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DNA Barcoding and Phylogenetics of *Betta cf anabatoides* Based on Cytochrome Oxydase Sub Unit 1 (CO1) Gene Markers from Tangkit Village, Jambi Province, Indonesia

Khairul Khairul[1*], Rivo Hasper Dimenta[1], Rusdi Machrizal[1], and Syahrial[2]
[1]Department of Biology Education, Universitas Labuhan Batu, Rantauprapat, North Sumatera Province 21415, Indonesia
[2]Department of Marine Science, Malikussaleh University, Aceh Utara, Aceh Province 20155, Indonesia

[*]**Corresponding Author: khairulbiologi75@gmail.com**

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ABSTRACT
One of the wild *Betta* species found in the swamps of Jambi Province is locally referred to as *Betta cf. raja*. However, genetic information on wild *Betta* species from this region is currently lacking. Molecular studies involving DNA barcoding and phylogenetic analysis are necessary for accurate taxonomic validation. This research employed the Polymerase Chain Reaction (PCR) method targeting the mitochondrial Cytochrome Oxidase Subunit I (COI) gene—a widely used genetic marker for fish species identification due to its stability and low likelihood of insertions or deletions. Muscle tissue samples from wild *Betta* specimens collected in Tangkit Village, Jambi Province (coded TJ 01 and TJ 02) were analyzed. DNA was isolated using the Magnetic Beads g-SYnC DNA Kit protocol. The resulting DNA amplification produced a fragment length of 679 base pairs. BLAST analysis of the nucleotide sequences identified a strong similarity with *Betta cf. anabantoides*. Phylogenetic analysis further supported this identification, grouping the Jambi specimens within the *Betta cf. anabantoides* clade with a bootstrap value of 96%. In conclusion, the results of DNA barcoding and phylogenetic analysis validate the taxonomic status of the wild *Betta* species from Jambi as *Betta cf. anabantoides*.

INTRODUCTION

Indonesia is the largest distribution area of endemic wild *Betta* species in the world **(Lindiantika *et al*., 2023)**. However, many of these species have yet to be identified at the species level. Wild *Betta* can be found in freshwater habitats ranging from lowland regions to highland areas across Asia. These so-called “hippopotamus fish” are prized for their distinctive colors and shapes, making them a popular ornamental fish export commodity **(Putri *et al*., 2021)**. According to **Fahmi *et al.* (2020)**, wild *Betta* are sold as ornamental fish and enjoy considerable demand in the market.

One population of wild *Betta* was discovered in the swampy waters of Tangkit Village, Jambi Province, where it is locally referred to as *Betta cf. raja*. Morphological identification conducted by **Khairul *et al.* (2024)** suggests that the population belongs to *Betta cf. raja*. However, the identification remains uncertain due to differences in the dark margin coloration of the anal fin and the lower part of the caudal fin. As **Hui and Ng (2005)** noted, species identification based solely on morphological traits may be unreliable and subject to misinterpretation.

Molecular taxonomic validation of wild *Betta* species is therefore critical for genetic conservation and systematic classification. Markers such as COI or Cyt *b* genes enable the detection of cryptic lineages, help prevent hybridization with other species, and contribute to strengthening local reference genome databases **(Syarif *et al.*, 2025)**. A genetic divergence of more than 3% in DNA barcoding typically indicates substantial evolutionary differentiation. According to **Schenekar *et al.* (2020)**, molecular validation enhances the accuracy of species identification, preserves genetic diversity, and supports conservation strategies based on precise systematics.

Fig. 1. Wild *betta* species from Tangkit Village (Jambi Province)

Endemic wild *Betta* species from Sumatra Island are increasingly vulnerable to extinction due to environmental degradation, land-use conversion, and overexploitation **(Dahruddin *et al.*, 2021)**. Despite this, the Indonesian government has yet to implement effective measures for their protection and conservation **(Prianto *et al.*, 2017)**. According to **Fahmi *et al.* (2020)**, molecular identification of wild *Betta* species in Indonesia is urgently needed to support conservation efforts.

One widely used molecular technique for species identification is DNA barcoding. This method offers significant potential for taxonomic validation and genetic resource conservation **(Haldar & Nath, 2020)**. With the advancement of molecular technologies, species classification can now be conducted using DNA data alone or in combination with morphological characteristics **(Kainama *et al.*, 2023; de Freitas *et al.*, 2024)**.

DNA barcoding involves the use of genetic primer segments ranging from 600–800 base pairs from the mitochondrial Cytochrome Oxidase Subunit 1 (CO1) gene **(Hebert &**

Gregory, 2005; Imtiaz *et al.*, 2017; Ward *et al*., 2005). The CO1 gene is internationally recognized as a standard marker for molecular taxonomy and species identification **(Bipeng *et al*., 2018)**.

To date, no DNA barcoding studies have been conducted on wild *Betta* populations from Tangkit Village, Jambi Province. Accurate species identification is essential for informing future monitoring, conservation, and genetic resource management. Therefore, this study aimed to identify the species of wild *Betta* from Tangkit using DNA barcoding and phylogenetic approaches.

MATERIALS AND METHODS

The specimen of wild *Betta* was collected from Tangkit Village, Jambi Province (on 16 August 2024. The fish were caught using a fishing rod and the bait used was earthworm. A small portion of muscle tissue was taken from the fish while it was still alive **(Hajibabaei *et al.*, 2005; Rahmah *et al.*, 2024)**. The tissue was then placed into a 2 mL microtube containing 96% pro-analysis ethanol **(Machrizal *et al*., 2023)**.

Muscle tissue samples were sent to Genetics Science Indonesia (GSI) for further work procedures as follows:

1. DNA extraction was performed using Magnetic Beads g-SYnc DNA kit (Geneaid, MGS096).
2. DNA amplification using MyTaq HS Red Mix 2X kit (Bioline, BIO-25048).
3. Electrophoresis of COI amplification products (B/7.2.1/IKP/005)
4. Bidirectional sequencing using the Sanger method DNA Sequencing by using Capillary Electrophoresis (1st BASE Subcontracted Lab Testing)
5. Bioinformatics analysis of Sanger Sequencing results (B/7.2.1/IKP/006)

The PCR reaction is as follows:

Table 1. PCR procedure

Component	1 x 25µl	Final Concentration
dd H_2O	to 25 µl	1 X
MyTaq HS Red Mix, 2 X	12,5	1 X
10 µM Primer VF2_t1	1	0,4 µl
10 µM Primer FishF2_t1	1	0,4 µl
10 µM Primer FishR2_t1	1	0,4 µl
10 µM Primer FR1d_t1	1	0,4 µl
DNA cetakan (*templete*)	1	≥ 50 ng/ µl

The temperature conditions in the PCR method can be seen as follows:

Table 2. PCR stages

Stages	Temperature (ºC)	Duration	Cycle
Initial Denaturation	95	1 minutes	1
Denaturation	95	15 minutes	35
Annealing	50	30 minutes	35
Extension	72	15 minutes	35
Hold	4	∞	1

In mitochondrial DNA isolation and amplification of the COI gene, the most commonly used primers are Folmer primers. These are universal primers designed to amplify a fragment of about 650 bp from the COI gene in a wide variety of fishes, mainly for DNA barcoding purposes.

Here are the standard folmer primer sequences:

- Forward primer: 5'-GGTCAACAAATCATAAAGATATTGG-3'
- Reverse primer: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' **(Ward *et al.*, 2005)**.

Additional sequences were obtained using BLAST searches based on databases in Genbank that are useful for species identification. Furthermore, the DNA barcode of specimen was registered to Genbank at the site: https://www.ncbi.nlm.nih.gov/genbank/ **(Syarif *et al.*, 2023)**. The genetic distance for each sequence was calculated using Molecular Evolutionary Genetics Analysis (MEGA) XI by applying the Kimura two-parameter (K2P) model and 1000 bootstraps **(Tamura *et al.*, 2021)**. Furthermore, according to **Yuan *et al*. (2014)** mathematical models are used to analyse molecular data to draw conclusions about the evolutionary history of species.

RESULTS

DNA barcode

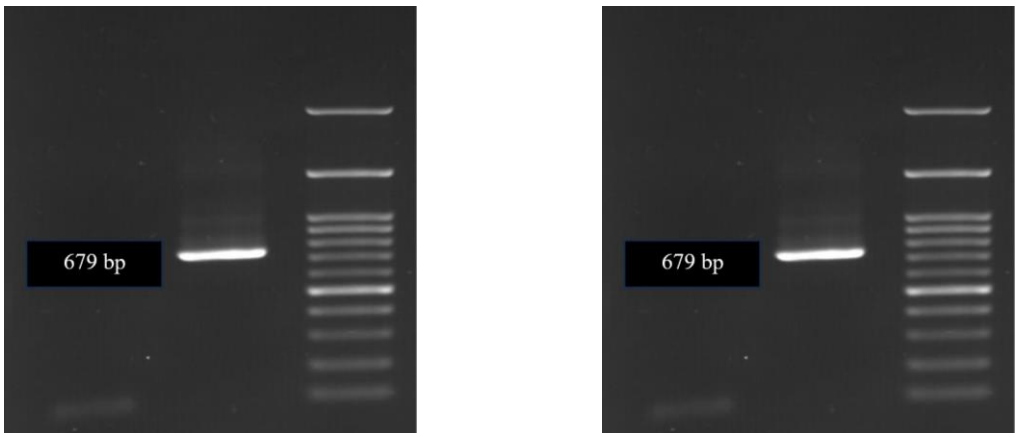
PCR amplification of the mitochondrial COI gene of wild betta species from the swamp habitat of Tangkit Village resulted in a fragment length of 679 bp. The amplification results were migrated in 1% agarose gel electrophoresis.

Table 3. DNA barcoding of sample (TJ 01) from Tangkit village, Jambi, Indonesia

GGCACCCTTT ACTTAATTTT TGGTGCATGG GCCGGAATGG TCGGTACCGC TTTAAGCCTG
CTTATCCGGG CAGAACTAAG TCAACCAGGA GCTCTTCTTG GAAACGACCA AATTTATAAT
GTAATTGTTA CGGCACACGC TTTTGTAATA ATTTTCTTTA TAGTCATACC CGTTATGATC
GGAGGTTTCG GAAATTGACT TGTCCCCCTT ATGATTGGGG CACCAGACAT GGCTTTCCCT
CGAATAAATA ACATAAGCTT CTGACTTTTA CCCCCATCCT TCCTTTTACT ATTAACATCC
TCCGGGGTAG AGGCCGGAGC CGGGACTGGT TGAACCGTAT ACCCACCCCT AGCCAGCAAC
TTAGCACACG CAGGTGCCTC TGTAGACCTG ACAATTTTCT CGCTTCACCT GGCAGGTGTT
TCATCTATTC TAGGGGCCAT TAATTTTATT ACCACAATTA TCAACATAAA ACCACCCGCA
ATCTCCCAAT ATCAAACACC CTTGTTTGTC TGAGCTGTTT TAGTAACAGC GGTATTACTA
AACACAACCT TCTTTGACCC TGCAGGAGGC GGAGACCCAA TCTTATACCA ACACCTGTTC
AACACAACCT TCTTTGACCC CGCAGGGGGC GGAGACCCAA TCTTATATCA ACACCTGTTC
TGATTCTTCG GACACCCTG

Table 4. DNA barcoding of sample (TJ 02) from Tangkit village, Jambi, Indonesia

GGCACCCTTT ACTTAATTTT TGGTGCATGG GCCGGAATGG TCGGTACCGC TTTAAGCCTG CTTATCCGGG CAGAACTAAG TCAACCAGGA GCTCTTCTTG GAAACGACCA AATTTATAAT GTAATTGTTA CGGCACACGC TTTTGTAATA ATTTTCTTTA TAGTCATACC CGTTATGATC GGAGGTTTCG GAAATTGACT TGTCCCCCTT ATGATTGGGG CACCAGACAT GGCTTTCCCT CGAATAAATA ACATAAGCTT CTGACTTTTA CCCCCATCCT TCCTTTTACT ATTAACATCC TCCGGGGTAG AGGCCGGAGC CGGGACTGGT TGAACCGTAT ACCCACCCCT AGCCAGCAAC TTAGCACACG CAGGTGCCTC TGTAGACCTG ACAATTTTCT CGCTTCACCT GGCAGGTGTT TCATCTATTC TAGGGGCCAT TAATTTTATT ACCACAATTA TCAACATAAA ACCACCCGCA ATCTCCCAAT ATCAAACACC CTTGTTTGTC TGAGCTGTTT TAGTAACAGC GGTATTACTA AACACAACCT TCTTTGACCC TGCAGGAGGC GGAGACCCAA TCTTATACCA ACACCTGTTC AACACAACCT TCTTTGACCC CGCAGGGGGC GGAGACCCAA TCTTATATCA ACACCTGTTC TGATTCTTCG GACACCCTG



(A) (B)

Fig. 2. The bands of COI gene amplification. (A) = TJ 01, (B) = TJ 02

Genetic distance

Genetic distance analysis was conducted using the Kimura 2-parameter (K2P) model. The CO1 gene fragment sequences from wild *Betta* samples were compared with reference sequences from the family *Osphronemidae* and genus *Betta* spp., based on data obtained from NCBI. Samples TJ 01 and TJ 02 showed the closest genetic distance (0.0046) to *Betta cf. anabantoides* (accession no. GQ911722.1) and the furthest distance (0.2374) to *Trichogaster trichopterus* (accession no. KM213048.1)..1) and the furthest distance of 0.2374 (KM213048.1) with Trichogaster trichopterus.

Table 5. Distribution of sequence divergence values based on CO1 gene markers in Samples (TJ 01 & TJ 02) with other species in %

No		1	2	3	4	5	6	7	8	9
1	Fish Tissue_TJ 01	*	*	*	*	*	*	*	*	*
2	Fish Tissue_TJ 02	0.0000	*	*	*	*	*	*	*	*
3	PQ676067.1_B. schalleri	0.1008	0.1008	*	*	*	*	*	*	*
4	MW591021.1_B. pugnax	0.0544	0.0544	0.1062	*	*	*	*	*	*
5	KM485447.1_B. cf pulchra	0.0496	0.0496	0.1062	0.0311	*	*	*	*	*
6	KM485420.1_B. apollon	0.1235	0.1235	0.1029	0.1272	0.1269	*	*	*	*
7	KM213048.1_T. trichopterus	0.2374	0.2374	0.2698	0.2526	0.2415	0.2545	*	*	*
8	GQ911731.1_B. fusca	0.0789	0.0789	0.0820	0.1059	0.1003	0.1046	0.2764	*	*
9	GQ911722.1_B. cf anabatoides	0.0046	0.0046	0.1064	0.0530	0.0512	0.1251	0.2385	0.0840	*

Phylogeny tree

Based on the results of phylogenetic analysis, the wild *Betta* sample from Tangkit Village (Jambi Province) was identified as *Betta cf. anabantoides* with a bootstrap value of 96%, indicating that 960 out of 1000 replicates supported the same grouping. Phylogenetic reconstruction was performed using the neighbor-joining method with the Kimura 2-parameter (K2P) model and 1000 bootstrap replications. The resulting phylogenetic tree shows that *Betta cf. anabantoides* clusters with the analyzed sample, forming a distinct clade based on genetic distance and DNA sequence similarity, supported by a 96% bootstrap value. A higher bootstrap value indicates greater confidence and reliability in the phylogenetic tree reconstruction. The complete results of the analysis are presented in **Fig. (3**).

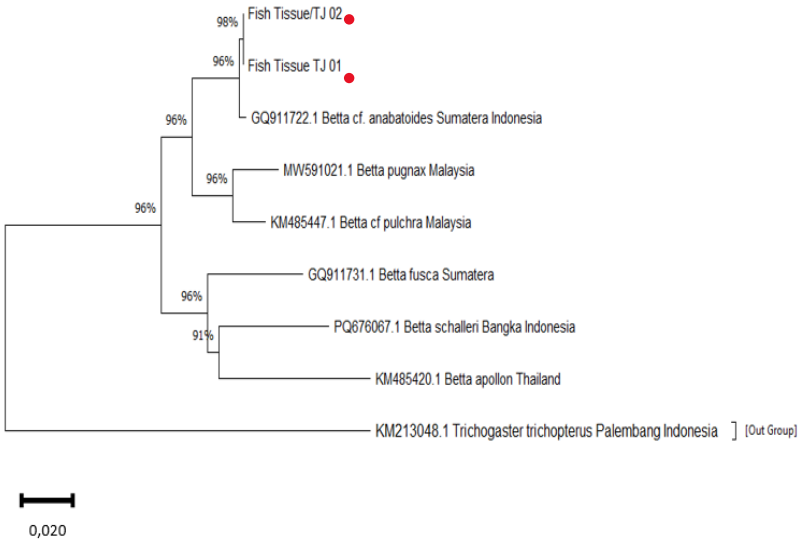


Fig. 3. Evolutionary relationships of the *Betta* genus based on COI Gene

DISCUSSION

Previous research by **Khairul *et al*. (2024)** revealed significant morphological differences among *Betta cf. raja* populations from three observation sites. These findings highlight the need for taxonomic re-evaluation, as morphological data alone had previously led to uncertainty in species identification. The DNA sequences obtained in this study serve as a molecular reference for species identification through DNA barcoding using COI gene markers. According to **Valen *et al*. (2023)**, DNA barcoding plays a vital role in advancing taxonomic knowledge. It not only aids species identification but also provides a contextual framework for reconstructing phylogenies and understanding population genetic structures **(Hajibabaei *et al.*, 2007; Andriyono & Suciyono, 2020; Panprommin *et al*., 2023)**. Furthermore, **Sirisha *et al*. (2018)** emphasized that DNA barcoding enhances molecular identification practices and offers broad standardization through PCR-based techniques.

Genetic distance values are influenced by various ecological, geographical, and evolutionary factors that shape natural patterns of genetic differentiation **(Martinez *et al.*, 2018)**. Habitat variation, life history traits, physiological adaptations, demographic structure, migration, and anthropogenic pressures can all contribute to genetic diversity loss **(Sadler *et al.*, 2023)**. As noted by **Fahmi *et al.* (2020)**, the sedentary or non-migratory behavior of fish species in the *Betta* genus contributes to their high speciation rates and distinctiveness in the wild.

The molecular results of this study confirm that samples TJ 01 and TJ 02 are genetically similar to *Betta cf. anabantoides*, and show no similarity to *Trichogaster trichopterus*, which was used as an outgroup. Genetic relatedness among fish species can be inferred from genetic distance values—smaller values indicate closer kinship, while larger values reflect greater divergence **(Rasmussen *et al*., 2009)**. According to **Achmad *et al*. (2019)**, close genetic relationships are often observed among populations originating from a common ancestor.

Supporting this, **Fahmi *et al*. (2020)** found in their phylogenetic analysis that *Betta raja* and *Betta cf. anabantoides* grouped together with a bootstrap value of 100%, reinforcing their close evolutionary relationship. *Betta cf. anabantoides* belongs to the *Betta pugnax* group and is characterized as a mouthbrooder. Phylogenetic studies by **Valen *et al*. (2023)** involving *Betta edithae* and other *Betta* species also demonstrated strong evolutionary relationships and genetic closeness. Additionally, **Zhang *et al.* (2022)** noted that shared behaviors, such as mouthbrooding, may reflect underlying polygenic traits. This phenomenon supports the concept of monophyly, where a group of taxa shares a single common ancestor **(Syaifudin *et al.*, 2020)**.

CONCLUSION

The results of molecular analysis have successfully validated the taxonomic identification of wild *Betta* from Tangkit Village, Jambi Province, as *Betta cf. anabantoides*. This conclusion is supported by the closest genetic distance value of 0.0046 and a bootstrap value of 96% obtained from phylogenetic analysis. Further studies involving DNA barcoding and phylogenetic analysis of *Betta raja* populations in Jambi Province are recommended to better understand their genetic relationship with *Betta cf. anabantoides*.

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