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Revolutionary Breakthrough: Unveiling the first DNA Barcoding of the Endemic wild *Betta burdigala* (Kottelat and Ng 1994) (Anabantiformes: Osphronemidae): A Critically Endangered Wild Betta from Bangka Island, Indonesia

Fitri Sil Valen[1*†], Hartoyo Notonegoro[2], Aditya Pamungkas[3†], Swarlanda[4] and Veryl Hasan[5]
[1]*Department of Aquaculture, Faculty of Agriculture Fisheries and Biology, University of Bangka Belitung, Jl Kampus terpadu UBB, Balunijuk33127, Bangka Belitung, Indonesia*
[2]*Department of Capture Fisheries, Faculty of Agriculture Fisheries and Biology, University of Bangka Belitung, Jl Kampus terpadu UBB, Balunijuk 33127, Bangka Belitung, Indonesia*
[3]*Marine Science Department, Faculty of Agriculture Fisheries and Biology, University of Bangka Belitung Jl. Kampus Terpadu UBB, Kepulauan Bangka Belitung 33172, Indonesia*
[4]*Yayasan Ikan Endemik Bangka Belitung, Indonesia*
[5]*Department of Aquaculture, Faculty of Fisheries and Marine Science, Universitas Airlangga, Surabaya 60115, East Java, Indonesia*
[*]For correspondence: fitrisilvalen11@gmail.com; fitrisilvalen@ubb.ac.id
[†]Contribution equally to this work and are co-first authors
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Abstract

Betta burdigala is an endemic Wild *Betta* which only known from Bangka Island, Indonesia. This species was listed as a Critically Endangered (CR) species based on the IUCN Red List of threatened species where the population of this species was significantly decreasing. We collect fivespecimens of *B. burdigala* from Bangka Island, Indonesia with 36–37 mm of Total Lenght (TL) from 7 to 13 April 2023 in Bikang River, Toboali District, South Bangka Regency, Bangka Island, Indonesia. In this research, we report the first record of the DNA Barcoding of *B. burdigala* based on the Cytochrome C oxidase Subunit I (COI) gene. We have registered the DNA Barcode of *B. burdigala* to the Genbank with the accession code OQ281707. DNA barcoding is a solution to provide accurate, fast and automatable identify species and species discovery. *B. burdigala* Based on the COI gene, *B. burdigala* and *B. uberis* have a close genetic distance and a DNA similarity of 96.66%, much higher than other bettas. Their genetic distance is 0.04, while a genetic distance between 0.010 and 0.099 is considered to be low and indicative of high similarity. According to the phylogenetic tree, these species are descended from a single, closely related ancestor on the same branch. Based on the COI gene, we assume that they are identical. Additionally, we advise conducting additional research using the mitochondrial DNA complex and in-depth morphological examination to confirm the additional of the study's findings.

Keywords: Biodiversity; Biogeography; DNA Barcoding; Endemic; Freshwater fish

Introduction

Freshwater fish species in Indonesia are extraordinarily diverse, as many as 1,266 species had been identified in Indonesian inland waters in 2022 (Robin *et al.* 2023a) and about 8.500 fish species have been classified based on habitat traits (*e.g.*, salty, brackish and freshwater). The diversity of Indonesian freshwater fish consists of endemic, native, introduced, and reintroduced (Gani *et al.* 2021; Ndobe *et al.* 2022; Hasan *et al.* 2023a).

Betta Bleeker 1849, is the most diverse genus in the Osphronemidae family. More than 65 species of Betta are found in South-East Asia, they are divided into several species groups (Kottelat 2013; Schindler and Linke 2013). *B. burigala* is an endemic wild betta original from Bangka Island Indonesia which is a member of the *B. coccina* group. Like wild bettas in general, *B. burigala* can be able to survive at pH 3.0 or 4.0. (Tan and Ng 2005) and inhabit peat swamp woodlands in shallow pools of buried leaf litter.

The existence of *B. burdigala* in the Bangka Islands was recorded as Critically Endangered (CR) based on the IUCN Red List of threatened species (Low 2019). The population of *B. burdigala* on Bangka Island has declined dramatically due to the effects of tin mining causing

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environmental damage and land conversion (Kusumah *et al.* 2023), as well as the presence of invasive fish causing competition for resources, predation, habitat modification, and disease transmission (Hasan *et al.* 2020; Ihwan *et al.* 2020; Insani *et al.* 2020; Robin *et al.* 2023a; Serdiati *et al.* 2020;). Despite being highly endangered, there are no known conservation activities for this species. To manage the conservation plan, we need to conduct a study on its life history, population, habitat trends and development.

In particular, understanding the life history of the species can be followed through molecular analysis utilizing the DNA Barcoding method, including comprehending evolution and speciation. In addition, DNA barcoding can also be used as a tool for rapid identification of species by using one or several genes in mitochondrial DNA which are standardized gene regions (Valen *et al.* 2021; Robin *et al.* 2023b). One of the standard genes as a tool for species identification in fish is the Cytochrome C oxidase Subunit I (COI) gene which is found in mitochondrial DNA. The COI gene has been chosen as a standard tool for molecular taxonomy and identification worldwide (Bingpeng *et al.* 2018) and can be relied upon as a basis for differentiation between animals (Liu *et al.* 2020). The use of the COI gene as a species identification tool has been successfully carried out for the identification of freshwater fish in Indonesia (Valen *et al.* 2022a; Insani *et al.* 2023).

In this research, we expose the first DNA barcoding of the *B. burdigala* based on the COI gene from Bangka Island, Indonesia. The DNA Barcoding will be uploaded to the Genbank NCBI database as a standard for recognizing Wild *B. burdigala* species based on the COI gene. This research will support information about the rapid species identification, understanding of biodiversity, increasing species genetic diversity and comprehension life history of species, and in the long term will support conservation actions and development.

Materials And Methods

Sampling site

The study was conducted from 7 to 13 April 2023 in Bikang River, Toboali District, South Bangka Regency, Bangka Island, Bangka Belitung Province, Indonesia. The sites in underground rivers cross extensive tree roots in natural primary woods, peat swamps, and blackwater streams where the bottom is covered by fallen leaves.

Fish samples collection and water quality

We collect samples using environmentally acceptable fishing equipment, *i.e*. traps with dimensions of 90 × 90 × 40 $cm^{3,}$ made of wire and mesh (Hasan *et al.* 2021). A total of two trap units were installed at two different depths, specifically 0.5 m and 1.0 m from the river surface. The fish caught in the traps were examined each day for a week after they were set. From both traps, all of the fish that had been caught were gathered and taken to the lab for the next research stage To research the wild betta burdigala's native habitat, we also conduct *in situ* analyses of water quality, including temperature, pH and dissolved oxygen.

Preserve fish and morphological analysis

A total of five (5) specimens of *B. burdigala* were caught using the fish trap during a week of traping. In fact, The wild *B. burdigala* was listed as a Critically Endangered (CR) based on the IUCN Red List of threatened species, and large-scale sample collecting is illegal under the law. As a result, we returned four specimens to their original habitat. However, at least one wild *B. burdigala* specimen was killed before being identified. Killing fish is viewed as an ethical method of using live fish for research (Metcalfe and Craig 2011). The right body's pectoral fin was removed for DNA extraction. Because the left side was used for morphometric data collection and is frequently visible in photographs. The sample was preserved in a 96% alcohol solution (Valen *et al.* 2022b). Materials examined were preserved in a 7% formalin solution (Valen *et al.* 2020) and deposited at the zoology laboratory, at Bangka Belitung University. Diagnostic morphological characters of the specimen were analyzed following Tan and Ng (2005). The determined meristic characters include dorsal fin rays, ventral fin rays, pectoral fin rays and anal fin rays. Other morphological characteristics of the specimen were measured using digital calipers to the nearest 0.1 mm.

DNA extraction and amplification

DNA was extracted using the 10% Chelex protocol (Walsh *et al*. 1991). 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 m*M* 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 a Thermal Cycler machine (Applied BiosystemsTM 2720). PCR cycling parameters included an initial denaturing phase of 3 min, denaturing at 94°C for 30 s, annealing at 48°C for 30 s and extension at 72°C for 45 s for 38 cycles. The PCR results were then visualized in 1% agarose gel *via* electrophoresis by staining Nucleic Acid Gel Stain (GelRed®) (Insani *et al.* 2023; Robin *et al.* 2023c). positive sample (sparkling DNA bands) was then processed for DNA reading (sequencing) using the Sanger dideoxy method at PT. Genetics Science Jakarta.

Data Analysis

Additional sequences were acquired using BLAST searches of the Genbank database, and they were used to identify specimens. We register the DNA Barcode of the sequence to the Genbank (National Center for Biotechnology Information) (https://www.ncbi.nlm.nih.gov/genbank/) with the accession number OQ281707. We also present the evolutionary history of the species by using the Neighbor-Joining method (Saitou and Nei 1987) with the bootstrap test (1000 replicates) (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) in MEGAX software (Kumar *et al.* 2018).

Results

Morphological identification

We identified specimen *B. burdigala* (Fig. 1) based on characters proposed by Tan and Ng (2005) (Table 1). This species is only known from Bangka Island, Indonesia by combining the following characteristics (Table 1): Red body colour; the presence of iridescent green spots on median fins.

DNA Barcoding of *B. burdigala*

DNA-Barcode of *B. burdigala* from Bangka Island was successfully sequenced with a base-pair length of 670 bp (Table 2) using Fish_F1 and Fish_R1 primers (Ward *et al.* 2005).

Molecular identification

The DNA Barcoding of the sequence from the Belitung Island was analyzed and compared to the NCBI GenBank (National Center for Biotechnology Information) *via* BLAST (Basic Local Alignment Search Tool-nucleotide) method (https://blast.ncbi.nlm.nih.gov) and the BOLD SYSTEM *via* Identification Engine to find out and analyze a sequence homology (Table 3).

Phylogenetic of *B. burdigala*

We calculated the evolutionary history of *B. burdigala* using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) (Fig. 2).

Genetic distances of the *Betta* genus

Analyses of the estimates of evolutionary divergence between the *Betta* genus based on the COI gene were conducted using the Maximum Composite Likelihood model (Tamura *et al.* 2004) in MEGA X (Kumar *et al.* 2018). The analysis involved 12 nucleotide sequences of the *B. genus* (Table 4). There were a total of 691 positions in the final dataset and ambiguous positions were removed for each sequence pair.

Table 1: Morphological characters of *B. Burdigala*

Morphometric data	Present study (n = 1)	Tan and Ng 2005
Standard length, SL	28-29 mm	23.8 mm
Total Lenght	128.5% SL	131.0-132.2% SL
Predorsal lenght	53.2% SL	52.8-54.9% SL
Postdorsal length	17.0% SL	16.7-17.4% SL
Dorsal fin base length	27.1% SL	26.8-28.6% SL
Preanal lenght	43.8% SL	42.5-45.5% SL
Dorsal fin rays	14	14-15
Predorsal scales	15	15-16
Subdorsal scales	11	11-11,5
Anal fin rays	25	24-26

Fig. 1: *Betta burdigala* collected from Bikang River, Bangka Island, Indonesia

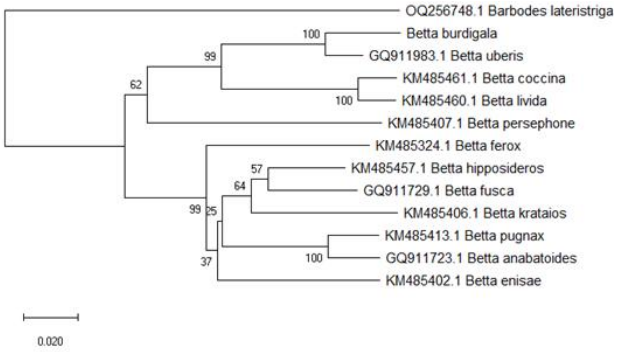

Fig. 2: Evolutionary relationships of the *Betta* genus based on COI Gene

Discussion

Based on characters proposed by Tan and Ng (2005), we confirm the specimen from Bikang River, Bangka Island, Indonesia identified as Wild *B. burdigala*. This species is only known from Bangka Island, Indonesia, and is recorded as an endemic species (Kottelat 2013). Moreover, the DNA-Barcode of WIld *B. burdigala* from Bangka Island with a base-pair length of 670 bp using Fish_F1 and Fish_R1

Table 2: DNA Barcoding of *Betta burdigala* from Bikang River, Bangka Island, Indonesia

DNA Barcoding of *B. burdigala*
CCGGAATGGTTGGTACCGCTCTAAGCCTGCTTATTCGAGCCGAGCTGAGCCAACCAGGAACTCTCCTTGGGGATGACC
AGATCTACAATGTAATTGTTACGGCGCACGCTTTTGTAATAATCTTCTTTATGGTAATACCTGTAATGATTGGGGGTTT
CGGGAACTGGCTTGTCCCCCTTATGATTGGTGGCACCAGACATGGCGCTTGTCCCCGGATGAATAATATGAGCTTCTG
GCTCCTACCTCCCTCTCTTTTTTACTACTATTAACATCTTCTGGGGTAGAAGCTGGTGCTGGTACTGGTTGAACCGTGT
ATCCCCCATTAGCCAGCAACTTAGCCCATGCGGGCGCATCTGTAGATTTGACAATTTTTTCACTTCACCTGGCGGGTGT
ATCATCTATCTTGGGGGCTATTAACTTTATTACCACAATTATCAACATGAAACCACCTGCAATTTCCCAATATCAAACA
CCTTTGTTTGTATGAGCCGTATTAGTCACAGCTGTACTACTCCTTCTATCACTTCCCGTCTTAGCTGCCGGAATCACAA
TGCTTTAACAGACCGAAATCTAAACACAACCTTTTTTGACCCTGCAGGGGGTGGTGACCCTACTTATACCAACACCTA
TTTTGATTCTTTGGCCACCAAAAAGTCTAA

Table 3: Species identification and similarity

Species Outcome	Family	Accession IDE	Query Coverage (%)	Percent Identity (%)
Betta uberis	Osphronemidae	GQ911983.1	94	96.66
Betta coccina	Osphronemidae	OQ784935.1	99	87.98
Betta livida	Osphronemidae	KM485461.1	94	88.30
Betta persephone	Osphronemidae	KM485407.1	94	84.98

Table 4: Estimates of Evolutionary Divergence between *Betta* genus based on COI gene

		1	2	3	4	5	6	7	8	9	10	11	12
1	*B. burdigala*												
2	*B. uberis*	0.04											
3	*B. coccina*	0.13	0.11										
4	*B. livida*	0.14	0.11	0.03									
5	*B. hipposideros*	0.17	0.15	0.17	0.17								
6	*B. fusca*	0.18	0.16	0.17	0.17	0.06							
7	*B. persephone*	0.18	0.16	0.18	0.18	0.18	0.18						
8	*B. ferox*	0.18	0.17	0.18	0.18	0.10	0.11	0.19					
9	*B. enisae*	0.19	0.18	0.20	0.21	0.12	0.12	0.17	0.13				
10	*B. krataios*	0.20	0.19	0.21	0.21	0.10	0.08	0.19	0.14	0.11			
11	*B. pugnax*	0.21	0.21	0.21	0.20	0.11	0.11	0.19	0.12	0.12	0.12		
12	*B. anabatoides*	0.22	0.21	0.21	0.19	0.10	0.11	0.20	0.12	0.12	0.12	0.04	

primers (Ward *et al*. 2005). The BLAST analysis shows that the Wild *B. burdigala* sequence is very close to *B. uberis* with a similarity identity of up to 96.66%. According to Hebert *et al*. (2003), species with 97–100% similarity levels are identical and species with differences above 3% based on COI genes are a different species. Based on the result, we confirm that the sequence we obtained from Bikang Village, Bangka Island, Indonesia is Wild *B. burdigala*, however we cannot show genetic similarity between our sequence with th Wild *B. burdigala* because the Wild *B. burdigala* sequence is not yet in the NCBI GenBank database. Furthermore, we confirmed this through morphological identification and genetic closeness to *B. uberis* because these two species are indeed in the same group, namely the Coccina group. Being in one group means that these species are morphologically similar and genetically close.

We also register the COI gene of *B. burdigala* to the Genbank (National Center for Biotechnology Information) (https://www.ncbi.nlm.nih.gov/genbank/) with the access code OR167622. The fragments COI gene can be used as a DNA Barcode, a standard for differentiating between animals and species identification (Dhar and Ghosh 2017; Bingpeng *et al.* 2018; Liu *et al.* 2020; Guo *et al*. 2022). This sequence will be used as a standard for species identification using DNA Barcoding identification with COI gene markers. Moreover, this information is very important to enrich science, especially to understand the taxonomy (Bhattacharya *et al*. 2016; Tadmor-Levi *et al.* 2022) and improving knowledge in biotechnology. The DNA Barcode of *B. burdigala* is the very first report for Indonesia and the first barcode registered in the Genbank.

The DNA Barcode of *B. burdigala* based on the COI gene had high average amounts of adenine and thymine called A-T rich group (A-T rich) around 60% and C-T Group around 40%. The A-T hydrogen bond consists of 2 hydrogen bonds which are weaker than the G-C hydrogen bond which has 3 hydrogen bonds, so the possibility of species mutation is higher. The complete percentage of nucleotide composition of Wild *B. burdigala* was T (30.7%), C (25.4%), A (23,3.0%) and G (20.6%). Based on the analysis of the evolutionary history of Wild *B. burdigala* involved 13 nucleotide sequences. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree, and are in the units of the number of base substitutions per site. There was a total of 691 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.* 2018).

Fig. 3: Habitat of an endemic *B. burdigala*, Bikang River, Toboali District, South Bangka Regency, Bangka Island, Indonesia

As can be seen, the phylogenetic tree of *B. burdigala* was clustered in a clade in the *Betta* genus and was close to *B. uberis* with a genetic distance of around 0.04 which. *B. uberis is* a wild betta from Borneo, Indonesia (Tan and Ng 2006). Borneo and Bangka Island were connected to the Ancient river, Sundaland before the last glacial maximum. Sundaland was a large single landmass with connected rivers, in the glacial period, Sundaland dispersed into Java, Borneo, Sumatra, Malay Peninsula and some adjacent islands including Bangka and Belitung Islands Islands (Hasan *et al.* 2023b, c). Thereby, Sundaland has the same biodiversity called is native to Sundaland. In some cases, species evolve into morphologically and genetically distinct forms to become distinct species.

Moreover, The closest distance between species occurs in *B. burdigala* and *B. uberis* about 0.04, which means that out of 100 base pairs, there are 4 different base pairs. According to Nei (1972), a genetic distance of 0.010–0.099 is included in the low category, 0.1–0.99 is included in the medium category, and a genetic distance of 1.00–2.00 is included in the high category. The genetic distance is the degree of difference in a gene which is calculated based on differences between species or populations.

Wild *B. burdigala* is a species which only known from Bangka Island, in this research the Wild *B. burdigala* was found in Bikang River, Bangka Island, Indonesia (Fig. 3), a peat swamps and the nearby blackwater streams in underground rivers crossing dense tree roots in natural primary forests (Tan and Ng 2005). This habitat contains humic acids and other compounds generated by decomposing organic matter usually stain the water a dark color with very few dissolved minerals present, and the pH can be as low as 3.0 or 4.0. Less light is emitted from the Bikang River, that reaches the surface of these environments due to the dense canopy of branches above, and riparian vegetation also tends to grow thickly in these environments. The substrate is typically covered by branches, fallen leaves, and tree roots that have sunk to the bottom. Depending on the time of year, the fish may have to spend several weeks surviving in the moist leaf litter because permanent water isn't always accessible.

Despite the Bikang River having high acidity water quality (pH 3.0 or 4.0.), however, the Wild *B. burdigala* is just like wild bettas in general, where this species can endure in environments with low pH with the blackwater streams enrich by decomposing organic matter. During the sampling, we also discovered several additional betta species coexisting in the Bikang River with also have high tolerant to the low pH like the *B. burdigala* including *B. simorum, B. chloropharynx.* In addition to *Betta* species, we discovered a number of other regional species that can adapt to the Bikang River's acidic pH circumstances, including *Barbodes selifer, Rasbora einthovenii, Rasbora chepalottenia, Nemacheilus selangoricus, Neohomaloptera johorensis, Trigonospoma pauciperforatum, Desmopuntius hexazona, Clarias leeiachanthus, Pristolepis fasciata, Channa bankanensis* and *Hamirhampodon pogonatus.*

B. burdigala which endemic to Bangka Island, Indonesia has extremely decreased of population. As is observed, *B. burdigala* was listed as a Critically Endangered (CR) based on the IUCN Red List of Threatened Species (Low 2019). The conservation status of this species may dramatically increase to become extinct in the next few years as a result because of habitat and environmental damage, especially because of the bad impact of open pit tin mining. As can be seen, the *B. burdigala* inhabits underground rivers crossing dense tree roots in natural primary forests, but now the existence of premier forest has started to be damaged due to land conversion and the negative effects of tin mining (Bernhardt and Palmer 2011). It is clear, mining activity involves deforestation and soil excavation (Asner and Tupayachi 2017), which causes forest degradation and *B. burdigala* loses its natural habitat. Extremely this species is starting to be threatened and unable to survive the decline in environmental quality due to chemical contamination and physical alteration of the river ecosystems by making the rivers susceptible to degradation and biodiversity loss.

Generally speaking, there were around 10,336 freshwater fishes which conservation status was assessed by the IUCN and 10 fish species were extinct in the wild as of 2021. Nearly 30 percent of the freshwater species are considered at risk of extinction (Jaganmohan 2022). As researchers we care about the existence of species in nature, especially endemic fish which are the identity of an area. We want to widely introduce the existence of *B. burdigala* and the current conservation status by reporting research results which can also serve as supporting data for the development and sustainability of this species. One of the data which will support future research is the first reporting of DNA barcoding of *B. burdigala* an endemic fish from Bangka Island based on the COI gene. This research is designed to be the solution to speed up the pace of species identification so that it can answer the

global challenge of identifying quickly and accurately for future biodiversity data collection and can continue to provide information on biodiversity and its latest conservation status.

Conclusion

The specimen Wild *B. burdigala* was identified using morphological and molecular analyses in this study. DNA barcode of *B. burdigala* using the COI gene with a base-pair length of 670 bp were registered in the Genbank with the access code OQ281707. This DNA barcode serves as a reference for species identification and contributes to taxonomy and biotechnology knowledge. The analysis of the evolutionary history of *B. burdigala* revealed its close relationship with *B. uberis*, a wild betta from Borneo, Indonesia. The genetic distance between the two species was found to be around 0.04. The study also highlighted the connection between Borneo and Bangka Island through the Ancient river, Sundaland, which explains the shared biodiversity between the two regions. In conclusion, this study provides valuable insights into the identification, conservation status, DNA sequencing, and evolutionary history of *B. burdigala*, highlighting its critically endangered status and the threats it faces due to habitat degradation and mining activities. According to the phylogenetic tree, these species are descended from a single, closely related ancestor on the same branch. Based on the COI gene, we assume that they are identical. Additionally, we advise conducting additional research using the mitochondrial DNA complex and in-depth morphological examination to confirm the additional of the study's findings.

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Author Contributions

FSV and HN planned the research, AP and S Collected the specimen, FSV and VH Identified the specimen.

Conflict of Interest

All authors declare No conflict of interest.

Data Avaibility

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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