First report of the Olive flathead gudgeon, Butis gymnopomus (Bleeker 1853) from Kerala waters with taxonomic notes on B. butis (Hamilton 1822) and B. koilomatodon (Bleeker 1849)

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Abstract

This article provides information on the taxonomy and distribution of the poorly known gobioid genus *Butis*from a tropical Ramsar site, Vembanad-Kole in Kerala state, India. The results of integrative taxonomy confirm the presence of *B. gymnopomus* in Kerala. Identity of the other two species – *B. butis* and *B. koilomatodon* in the study area was also confirmed through morphology and mitochondrial Cytochrome C Oxidase 1 gene. *Butis gymnopomus* is distinguished from *B. butis* by the absence of scales on the interorbital region and the absence of axillary scales. The interorbital region is narrow in *B. gymnopomus* compared to *B. butis*. The overall K2P genetic distance between the species was found to be 14%. The study highlights the necessity of a detailed study on systematics of gobioids based on large regional sampling.

Introduction

The family Butidae Bleeker of the order Gobiiformes (Thacker 2015) comprises 'butid sleepers or gudgeons 'having restricted distribution in the fresh and brackish waters of tropical, Indo- Pacific, and West Africa (Nelson et al 2016; Froese and Pauly 2017). Formerly treated as a subfamily (Butinae) under Eleotridae, were then elevated to family Butidae based on molecular phylogenetic analysis (Thacker 2009). Comprising 47 species in 10 genera, Butids are separated from the eleotrids in having the sensory papillae in a transverse pattern (about half the eleotrids have a transverse pattern), head pores usually well developed (reduced in some taxa such as *Kribia* and *Oxyeleotris paucipora*; head pores absent altogether in *Milyeringa, Oxyeleotris nullipora* and *Typhleotris*), usually 17 segmented caudal fin rays, the bony preopercular canal usually follows the full length of the preopercular bone, extrascapulae and nasal bones are usually present, the upper caudal cartilage plate is elongate anteriorly but not reaching posteriorly over the epural(s), and the adductor membrane tendon from A1-β muscle segment attaches to an anterior process on the maxilla (Hoese and Larson in press).Among the genera, *Oxyeleotris* is the most species-rich (n = 19) followed by *Bostrychus* (8) and *Butis* (6) (Fricke et al. 2022). Owing to their small size and cryptic lifestyles in different habitats, proper identification and categorization of gobiiform fishes solely on morphology is challenging (Winterbottom 1993), and can be assisted with DNA barcoding (Huang et al. 2013; Linh et al. 2018; Islam et al. 2021).

Family Butidae in Indian waters is represented by nine species in five genera- *Butis, Bostrychus, Odonteleotris, Ophiocara*, and *Incara.* Of the genera, *Butis* is the most species diverse with five species - *B. butis* (Hamilton 1822), *Butis humeralis* (Valenciennes 1837), *B. koilomatodon* (Bleeker 1849), *B. gymnopomus* (Bleeker 1853) and *Butis amboinensis* (Bleeker 1853) (Nair and Dineshkumar 2018; Venkatesaerlu 1967; Remadevi 2010; Geevarghese 1981; Kurup 1994). No other species of butids except *Butis butis* (Hamilton 1822) and *Butis koilomatodon* (Bleeker 1849) have been reported from Kerala waters (Geevarghese 1981; Raghunathan 2007), despite extensive species inventories carried out in the estuarine waters in Kerala.

The acceptance and usage of Cytochrome C Oxidase 1 gene (CO1) as a rapid and accurate barcoding marker complements conventional taxonomy for differentiating and describing novel species across the animal kingdom (Herbert et al. 2003; Ward et al. 2005; Thacker and Roje 2011). Taxonomic studies based on molecular techniques on gobioids were found to be few from waters except for a few reports from Ashtamudi Lake, Kerala (Viswambaran et al. 2013); Chindwin, Ganges and Kaladan River basins flowing into Indian territory (Laskar et al. 2017) and Andaman Nicobar Islands (Daniel et al. 2018).The current study aims to confirm the identity of the poorly known gudgeons of the genus *Butis* in Vembanad – Kole, a Ramsar site in Kerala, India using integrative taxonomic techniques.

Materials and Methods

Sampling

Extensive sampling was conducted in Vembanad- Kole wetlands in the South Indian state of Kerala from May 2021 to November 2021. Specimens were obtained from commercial gill and stake net (10mm, 12mm) fishery and through fishing operations using hand scoop net, and were preserved in 10% neutral buffered formalin. Identification of the species followed Miller et al., (1989). Tissue samples from representative specimens of each species were fixed in absolute alcohol and kept at -20°C for molecular analysis. The voucher specimens have been deposited at the referral museum of KUFOS (Table 1).

Morphomeristic analysis

Measurements and counts follow Miller et al. (1989) and were taken under a Meswox stereo microscope. Measurements were taken to the nearest 0.1 mm using a Yamayo- digital caliper. Measurements and counts were taken under a Meswox stereo microscope. Images of live and preserved specimens were photographed using Canon 5d and 550 d digital cameras and the distinguishing characters were observed under a Leica S9D stereo microscope and photographed using a Canon camera (550D) attached to it.

DNA isolation, PCR amplification, and sequencing

Total Genomic DNA was isolated from the muscle and/or fin tissues of each fish using NucleoSpin® Tissue Kit (Macherey-Nagel). The mitochondrial CO1 fragment were amplified using the primers LCO5' GGTCAACAAATCATAAAGATATTGG3' and HCO 5' TAAACTTCAGGGTGACC AAAAAATCA3' (Folmer et al. 1994). The PCR cycles were carried out in a Master cycler PCR System (Eppendorf) with an initial pre-denaturation at 98ºC for 30 sec, followed by 10cycles of 98ºC for 5 sec, 50ºC to 72ºC annealing temperature for 15 sec, extension at 72ºC for 15 sec, and a final extension step at 72ºC for 60sec.

The PCR products were purified usingExoSAP-IT (GE Healthcare). The sequencing reaction was done in a PCR

thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following Manufacturer’s protocol. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems) and required editing of the raw DNA sequences was done using Bio Edit sequence alignment version 7.0.5.2. (Hall1999) and were successfully submitted to NCBI GenBank.

Table 1
List of species with habitat features, voucher and GenBank accession number

SI No.	Species	Habitat	Location	Voucher	GenBank Accession No.
01	*B. butis*	Muddy	Panangad 9.9120773, 76.3151056	KUFOS.FV.2021.1023	MW877710
02	*B. koilomatodon*	Muddy Mangrove shore	Anapuzha 10.2100032, 76.219317	KUFOS.FV.2021.1003	OM442976
03	*B. gymnopomus*	Rocky, Muddy Mangrove shore	Biyyam 10.78755556, 75.96575 Idiyanchira 10.53833333, 76.06816667 Panangad 9.9120773, 76.3151056	KUFOS.FV.2021.1002	MW881237

Molecular phylogenetic analysis

Multiple Sequence Alignment (MSA) was performed using MUSCLE (Edgar 2004) and implemented in MEGAX (Molecular Evolutionary Genetics Analysis) (Kumaretal.2018). The parameters include the number of Conserved sites (C sites), Variable (Polymorphic) sites (V sites), Parsim-informative sites (Pi sites), Singleton sites (S sites), the number of identical pairs (ii), transitional pairs (si) and transversional pairs (sv) and nucleotide composition were determined for the homologous end trimmed COI sequences in MEGA X. The evolutionary divergence between sequences was estimated and the Kimura 2-parameter (K2P) model (Kimura 1980) was adapted to estimate the genetic distance between the species of the family Butidae.Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

Analysis of the CO1 gene dataset involved, three original sequences and 12 related sequences of butids retrieved from NCBI-GenBank such as *Butis butis* (MT765266, JX193741, MH827972, MW498535), *Butis humeralis* (MT765262, MF594611), *Butis koilomatodon* (MW379734, MG574474), *Butis gymnopomus*

(KU692407, KU692389). *Bostrychus sinensis* (KT951786) and *Ophiocara porocephala* (MW322096) were out-groups for COI tree construction.

A best-fit model for nucleotide substitution was selected from 24 models using MEGA X (Kumar et al. 2018) based on a minimum Bayesian Information Criterion (BIC) value (Nei and Kumar 2000; Kumar et al. 2018). The evolutionary history was inferred using the Maximum Likelihood method, constructed based on the best-fit model of Hasegawa-Kishino-Yano (Hasegawa et al. 1985), and the reliability of the tree was estimated using 1000 bootstrap replications (Felsenstein, 1985). The phylogenetic tree was visualized using FigTree v1.4.4 (Rambaut 1999) and edited by Adobe illustrator.

Results

Three species of butids –*Butis gymnopomus, B. butis* and *B. koilomatodon* were obtained from different locations in Vembanad- Kole wetland (Fig. 1& 3).

Morphometric data of the three species of Butis have been provided in Table 2.

Table 2

Morphometric data of *Butis koilomatodon, B. gymnopomus* and *B. butis*.- HL (Head length), Hw (Head width), SN/D1 (Distance from snout to origin of the first dorsal fin), SN/D2 (Distance from snout to origin of the second dorsal fin), SN/A (Distance from snout to vertical of anal fin origin), SN/AN (distance from snout to vertical of the anus), SN/V (Length from snout to pelvic fin origin), CP (Caudal peduncle length), CPd (Caudal peduncle depth), D1b and D2b (Length of first and second dorsal fin bases), Ab (Length of anal fin base), P1 (Pectoral fin length), V1 (Pelvic fin length), Ad (Body depth at anal-fin origin), SN (Snout length), E (Eye diameter), PO (Post orbital length), CHd (Cheek depth), I (Interorbital length), SL (Standard length).

	***Butis koilomatodon* (n = 10)**			***Butis gymnopomus* (n = 7)**			***Butis butis* (n = 5)**		
	Range (%)	Mean	SD	Range (%)	Mean	SD	Range (%)	Mean	SD
% Of SL.									
HL	29.5–36.2	31.9	2.02	30.9–36.4	33.8	1.6	33.8–36.1	35.1	0.8
Hw	18.8–28.3	21.83	3.5	50.1–68.8	57.1	7.09	52.7–60.3	56.3	3.5
SN/D1	34-43.6	37.1	2.7	36.6–42.8	39.1	2	41.7–43.5	42.6	0.7
SN/D2	51.2–59.2	54.9	2.06	52.2–62	55.7	3.4	57.2–60.6	59.2	1.12
SN/AN	41.8–60	51.23	5.29	48.2–54.4	50.6	1.97	53.04–54.4	53.6	0.5
SN/A	54-62.3	57.7	2.5	52.4–58.1	55.8	1.9	58.9–60.9	60.4	0.9
SN/V	26.3–34.2	28.7	2.26	29.5–32.9	31.4	1.12	31.7–34	32.6	0.9
CP	25.3–32	28.5	2.3	13.68–28.4	23.9	5.5	25.8–29.8	27.7	1.5
D1b	11.7–15.8	14.11	1.09	11.6–14.4	12.98	1.03	12.3–16.1	14.2	1.46
D2b	15.89–22.5	19.03	2.21	14.2–15.9	15.13	0.5	13.4–16.6	14.9	1.16
Ab	14.6–19.6	16.6	1.59	11.7–15.7	14.4	1.3	11.8–15.4	13.9	1.32
C1	21.4–24.6	23.45	1.17	17.8–23.6	20.1	1.8	24.4–36.2	28.07	4.77
P1	23.6–28.2	26.23	167	19.4–22.5	21.5	1.09	23.9–24.9	24.4	0.7
V1	21.6–26.2	23.26	1.44	14.03–21.3	16.5	2.3	16.8–18.3	17.6	0.5
Ad	19.5–23.9	21.8	1.12	14.3–15.8	15.01	0.6	18.1–22.8	19.3	1.9
Aw	12.6–17.1	14.7	1.7	10.7–13.1	11.6	0.8	16.1–18.7	17.3	1.2
% Of CP									

	Butis koilomatodon (n = 10)			*Butis gymnopomus* (n = 7)			*Butis butis* (n = 5)		
CPd	31.6–60.1	40.12	7.9	30.5–57	37.9	9.3	36-41.2	39.01	2.1
% Of H									
SN	25.9–30.8	28.37	1.59	23.4–40.7	30.8	6.03	32.3–39.1	35.86	2.46
E	20.4–29.4	24.4	2.86	23.07–30.8	26.5	3.09	17.5–20.8	19.5	1.4
CHd	23.7–34.1	26.7	3.27	7.86–16.04	11.5	2.6	10.3–14.6	13.17	2.3
I	10.1–13.5	11.7	1.31	3.9-8	6.2	1.4	16.02–21.5	18.85	2.27

B. koilomatodon (Bleeker 1849) can be distinguished from its congeners morphologically, having a short deep body and head, without the long jaws and snout of its congeners. It has a head width of 18–28% of its standard length (SL), while *B. gymnopomus* (Bleeker 1853) and *B. butis* (Hamilton1822) have head widths of 50–68% and 52–60% of their SL, respectively. The cheek depth of *B. koilomatodon* is 23–34% of its head length but congeners have relatively less cheek depth as a percent of head length. In appearance, *B. gymnopomus* resembles *B. butis*, and can be distinguished from *B. butis* by the naked interorbital region and snout (Fig. 2-A), lack of axillary scales (Fig. 2-C), and absence of a dark spot on the base of the pectoral fin. Body depth is 14–15% of SL for *B. gymnopomus*18-22% of SL for *B. butis*. Eye diameter is 23–30% of HL for the former and 17–20% of HL for the latter. There is a relatively wide interorbital region in *B. butis* (16–21% of HL vs 3–9% of HL in *B. gymnopomus*) (Table 2) (Fig. 2-B).

Key to the butid species occurring in Central Kerala.

1a. Head more compressed (Head width 18–28% of standard length) and cheek depth 24% of head length ……………………………………………………………………………......**Butis koilomatodon**

1b. Head less compressed (Head width 50–68% of standard length) and cheek depth 7–16% of head length…………………………………………………………………………………………...**2**

2a. Axillary scales present (Fig. 2D), wide and scaled inter-orbital region (Fig. 2B), relatively small eyes (Eye diameter 17–20% of head length) ……………………………………………… **Butis butis**

2b. Axillary scales absent (Fig. 2C), narrow interorbital region without scales (Fig. 2A), relatively large eyes (eye diameter 23–30% of head length…………………………………..........**Butis gymnopomus**

Molecular phylogeny

Barcodes were generated for three butid species (*B. butis*, *B. koilomatodon, B. gymnopomus* (Table 1). There was a total of 354 positions in the final dataset and the overall genetic distance between the studied species was 14%. The sequence alignment of the entire dataset of COI gene sequences included 658 base pairs and the average nucleotide frequencies for all butid species were observed as T = 28.1, C = 28.1, A = 25.3, and G = 18.6. The overall GC content was 46.7%The nucleotide pair frequency analysis of *Butis* species CO1 gene revealed that out of 658 pairs, 497 were conserved, 160 were variable, 148 were parsimony informative, and 12 were singleton, respectively. In the present study, the average number of transitional pairs (Si = 51) for the CO1 gene dataset was more frequent than the average number transversional pairs (Sv = 19) which indicates that the sequences of butids are not saturated and the species can be discriminated.

The phylogenetic analysis resulted in well-resolved three clades at species level, with each clade belonging to a separate species (Fig. 4), and the relationship among the species of the tree is supported by high bootstrap support values. The CO1 sequences of *B. butis* formed a single clade with the highest support values. Likewise, *B. koilomatodon* clustered under the same clade as *B. humeralis* and formed a sister group relationship, with 100 bootstrap values. The CO1 sequences of all three *Butis* species were found to be share a common ancestor revealing the monophyletic origin. The phylogenetic inference showed a clear-cut separate cluster, facilitating the accurate identification of target butid species.

Discussion & Conclusion

The present study represents the first morphological and molecular phylogenetic analysis of the *Butis* species in Indian waters and highlights the effectiveness of the CO1 gene for confirmation of the *Butis* species as reported for other gobioid genera. (Huanget al. 2013; Knebelsberger and Thiel2014; Islam et al.2021). The integrative taxonomy results, uncover the under-estimated species diversity of Butids in Kerala waters and reports for the first time, the presence of *B. gymnopomus*, which is often misidentified as *B. butis* (Batuwita et al. 2015). The study results, justifies the inclusion of *Butis koilomatodon* in the genus *Butis* by Larson and Murdy (2001) that was previously placed under the genus *Prionobutis* (Koumans 1941). As the two species (*B. butis* and *B. gymnopomus*) occur in the Vembanad Lake system, dichotomous keys generated out of the study will be useful for their precise identification.

The sequence analysis of all butid species estimated agrees with the ranges obtained in the studies of Viswambharan et al. (2013) and Linh et al. (2018) in gobioids. Also, the transition versus transversion ratio was comparable to the previous observations made by Ward et al. (2005), Lakra et al. (2011), and Rathipriya et al. (2016) in different fish species. The COI gene datasets revealed strong relationships among the butid fish species and solidified the monophyly of the family Butidae (Thacker 2009; Agorreta et al. 2013. According to Agorreta et al. (2013), Butidae is currently the only recognized family without any morphological synapomorphies, but Gierl et al. (2013) proposed two potential morphological synapomorphies for this group. The species of the genus *Butis* were delimitated by CO1 sequences and the phylogenetic relationships were highly concordant with the previous morphological findings (Wang et al. 2001; Agorreta and Ruber 2012).

Declarations

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Authors’ Contributions The specimens were collected by Krishnaprasad PH and Melbin Lal. The molecular analysis was performed by Mugda Sukumaran. The manuscript was prepared by Krishnaprasad PH and Mugda Sukumaran, while Dr. Anvar Ali P. H. reviewed the whole project. All authors read at approved the manuscript.

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Data availability The specimens are available at the Kerala University of Fisheries and Ocean Studies (KUFOS) Museum.

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Figures

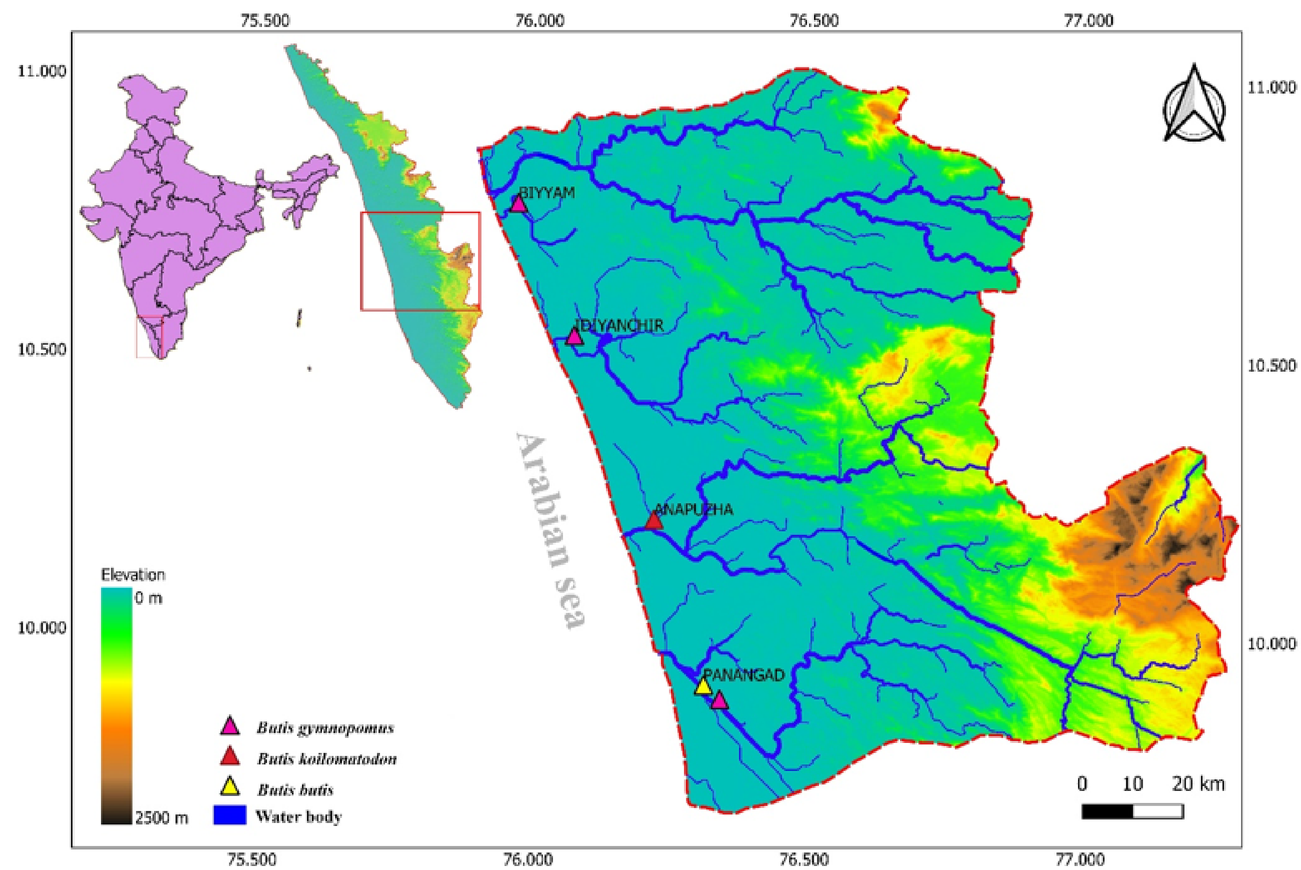

Figure 1

Distribution of butid species in Vembanad-Kole wetland.

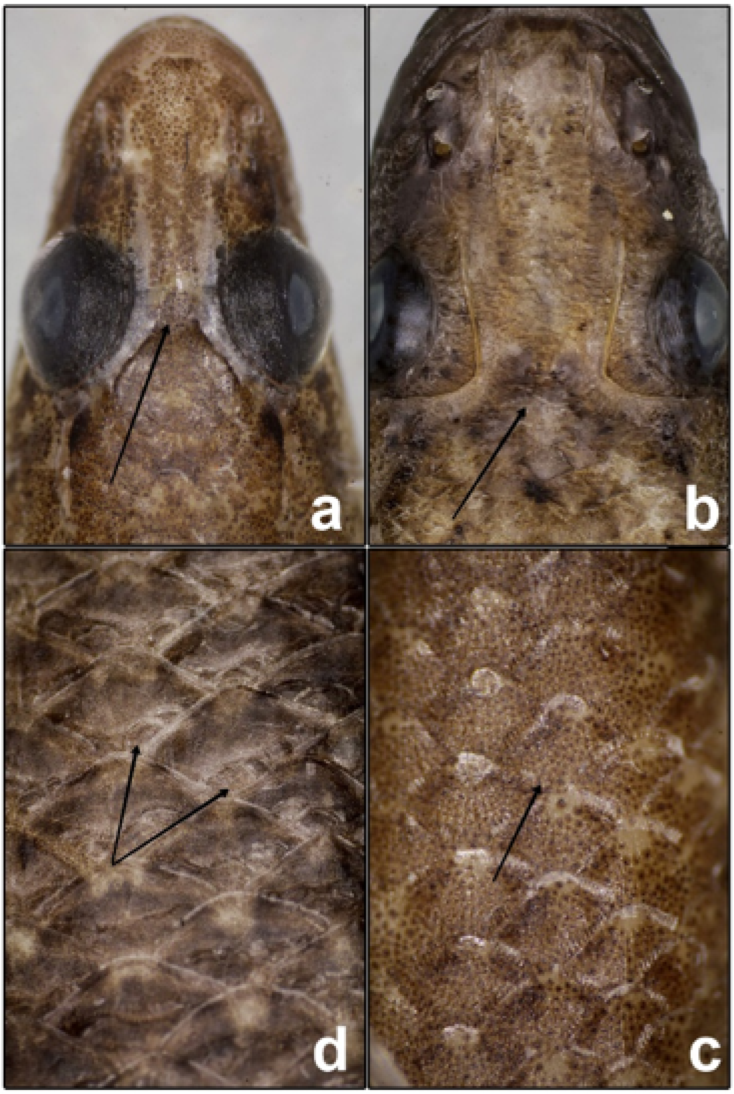

Figure 2

Morphological characters for distinguishing B. gymnopomus and B. butis **a**. B. gymnopomus; Naked and narrow interorbital region. **b**. Scaled interorbital region of B. butis. **c**. Lack of Axillary scales in B. gymnopomus. **d.**Axillary scales of B. butis

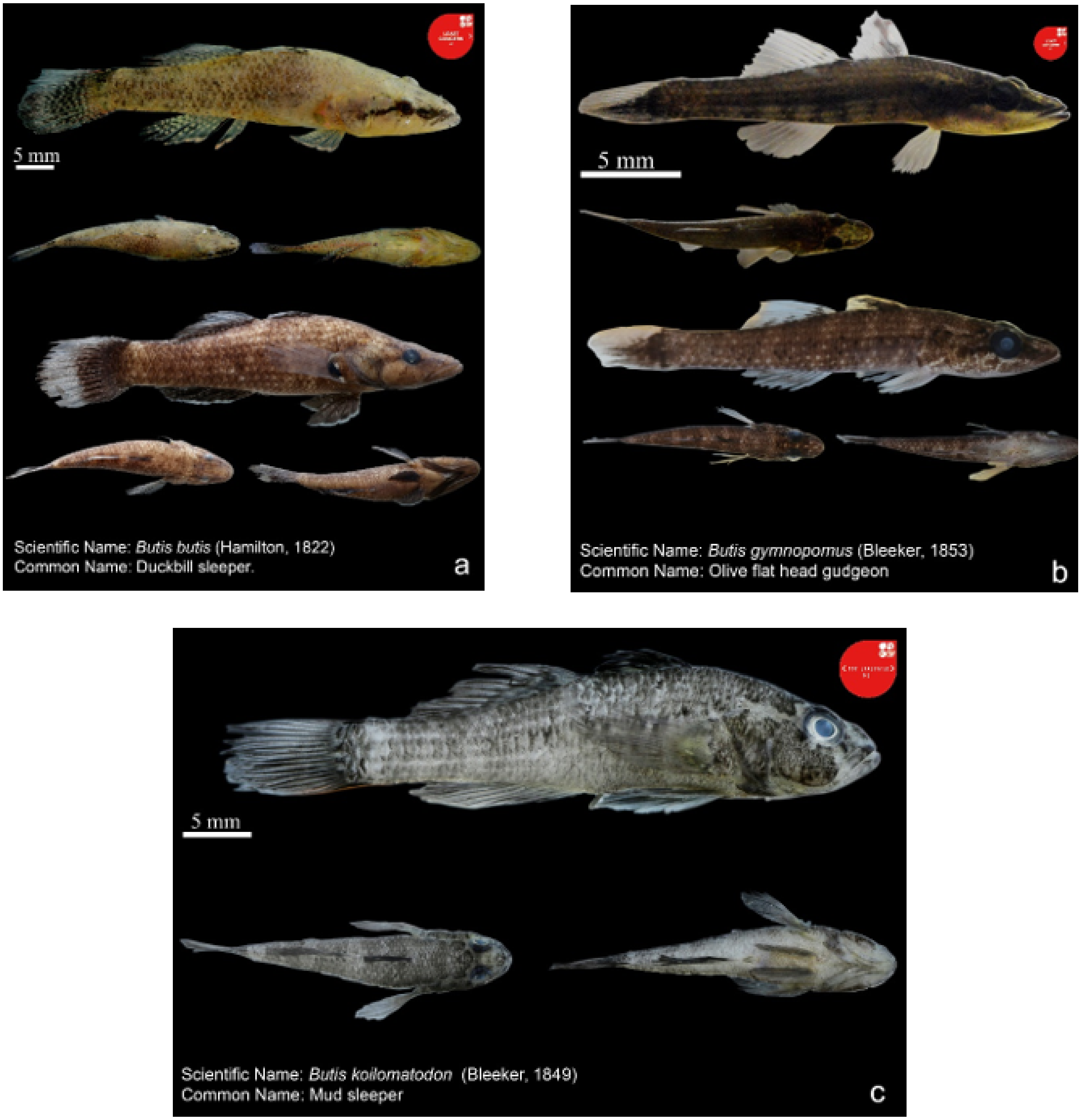

Figure 3

Images of live and preserved specimens **a.** *Butis butis*; **b**. *B. gymnopomus*; **c**. *B. koilomatodon*.

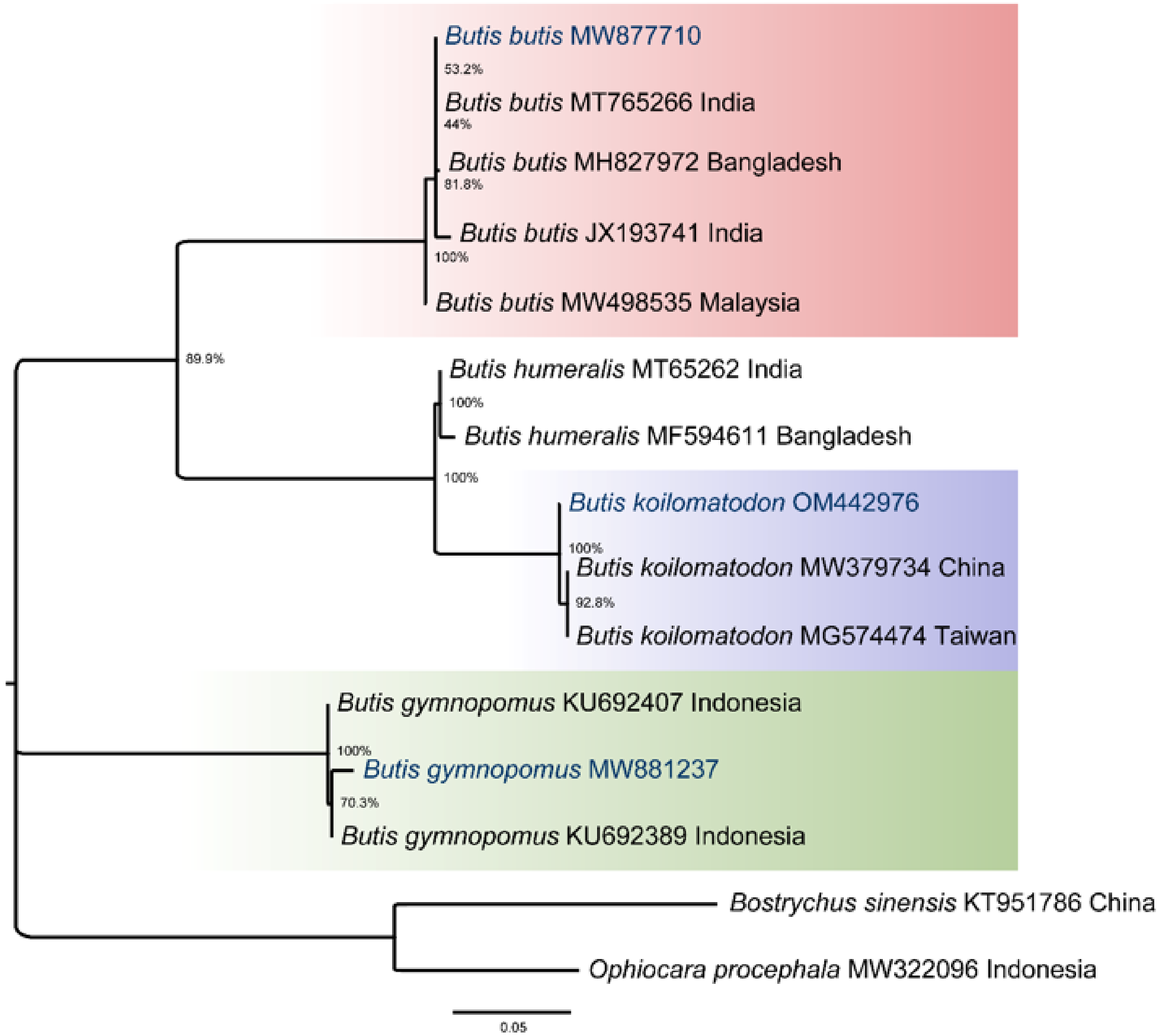

Figure 4

Molecular Phylogenetic Analysis by Maximum Likelihood Method using partial CO1 gene dataset of butids.