020; Hebert *et al.,* 2003). Notably, the COI gene has been successfully employed for the identification of freshwater fish species in Indonesia (Insani *et al.,* 2022; Roesma *et al.,* 2022).

This research aims to record the new distribution record for *A. molobrion* on Bangka Island, Indonesia and update the geographic distribution of this species. Additionally in this study, the new finding of of the DNA sequence of *A. molobrion* based on the COI gene was registered into NCBI Genbank in order to increase the Barcode of Life (BOL). In the future, this DNA barcode will benefit as a standard for identifying *A. molobrion* and assist in DNA and biotechnology-based research.

METHODS

Sampling sites and fish collection

Two specimens of *A. molobrion* were collected using a fish trap on 20th January 2023 in the Bumang Kemuja River, Bangka Island (Indonesia). The sampled habitat was in the central channel of the river (ca.10-20 m wide), along a river bend with a rocky and sandy substrate and fast-flowing water. One specimen was preserved in 96% ethanol (Nuryanto *et al.,* 2018) for ensuing DNA examination other one specimen was fixed in formalin 10% and deposited in Laboratorium of Aquaculture, Bangka Belitung University. *Trigonospoma pauciperforatum, Trigonospoma gracile, Brevibora cheeya, Rasbora chepalottenia, Clarias leeiachanthus,* and *Mastacembelus notopthalmus* were also collected from the same habitat of *A. molobrion* during the sampling.

DNA extraction and amplification

The DNA was extracted by using the 10% Chelex protocol. Following the extraction process, amplification of the partial fragment of mitochondrial Cytochrome C Oxidase Subunit 1gene (CO1) was following 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 was 25 µL consisting of a mixture of 2 µL extracted DNA template, 1.25 µL of each primer in 10 mM concentration, 4.5 µL ddH2O, 1.5 µL 10x PCR Buffer, 2.5 µL dNTPs, 2.0 µL MgCl2, and 0.125 µL PE Amplitaq. The reaction mixture was then amplified using an Applied BiosystemsTM 2720 Thermal Cycler machine. PCR cycling parameters included an initial denaturing phase of 3 minutes, denaturing at 94oC for 30 seconds, annealing at 48oC for 30 seconds, and extension at 72oC for 45 seconds for 38 cycles. The PCR results were then visualized in 1% agarose gel via electrophoresis by staining Nucleic Acid Gel Stain (GelRed®) (Robin *et al.,* 2023b; Nuryanto *et al.,* 2022). A positive sample (sparkling DNA bands) was then processed for DNA reading (sequencing) using the Sanger dideoxy method (Al-Shuhaib *et al.,* 2023).

Data analysis

The Species Identification was calculated using the BOLD SYSTEM (https://www. boldsystems.org) and the BLASTn (Basic Local Alignment Search Tool-nucleotide) NCBI GenBank (https://blast.ncbi.nlm.nih.gov). Previously, the quality of the species DNA sequences is then visually assessed using Sequence Scanner software, which displays the DNA fragment nucleotide chromatograms. Graphs with well-separated, high-peak sequencing results showcase superior quality. Graphs with peaks that slope or are not separated

from one another, on the other hand, show low-quality sequencing findings. To create a base arrangement whose outcomes we can rely on, low-quality portions of the sequence are then removed. The superior quality of sequences was aligned using the Muscle algorithm. The evolutionary history was inferred using the Neighbor-Joining method (Li, 2015). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses, Nucleotide composition, and polymorphic sites were, were conducted in MEGA X (Kumar *et al.*, 2018).

RESULTS AND DISCUSSION

Morphological identification

The specimen collected in the Bumang Kemuja River, Bangka Island (Indonesia), was successfully identified as *Acanthopsoides molobrion* (Figure 1) based on characters proposed by Siebert (1991): The diagnostic features exhibited by the specimen include: Body terete; peduncle slender; eye small and usually in the anterior half of head; head scaleless; head length approximately 20% of SL; snout length moderate; lateral line usually incomplete; suborbital spine bifid; caudal-fin symmetrical and emarginate; dorsal-fin with 7 branched fin-rays. This is the same as the number of rays given by Siebert (1991) for Acanthopsoides (7, rarely 8, branched rays). The snout of *A. molobrion* is also quite lengthy, especially when compared to that of *A. robertsi.*

New record

INDONESIA – Bangka Island • Bangka Belitung Province; Bangka Induk District, Bumang Kemuja River; (2°05’08” S, 105°57’24”E) caught with a fish trap; 2 ♂ specimens (Figure 1).

Molecular identification

Molecular identification of *Acanthopsoides molobrion* was based on the DNA Barcoding method. The DNA-Barcode of *A. molobrion* from Bangka Island was successfully sequenced with a base-pair length of 670 bp (Table 1) using Fish_F1 and Fish_R1 primers (Ward et al 2005). Fragments that have more than 655 base pairs of COI genes can be used as a DNA Barcode, a standard for differentiating between animals (Guo *et al.*, 2022).

Figure 1. A live Specimen of *A. molobrion* from Bumang Kemuja River, Bangka Island, Indonesia.

Table 1. DNA Barcoding of *A. molobrion*, from Bumang Kemuja River, Bangka Island, Indonesia

DNA Barcoding of *A. molobrion* from Bumang Kemuja River, Bangka Island, Indonesia
CTGTATCTGGGTGCCTGAGCCGGGATAAGTAGGAACCGCCCTTAGCCTCCTCATTCGCGCCGAGCTTAGCCAGCC
CGGATCCCTTCTTGGTGATGACCAAATTTATAATGTAATCGTCACCGCCCACGCCTTCGTAATAATTTTCTTTAT
AGTAATGCCAATTCTTATTGGCGGGTTTGGCAACTGACTAATCCCACTTATGCTTGGTGCCCCTGATATGGCATT
CCCACGAATGAACAATATAAGCTTCTGACTCCTACCTCCATCATTTCTTCTACTATTAGCCTCTTCTGGCGTAGA
AGCTGGGGCAGGGACAGGTTGAACCGTATATCCACCCCTAGCGGGCAACCTCGCCCACGCAGGCGCATCCGTAGA
CTTAACCATTTTCTCCTTACATTTAGCAGGTGTGTCCTCTATTTTAGGGGCAATTAATTTTATTACTACAACAAT
TAATATGAAACCCCCTGCCATCTCTCAATACCAAACGCCCTTGTTTATCTGAGCTGTTTTAGTGACGGCGGTCCT
TCTCCTGCTATCCCTGCCCGTCCTGGCCGCCGGGATTACAATGCTGCTAACGGACCGAAACCTGAACACTACCTT
CTTTGACCCAGCCGGAGGAGGAGACCCAATCCTTTATCAACACCTCTTCTGATTCTTTGGCCACCAGAA

Additionally, the sequence information from *A. molobrion* from Bangka Island was uploaded to GenBank NCBI with the accession number OR144414. This barcode is the very first report for Indonesia and the first barcode registered in the Genbank. The DNA barcode of *A. molobrion* can be used as a reference for species identification in the next research (Tang *et al.*, 2023; Liu *et al.*, 2020). This information is very important to enrich science, especially to understand the taxonomy (Robin *et al.*, 2023b; Tadmor-Levi *et al.*, 2022) and improve knowledge in biotechnology. Furthermore, this sequence attempts to complement the Barcode of Life (BOL) project by registering eukaryotic biodiversity.

However, the DNA Barcode of *A. molobrion* contains Nucleotides bases (A, T, G, C) with the percentage of nucleotide composition being T (28.1%), C (29.2%), A (27.0%), and G (15.8%). The COI gene of this genus is classified as an A-T-rich group (A-T rich) due to the high average quantities of adenine and thymine found in the DNA Barcode of *A. molobrion*. There is a greater chance of a species mutation because the A-T hydrogen connection has two hydrogen bonds, whereas the G-C hydrogen bond has three hydrogen bonds (Valen *et al.*, 2023b).

According to research discoveries, the GenBank database does not have a DNA barcode for *A. molobrion*. Our DNA Barcode was the very first record of the NCBI Genbank. Subsequently, we identified the family level between the sequences from the study specimen and the sequences in GenBank to confirm the sequence; this revealed that our sequence is a member of the Actinopterygii class and the Cobitidae family (Table 2). The results of research based on morphology are by this. The results of this research revealed that the cytochrome C oxidase subunit I (COI) gene could accurately identify species, genera, and families.

Phylogenetic tree

The phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model in MEGA X (Kumar et al. 2018) to show the evolutionary *A. molobrion* within the family of Cobitidae (Figure 4). We took the sequences of Cobitidae (10 species, 4 genera) and Cyprinidae (10 species, 10 genera) from the Genbank and built the phylogenetic tree based on the COI gene.

Table 2. Species identification and similarity of the Cobitidae family

Species Outcome	Family	Accession ID	Query Coverage (%)	Percent Identity
Acantopsis rungthipae	Cobitidae	MF510006.1	95	85.13
Acantopsis rungthipae	Cobitidae	MF510005.1	95	85.13
Acantopsis rungthipae	Cobitidae	MF5100031	95	85.13
Paralepidocephalus yui	Cobitidae	NC068240.1	99	85.15

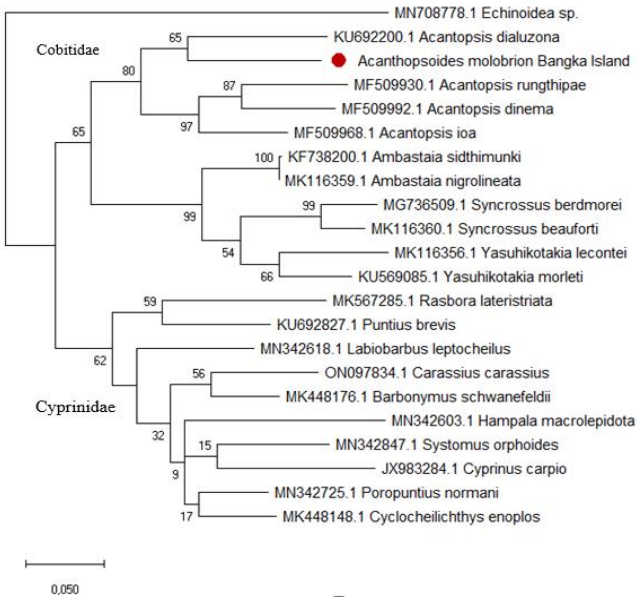

Figure 2. Phylogeny of the Cyprinidae and Cobitidae based on COI sequences.

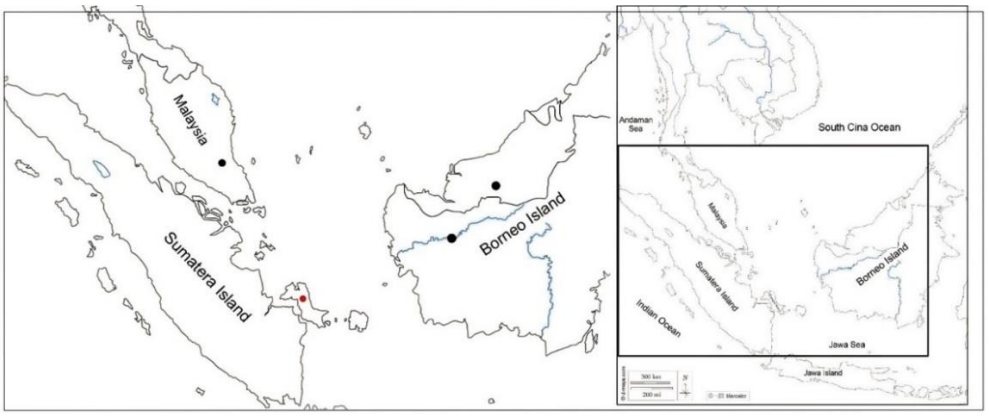

Figure 3. Map of the known distribution of *Acanthopsoides molobrion*. Bangka Island (red circle); publish records (black circle) based on Kottelat (2020); and Siebert (1991).

However, Cobitidae was clustered as the sister group to basal Cyprinidae. Cobititoidea and Cyprininoidea are monophyletic superfamilies from Cypriniformes Nelson (2006). Our current research confirmed that *A. molobrion* belongs to the Cobititoidea family (Page & Tangjitjaroen, 2015).

Distribution of Acanthopsoides molobrion

We confirmed that the first record of the *Acanthopsoides molobrion* was in Bumang Kemuja River, Bangka Island, Indonesia, and the second record for the country after the previous record was in Kapuas River, Borneo. A total of two specimens of *A. molobrion* in different sizes (46-50 mm) were found around January 2023. The new record of *A. molobrion* provided in Bangka Island is the southernmost record for this species, expanding its geographic distribution. In addition, the new record site is about 500 km south of the nearest locality in Peninsular Malaysia, and about 750 km southwest from the nearest locality in Borneo. New records of freshwater fish are essential contributions to the natural sciences to understanding species diversity and biogeography (Robin *et al.*, 2023c; Hasan *et al.*, 2023b). In particular, new records that expand the spatial range of a species are necessary to support appropriate conservation-related decisions and environmental impact assessments (Hasan *et al.*, 2024b).

Understanding the new distribution of the species is crucial in informing appropriate conservation-related decisions and conducting sustainable environmental impact assessments (Hariyanto, 2019). The previous presence of *A. molobrion* was known to inhabit regions in Borneo and Malaysia (Figure 3). This historical context highlights the importance of identifying current distribution patterns, as shifts in the species' range could indicate ecological changes, potential threats, or new conservation opportunities. Accurate, up-to-date distribution data will enable conservationists and environmental planners to implement effective protection measures, allocate resources efficiently, and mitigate any negative impacts on the species' habitat.

A. molobrion is probably naturally rare despite a conservation status of LC (Least Concern) in the International Union for Conservation of Nature (iuCn) Red List (Daniels & Allen 2020). this is due to the lack of clear information about the ecology, biology, range, and scale distribution of *A. molobrion* makes it difficult to draw meaningful conclusions about the actual conservation status, we emphasize the urgency for more comprehensive and accurate data on the ecology and distribution of native taxa, including this species, to facilitate species-specific conservation assessments and management in Indonesia. In particular, data collection on the existence of this species in various locations using the DNA metabarcoding technique, by relying only on water samples to see whether this species exists in certain waters, because this fish is difficult to catch, and traditional data collection may give inaccurate results. We also speculate that there is a strong possibility that *A. molobrion* is present in other islands close to the West Malaysia mainland, Sumatra, and Borneo.

In general, *A. molobrion* is only found in clean waters with good water quality, fine sand bottoms with pebbled rocks, and moderate to swift water currents. As it is presented, the Bumang Kemuja River still provides a good habitat for *A. molobrion* (Figure 4), with clean water, temperature of 20-26°C, pH 6.0-7.0 and dissolved

Figure 4. Sampling site of *A. molobrion* at Bumang Kemuja River, Bangka Island, Indonesia. The water is clean with good water quality, fine sand bottoms with pebbled rocks, and moderate to swift water currents.

oxygen was 6.5-9.1 mg/l. The presence of *A. molobrion* in Bumang Kemuja River, Bangka Island indicated that this ecosystem is still in a good state, especially for *A. molobrion* which as a demersal freshwater species feeds on detritus, the small animals in the bottom sediments such as daphnia, cyclops, and small crustaceans. The health of the bottom of the waters greatly determines the existence of *A. molobrion*. This information is also important for classifying rivers based on quality and planning efforts to conserve and protect ecosystems. Even more, currently, most of the waters in Bangka Island have been damaged due to the negative effects of open pit tin mining (Hasan *et al.*, 2023c; Kusumah *et al.*, 2023).

This study provides a new distribution record for *A. molobrion* on Bangka Island, whereas its previous distribution was only in Borneo and Malaysia. Understanding the species diversity and biogeography of the species is aided by new records. From this study, the new record of *A. molobrion* has expanded the species' recorded distribution range, which has expanded our knowledge of this species. Interestingly this record is new for the *A. molobrion* DNA sequence based on the COI gene, where this sequence is the first DNA barcode in Indonesia and the first barcode registered in the Genbank. Subsequently, to increase the Barcode of Life (BOL), the DNA sequence was registered into NCBI Genbank by naming access code OR144414. In the future, this DNA barcode will assist in DNA and biotechnology-based research and be utilized as a standard for identifying *A. molobrion*.

CONCLUSION

The discovery of *Acanthopsoides molobrion* in Bumang Kemuja River, Bangka Island, Indonesia represents the first new record for Bangka Island and the second record for the country. In particular, understanding the new distribution of the species is crucial in informing appropriate conservation-related decisions and conducting sustainable environmental impact assessments. Moreover, this update is the first record for the *A. molobrion* DNA barcode based on the COI gene, for Bangka Island, Indonesia. The DNA barcode of *A. molobrion* was registered into NCBI Genbank using access code OR144414. In addition, the DNA barcode of *A. molobrion* will be used as a standard for identifying species and will aid in DNA and biotechnology-based studies in the future. Despite the IUCN Red List's classification of this species as Least Concern (LC), finding an *A. molobrion* during our routine data collection of fish species is quite difficult. This species is only known to occur in waters with good water quality. However, the waters of Bangka Island have been severely damaged due to the negative impacts of open-pit tin mining. Therefore, conservation and domestication efforts are required to maintain *A. molobrion* survive on Bangka Island. Further detailed research on *A. molobrion*, including its reproduction, feeding habits, population size, and habitat, is necessary to update its development and conservation status.

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