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Morphological and molecular characterisation of eggs and larvae of dark sleeper goby, *Butis humeralis* (Valenciennes, 1837), collected from a tropical estuary

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Original Article

Abstract

Eggs of *Butis humeralis* (Valenciennes, 1837) were described for the first time from the west coast of India. A cluster of sparsely distributed eggs of *B. humeralis* was observed on a gritted glass panel submerged for biofouling studies in the Cochin estuary, Kerala. The egg mass covered an area of 62 cm^2 with an average abundance of 120 ± 30 eggs/cm^2. Molecular characterisation of the egg was performed to confirm species identification. The brownish-yellow, sticky, pyriform-shaped, transparent eggs of *B. humeralis* measured 0.41 ± 0.01 mm in diameter along the long axis and 0.36 ± 0.01 mm along the short axis. The newly hatched larvae of *B. humeralis* were slender and translucent with a total length of 1.01 ± 0.037 mm. The total length of the preflexion larvae was 2.08–2.12 mm. DNA barcoding using partial mitochondrial cytochrome oxidase subunit I gene sequences confirmed species identity, with 100% similarity to *B. humeralis* sequences from the Cochin estuary, which were deposited in GenBank. Genetic divergence analysis showed extremely low intraspecific variability (0.0%) and moderate interspecific divergence (5.7–24.7%), confirming genetic distinctness and clear taxonomic separation of *B. humeralis.* The first taxonomic description of the early life stages of this lesser-known species will facilitate future ichthyoplankton identification and contribute to assessing its conservation status within the ecosystem.

Keywords: *Goby, Cochin estuary, eggs, larvae, DNA barcoding*

Introduction

Sleeper gobies, or dark sleeper or olive flathead-gudgeon, belong to the family Eleotridae under the Order Gobiiformes (Fricke *et al.,* 2025). Eleotridae is composed of three subfamilies: Butinae, Eleotrinae and Milyeringinae (Fricke *et al.,* 2025). Eleotrids are primarily found in tropical and sub-tropical brackish and freshwater zones of Asia, Africa, and Australia (Thacker, 2011). Molecular phylogenetic examination of Gobiiformes suggests elevating the subfamilies Eleotrinae and Butinae to the families Eleotridae and Butidae, respectively, as new rank classifications (Thacker, 2009; Abdulmalik-Labe and Quilang, 2024). It is also native to Hong Kong, Thailand, and Papua New Guinea and has been reported from India, Vietnam, Bangladesh, Malaysia and Singapore. The subfamily Butinae within the family Eleotridae comprises nine species across five genera: *Butis*, *Bostrychus*, *Odonteleotris*, *Ophiocara* and *Incara*. The genus, *Butis,* includes seven valid species, such as *B. amboinensis* (Bleeker, 1853), *B. butis* (Hamilton, 1822), *B. gymnopomus* (Bleeker, 1853), *B. humeralis* (Valenciennes, 1837), *B. koilomatodon* (Bleeker, 1849), *B. melanostigma* (Bleeker, 1849) and *B. prismatica* (Bleeker, 1849). However, only five species, namely *B. amboinensis*, *B. butis*, *B. gymnopomus*, *B. humeralis*, and *B. koilomatodon*, were reported from India (Nair and Dineshkumar, 2018; Venkateswarlu, 1967; Geevarghese, 1981). *B. amboinensis* were only reported from the east coast of India, while *B. humeralis, B. butis, B. koilomatodon,* and *B. gymnopomous* were found in the Veli, Korapuzha-Kuttiadi, Ashtamudi, Kodungalloor-Azhikode, and

Vembanad estuarine waters of Kerala, on the south-west coast of India (Geevarghese, 1981; Gopi, 2006; Raghunathan, 2007; Zeena and Beevi, 2011; Nandan *et al.,* 2012; Krishnaprasad *et al.,* 2025).

The present study is the first description of *B. humeralis* eggs and early life stages. The study also provides a detailed description of egg morphology, hatching and changes associated with the growth and development of *B. humeralis* larvae under laboratory conditions, as well as the molecular characterisation of *B. humeralis* eggs from the Cochin estuary, Kerala.

Material and methods

Study area and sampling method

To examine the biofouling impacts on aquaculture cages and jetties, gritted glass panels (150 mm × 150 mm × 2 mm) were suspended at a depth of 1-1.5 m at the cage site near Kalamukku Fishing Harbour (9.59′ 11.7″ N 76.14′ 43.7″ E) in Cochin estuary, Kerala (Fig. 1). Numerous eggs were found adhered to the glass panel upon retrieval after twenty days in monsoon season. The glass panels with the eggs were carefully detached from the cages and transported to the laboratory for further investigation. A working-party zooplankton net with a mesh size of 300 μm was used to collect zooplankton during the post-monsoon (November 2021 and January 2022). Later, glass panels were dipped into a tray filled with filtered estuarine water (0.2 μm) collected from the study area to observe egg hatching and development in the laboratory. The physicochemical characteristics of surface water samples collected from the panel-immersed site were measured according to standard procedures (APHA, 1998).

Microscopic examination of the egg samples

A cluster of eggs was observed on the gritted glass panels and in zooplankton samples. Measurements of 150 eggs were taken under the stereo microscope (Nikon SMZ25 – NIS Elements D 5.30.00). The area of attachment of eggs on a gritted glass panel was calculated by counting the total number of grits containing eggs, multiplying it by the area of a single grit, and averaging the number of eggs per grit. These eggs were carefully detached from the panel using a brush and kept in a 1000 ml glass jar containing estuarine water collected from the cage site. They were observed every 24 hours to study the early developmental stages of the egg samples. A total of 6 and 25 eggs were counted from the zooplankton samples in November 2021 and January 2022, respectively. The taxonomic identification of *B. humeralis* eggs was carried out using similar morphologically identified

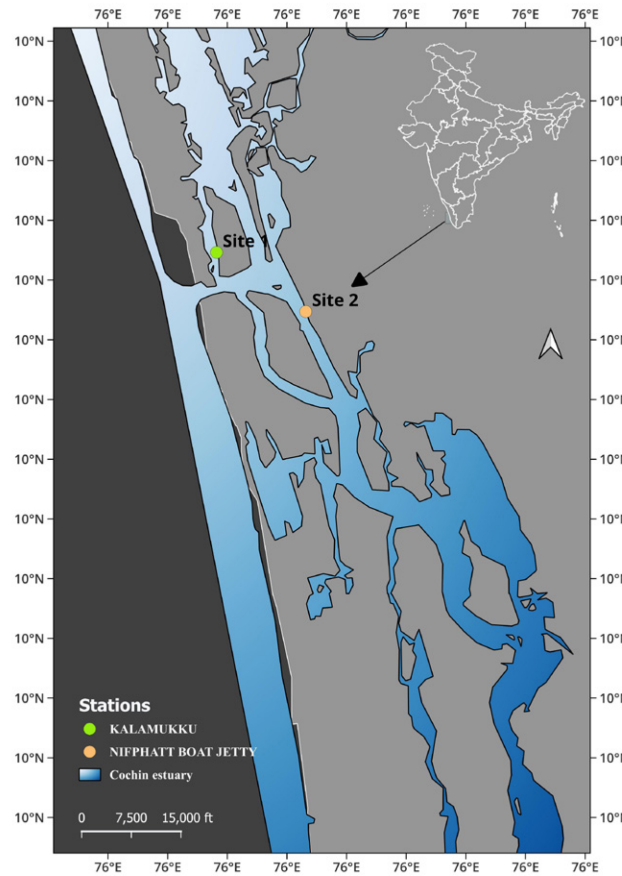

Fig. 1. Map showing the collection sites of *Butis humeralis* from Cochin estuary, Kerala, south-west coast of India

eggs from the same family, Eleotridae (Lindstrom, 1999; Maeda *et al.,* 2008). The volume of the yolk sac and oil globule of eggs and developing larvae was calculated (Bagarinao, 1986).

DNA barcoding of the egg samples

A subsample of the eggs collected and sorted both from the glass panel and total eggs from zooplankton samples was preserved in 95% ethanol. Total DNA was isolated using the DNeasy® Blood and Tissue kit (Qiagen, Germany), and its quality and quantity were assessed using the NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). Molecular phylogenetic analyses were carried out using mitochondrial Cytochrome Oxidase I (COI) sequencing.

PCR carried out in 25 μl reactions using 2X EmaraldAmp GT PCR Master mix (Takara, Japan) using 20 pM of primers and 20 ng of template DNA (i.e., 12.5 μl of EmaraldAmp GT PCR Master mix, 0.25 μl primers (Fish F1 5′-TCAACCAACCACAAAGACATTGGCAC-3′ and Fish R1 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) (Ward *et al.,* 2005) and 1 μl template DNA made up to final volume with nuclease-free water). The reaction mixture was preheated at 94 ^{0}C for 5 minutes, followed by 25 cycles (94 ^{0}C for 30 seconds, 50 ^{0}C for 30 seconds, and 72 ^{0}C for 35 seconds) and a final extension at 72 ^{0}C for 5 minutes. PCR products were visualised in a 1.5% agarose gel and compared with a NEX-GEN 100 bp DNA ladder (Genetix Biotech Asia Pvt. Ltd., New Delhi, India). PCR products were sent for sequencing to the sequencing facility at Enfys Lifesciences Pvt. Ltd. (Kerala, India). The forward and reverse DNA sequences were aligned and assembled using BioEdit sequence alignment editor, version 7.0.5.2 (Hall, 1999), and species-level morphological identifications were performed through sequence similarity searches using BLAST in GenBank.

Results

Observation of the gritted glass panel after 20 days of immersion showed that the egg mass covered an area of 61.80 cm^2 on the glass panel with an average of 120 ± 30 eggs/cm^2 (mean ± SD; n=10) and was irregularly distributed on the surface of the glass panel (Figs. 2a and b). The egg mass appeared to be a single clutch due to its homogenous growth and appearance. Owing to the sparse distribution, minute size of the eggs, and variability of the density within an egg mass, it was difficult to determine the accurate number of eggs within the clutch. Nevertheless, estimates of egg numbers from subsamples indicated that the clutch comprises 7,680 eggs / 61.80 cm^2. Morphologically similar eggs were also collected through seasonal zooplankton sampling from two different stations in the Cochin estuary during November 2021 and January 2022. The number of eggs per haul was 6 and 25, respectively (Fig. 3). The atmospheric temperature, water temperature, salinity, and pH of the study area during the retrieval of the gritted glass panels were 28 °C, 26 °C, 6 PSU, and 7.29, respectively. Other physicochemical parameters, such as turbidity, total suspended solids (TSS), and dissolved oxygen (DO), during the sampling period were 7.58 NTU, 28.67 mg L^{-1} and 5.43 mg L^{-1}, respectively. The nutrient concentrations, including inorganic nitrate, nitrite, inorganic phosphate, silicate, and ammonia, were 0.129 mg L^{-1}, 0.008 mg L^{-1}, 0.063 mg L^{-1}, 1.34 mg L^{-1}, and 0.03 mg L^{-1}, respectively.

Morphological characteristics of B. humeralis eggs

The egg mass appeared to be a single clutch due to its homogenous growth and appearance. Owing to the minute size and sparse distribution of the eggs and variability of the density within an

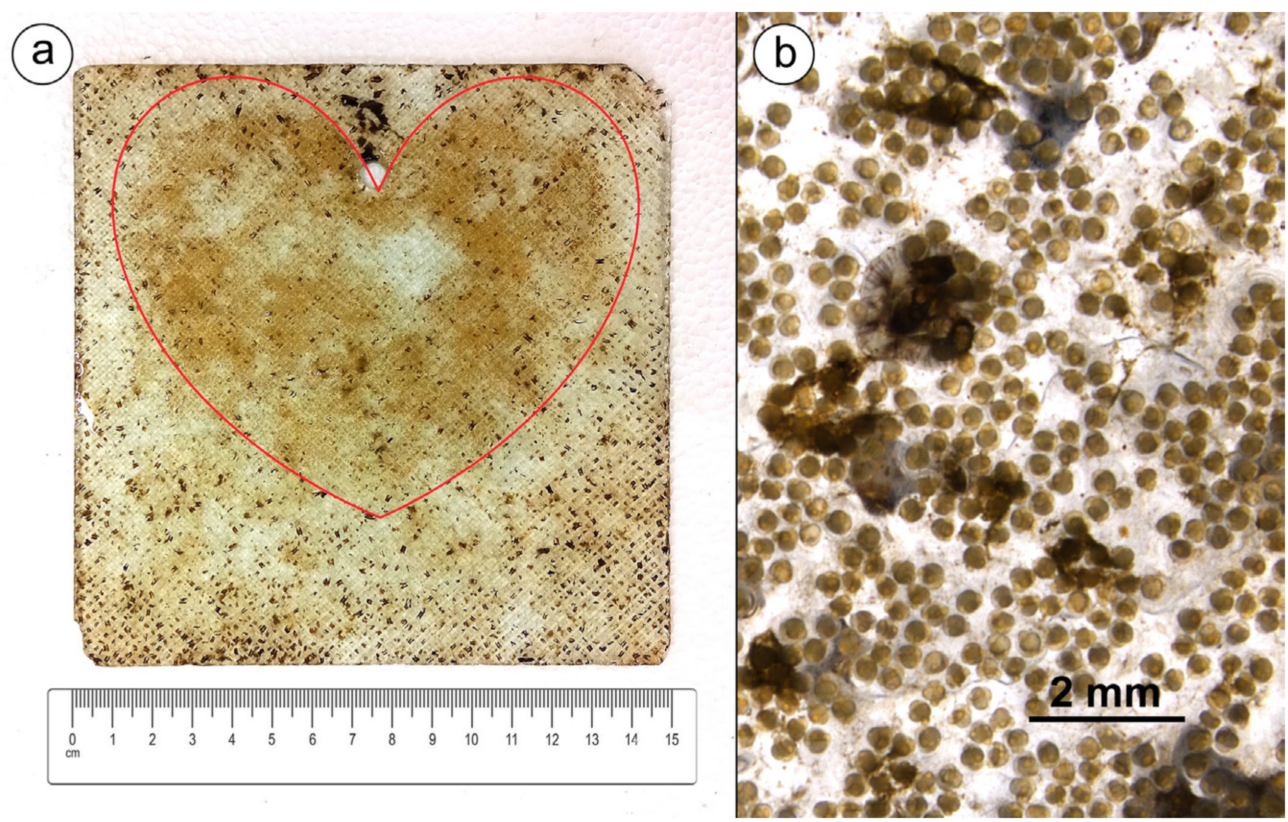

Fig. 2. a) Surface of the fouled gritted glass panel with egg mass of *B. humeralis* (shown within red heart shape area) after 20 days immersion near Kalamukku Fishing Harbour, Cochin Estuary, Kerala, b) Magnified picture of the centre of *B. humeralis* egg mass deposited on the surface of the gritted glass panel, showing the typical arrangement

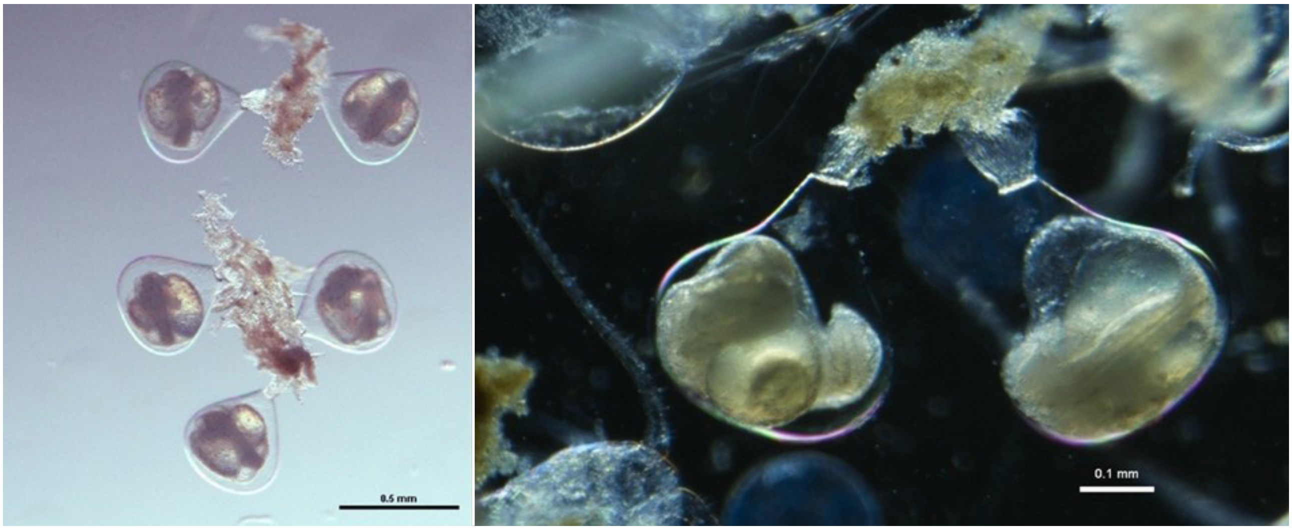

Fig. 3. Eggs of *B. humeralis* from zooplankton samples collected from NIFPHATT Jetty

egg mass, it was difficult to determine the accurate number of eggs within the clutch. The demersally attached eggs of *B. humeralis* attached to gritted glass panels were brownish in colour, pyriform in shape and measured 0.41 mm × 0.36 mm (Fig. 4a). The perivitelline space was very narrow, with the embryo occupying almost the entire vitelline space. The pointed end of the egg comprised a bundle of adhesive filaments that held the neighbouring eggs together on the surface in a monolayer. The yolk was clear, unsegmented, and golden in colour, with 4-6 oil globules present 5 min before hatching at the embryonic stage. The chorion appeared clear and smooth under stereomicroscopic observation. The eggs showed twitching and heartbeat movements during the late segmentation or pre-hatching stage. The egg possessed a primitive optic vesicle, notochord, and yolk sac containing numerous oil globules, each measuring 0.076 ± 0.03 mm in diameter. Shortly before hatching, numerous tiny oil droplets coalesced into two globules. Twitching and wriggling movements became more vigorous just before hatching. The embryo ruptured the egg capsule, and the yolk sac larva came out within 2 to 3 hours after being brought to the laboratory (Fig. 4b). *B. humeralis* larvae were poorly developed at the time of hatching and lacked a functional mouth, smaller size of eggs with narrow perivitelline space and yolk diameters less than 1 mm. Volume of yolk sac and oil globule reduced with larval development (Table 2).

DNA barcoding of B. humeralis eggs

The three aligned sequences used for similarity search (BLAST) in the NCBI database showed 100% identity and query coverage to *B. humeralis.* The generated sequence was submitted to the NCBI

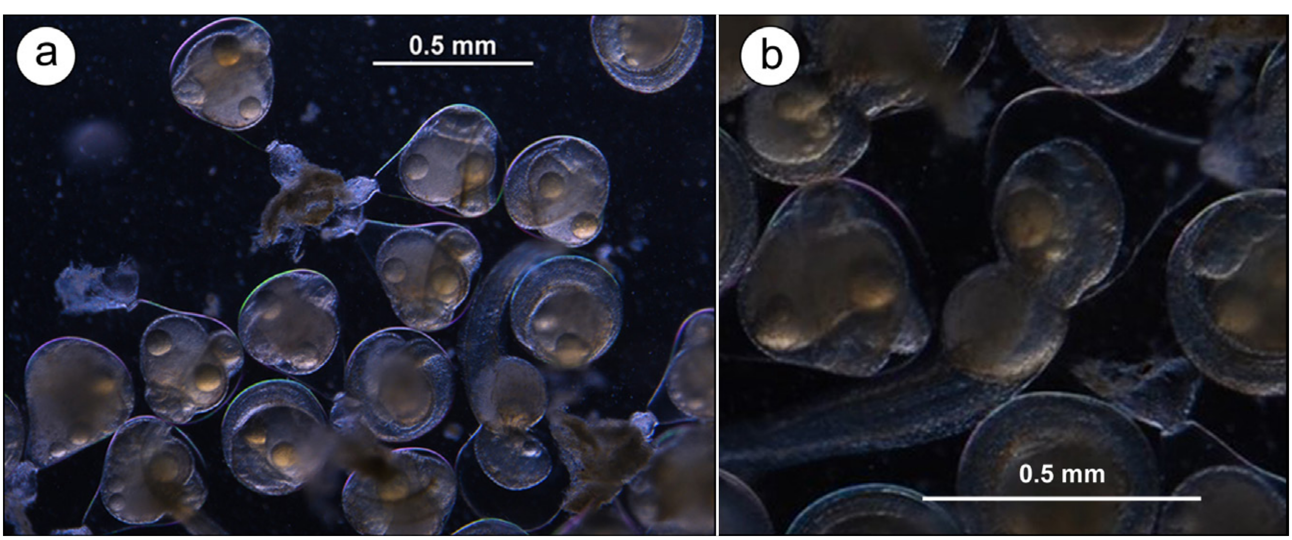

Fig. 4. a) Eggs of *B. humeralis* in the twitching and heart beating stage showing the orientation of embryos within the chorionic cavity. Adhesive filaments at the pointed end adhered the eggs to the surface of the spawning site, b) Yolk sac larval hatching of *B. humeralis* by penetrating through the egg membrane

GenBank with Accession Nos. OP872738, PZ256944, PZ256945. The genetic divergence values estimated using MEGA X among similar species of *Butis* (Table 3). The neighbour-joining (NJ) tree generated using the Kimura 2-parameter model showed three major distinct clades with high bootstrap support values. *O. marmorata* (PP085180) has been taken as an outgroup to root the tree (Fig. 6). The *B. humeralis* sequences (MK359460, MK359458, OP872738) formed a monophyletic cluster with 100% bootstrap support, indicating very low genetic divergence. Other *Butis* sequences subgroups (PZ256944, PZ256945) within the *Butis humeralis* cluster formed moderate to high bootstrap support values. The genetic differentiation from related species (*B. koilomatodon*, *B. butis*, *B. amboinensis*) within the family is also evident in the phylogeny. The outgroup *O. marmorata* confirmed the appropriate rooting of the phylogenetic tree. Pairwise genetic analysis using the Kimura 2-Parameter (K2P) revealed clear patterns of divergence within and between *Butis* species. Intraspecific distances were extremely low (0.000 to 0.008), showing minimal genetic variability. However, interspecific distances were higher (0.054 to 0.178), indicating strong divergence among most *Butis* species.

Systematics

Order: Gobiiformes (Günther, 1880)
Family: Eleotridae (Bonaparte, 1835)
Subfamily: Butinae (Bleeker, 1874)
Genus: *Butis* (Bleeker, 1856)
Species: *Butis humeralis* (Valenciennes, 1837)
Larval development of B. humeralis

The larval development of *B. humeralis* in the laboratory, with relation to the time period after hatching and characteristic features of each stage, is shown. (Table 1 and 2 and Figs. 5 a-g). Progressive morphological and behavioural changes in larval development occurred from egg hatching to 144 hours post-hatch (HPH). At hatching of eggs (0 HPH), the released yolk sac (0.55–0.62 mm) larvae were (0.97–1.09 mm notochord length (NL)) translucent, with initial pigmentation and oil globules visible. By 24 HPH, partial yolk absorption, embryo fin fold formation and 'swim-up and sink' movements were exhibited. At 48 HPH preflexion larvae (1.53–1.55 mm NL) showed prominent head development, full absorption

Table 2. Volume of yolk sac and oil globule from 0 to 96 HPH

After hatching (hours)	Volume of yolk sac ($V = \pi/6\ l\ h^2$) (mm^3)	Volume of oil globule ($V = \pi/6\ d^3$) (mm^3)
	Bagarinao (1986)	Bagarinao (1986)
0	0.05239 ± 0.01039	0.00026 ± 0.00024
24	0.00835 ± 0.00119	0.00015 ± 0.00008
48	0.00154 ± 0.00013	Completely absorbed
72	0.00034 ± 0.0000	Absent
96	Yolk exhausted	Absent

Table 1. Larval development of *B. humeralis* in the laboratory

Day	Hour Post-Hatch (HPH)	Length (mm)	Characteristics
1	0	0.97-1.09 NL 0.99-1.11 TL	Yolk sac larvae (Fig. 6a) were slender and translucent, having a large, slightly oval, brownish coloured yolk sac. Numerous oil globules coalesced into two in the yolk sac. Primitive optic vesicles and otoliths formed laterally on the head. Pigmentation appeared on the tip of the snout, above the eyes, on the anterior and posterior margins of the yolk sac, ventrally on the posterior half of the trunk, ventrally on the anterior two-thirds of the tail, and dorsally on the midtail.
2	24	1.15-1.2 NL 1.19-1.25 TL	The yolk sac was partially reduced in diameter. The embryonic fin fold formed. Myomeres were partially visible. Melanophores and xanthophores above the eyes disappeared, and portions of the eye margin turned pigmented. Ventral melanophores and xanthophores were reduced on the anterior margins of the yolk sac, and irregular bands of pigments appeared on its trunk and caudal region. The larvae exhibited slight movements using "swim up, sink" behaviour with their tail (Fig. 6b).
3	48	1.53-1.55 NL 1.55-1.57 TL	The head and unopened mouth appeared as a prominence or bump. The yolk sac was further reduced in diameter. Oil globules were completely absorbed. Lens and choroid fissures were formed on the eye located at the anterior-lateral position of the head. The Notochord became distinct. Myomeres were well-developed, with 15 pre-anal and 45 post-anal myomeres. Anus and pectoral fin bud were slightly visible. Melanophores appeared above the eye, yolk sac and head. Vertical melanophore bands prominently appeared on the body and caudal region. Larvae started swimming with constant tail movements (Fig. 6c).
4	72	1.53-1.55 NL 1.55-1.57 TL	Remnants of the yolk sac were apparent. The mouth cleft was prominent. The eyes became dark and fully pigmented. Pectoral fins developed (Fig. 6d).
5	96	1.54-1.56 NL 1.56-1.58 TL	Well-developed mouth formed with clearly distinct upper and lower jaws. The yolk sac was completely absorbed. Larvae displayed wandering movements with frequent opening of the upper and lower jaws. Melanophores were observed in series along the lower jaw, below the gut, and on the dorsal and ventral lines of the body. Dorsal, anal, and caudal fin folds were also present (Fig. 6e).
6	120	1.6-1.7 NL 1.67-1.69 TL	Mouth opened with a gap of 0.04–0.05 mm. Body depth at the anus was 0.25 – 0.26 mm. Pectoral fin length was 0.12 – 0.14 mm. Head length and head depth were 0.28 – 0.29 mm and 0.26 – 0.27 mm, respectively. Eye diameter was 0.12 – 0.13 mm (Fig. 6f).
7	144	1.96-2.0 NL 2.08-2.12 TL	The growth of the upper and lower jaw slowed compared to the previous days. The head length slightly increased (Fig. 6g). The pectoral fin is well developed, and the larva began stable swimming.

NL denotes Notochordal Length; TL denotes Total Length

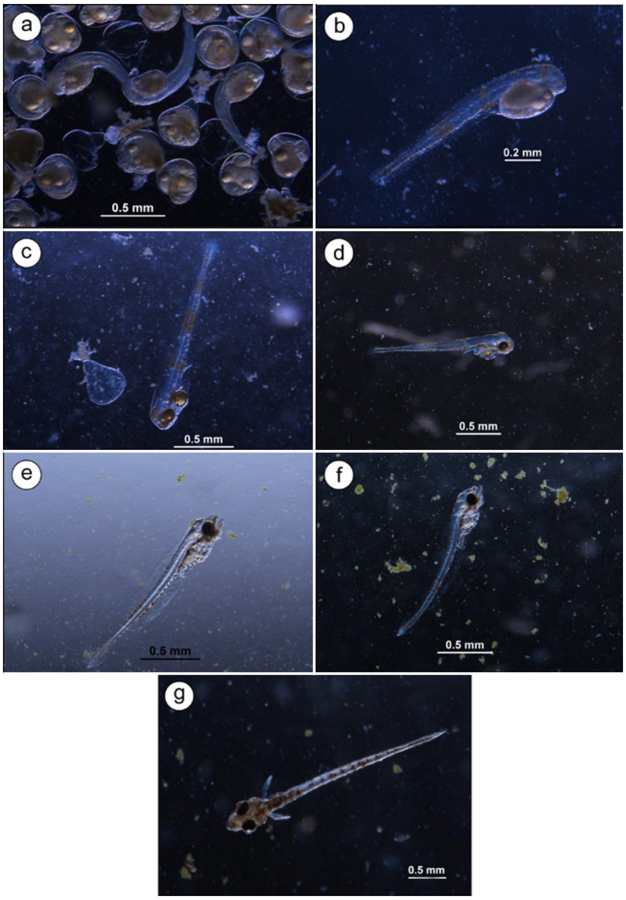

Fig. 5. Larval development of *B. humeralis* a) 0 HPH, b) 24 HPH, c) 48 HPH, d) 72 HPH, e) 96 HPH, f) 120 HPH, g) 144 HPH (dorsal view)

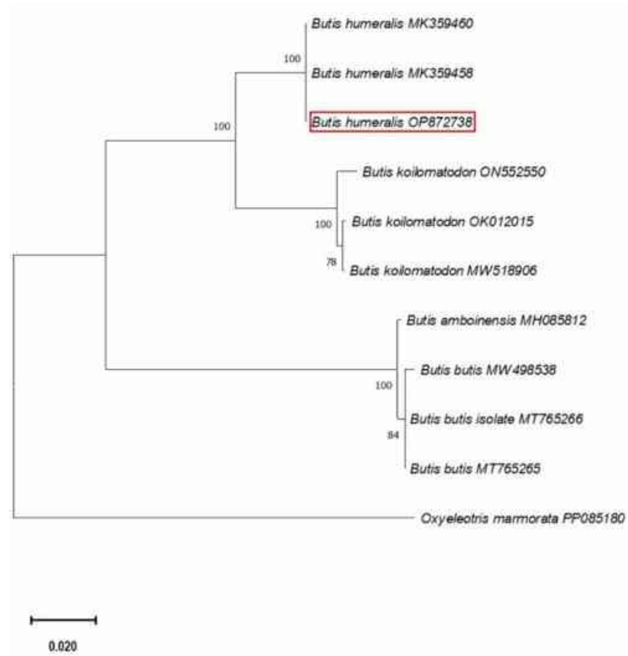

Fig. 6. Neighbour-Joining (NJ) Tree of COI sequences from four species of *Butis*. The sequence of *Oxyeleotris marmorata* was used as the outgroup. Accession number from the study

of the oil globule, a peculiar notochord, and well-developed myomeres. At 72 HPH, the yolk sac is nearly absorbed, the eyes are pigmented, and the pectoral fins are developed. At 96 HPH, yolk absorption is completed with active body movement; at 120 HPH (1.6–1.7 mm NL), mouth opening occurs. Eye diameter and body depth were clearly defined. At 144 HPH, larvae (1.96–2.12 mm) showed growth improvement, pectoral fin development and stable swimming behaviour (Table 1 and 2).

Discussion

B. humeralis is classified under the IUCN as Least Concern (LC). It was reported from the Indian Sundarbans and the Digha coast in West Bengal (Chatterjee *et al.,* 2013; Mishra and Gopi, 2017; Yennawar *et al.,* 2015), and from the coastal and estuarine waters of Tamil Nadu (Jeyaseelen and Krishnamurthy, 1981). The current study confirms the presence of *B. humeralis* in the waters of Kerala and provides the first description of its early developmental stages.

B. humeralis shares similarities in egg morphology with those of the two sleeper gobies of family Eleotridae, *Eleotris fusca* and *Eleotris acanthopoma*, as well as with *Lenticeps concolor* (indigenous Hawaiian goby) and *Stiphodon percnopterygionus* (sicydiine goby). The eggs of these gobies are pyriform and adhere to the substrate with a bundle of adhesive filaments. (Lindstrom, 1999; Maeda *et al.,* 2008; Yamasaki and Tachihara, 2006). Therefore, this study shows limitations in morpho-taxonomic identification due to the lack of standard literature. The *B. humeralis* sequence developed for this study showed 100% similarity and query coverage to the two sequences (Accession numbers: MK359460, MK359458), which were previously deposited from the Cochin estuary. DNA barcoding provides higher accuracy (94-99%) than the visual identification of fish eggs. (Mateos-Rivera *et al.,* 2020). Limitations of morphological taxonomy are overcome by molecular identification using COI genes (Hou *et al.,* 2022). DNA metabarcoding is a faster and more cost-effective method than individual barcoding, but individual barcoding yields better results (Breitbart *et al.,* 2023). Hence, DNA barcoding and molecular characterisation enabled more accurate species identification.

Although information on the early developmental stages of *B. humeralis* is currently lacking, the present study is relevant, as it details egg morphology, hatching, and the development of the preflexion larva up to 144 HPH by monitoring changes in eyes, pigmentation, fins, yolk sac size, oil globules, and mouth. Although identifying the early life stages of fish is challenging due to rapid developmental changes, DNA barcoding using

Table 3. Pairwise genetic distances between *Butis* species using the K2P model

	OP872738 *B. humeralis*	MK359458 *B. humeralis*	MK359460 *B. humeralis*	PZ256944 *B. humeralis*	PZ256945 *B. humeralis*	OK012015 *B. koilomatodon*	ON552550 *B. koilomatodon*	MW518906 *B. koilomatodon*	MT765265 *B. butis*	MT765266 *B. butis*	MW498538 *B. butis*	MH085812 *B. amboinensis*	PP085180 *O. marmorata*
OP872738 *B. humeralis*	****												
MK359458 *B. humeralis*	0.000	****											
MK359460 *B. humeralis*	0.000	0.000	****										
PZ256944 *B. humeralis*	0.000	0.000	0.000	****									
PZ256945 *B. humeralis*	0.000	0.000	0.000	0.000	****								
OK012015 *B. koilomatodon*	0.056	0.056	0.056	0.054	0.058	****							
ON552550 *B. koilomatodon*	0.061	0.061	0.061	0.060	0.064	0.008	****						
MW518906 *B. koilomatodon*	0.056	0.056	0.056	0.054	0.058	0.000	0.008	****					
MT765265 *B. butis*	0.160	0.160	0.160	0.144	0.142	0.169	0.174	0.169	****				
MT765266 *B. butis*	0.160	0.160	0.160	0.144	0.142	0.169	0.174	0.169	0.000	****			
MW498538 *B. butis*	0.164	0.164	0.164	0.144	0.142	0.173	0.178	0.173	0.003	0.003	****		
MH085812 *B. amboinensis*	0.157	0.157	0.157	0.144	0.142	0.166	0.171	0.166	0.003	0.003	0.007	****	
PP085180 *O. marmorata*	0.214	0.214	0.214	0.225	0.221	0.228	0.232	0.228	0.246	0.246	0.243	0.246	****

the COI gene improves taxonomic identification, supported by robust reference databases (Jiang *et al.,* 2024; Sruthy *et al.,* 2025). Many gobies hatch after eye pigmentation (Shinomiya *et al.,* 1981 a, b; Suzuki *et al.,* 1988; Suzuki *et al.,* 1989). However, eye pigmentation in *B. humeralis* started slightly at 24-hour post-hatch (HPH) and became densely pigmented at 72 HPH. The yolk sac stage begins at hatching and ends with yolk absorption (Kendall, 1984). In the present study, the absorption times of the yolk and oil globules were observed at 96 HPH, the same as in *Dormitator latrifrons* of the same family Eleotridae (Reyes-Mero *et al.,* 2022). During yolk and oil globule exhaustion, the larvae of *B. humeralis* developed pigmented eyes and differentiated fins, enabling them to survive progressive starvation as they transitioned from endogenous to exogenous feeding.

Gobies lay pear or pyriform-shaped eggs in demersal habitats, where they attach to surfaces in single layers (Russell, 1976). The egg masses of *Stiphodon percnopterygionus* (sicydiine goby) were guarded by the male and laid on the undersides of stones in freshwater (Yamasaki and Tachihara, 2006). Most gobiids exhibit an iteroparous reproductive pattern, in which females deposit eggs on vegetation or substrates, while males provide post-fertilisation parental care (Trujillo-García *et al.,* 2024).

According to Koumans (1953) and Miller *et al.* (1989), the habitat of *B. humeralis* ranges from marine to freshwater environments. Hui *et al.* (2010) reported that *B. humeralis* may survive in freshwater but may not breed successfully if access to the marine environment is restricted. In the present study, the *B. humeralis* eggs were found in an estuarine environment with a salinity of only 6 PSU at the time of collection. Although the two stations in the Cochin estuary have a direct connection to the sea through Cochin Barmouth, freshwater-seawater interactions occur regularly due to river water discharge and tidal forces. Therefore, the present scenario supports the previous investigations. The lunar cycle is crucial for their spawning and larval recruitment in estuaries (Miller, 1984; Thresher, 1984; Berra, 2001; Dinh *et al.,* 2016). In this study, the mature eggs of *B. humeralis* were collected five days before the new moon from Cochin Estuary. Here, hatching may also coincide with the lunar cycle, as it does in the wild.

The COI-based genetic distance analysis and the phylogeny of *B. humeralis* validate the genetic distinctiveness of most *Butis* species and reveal typical interspecific divergence consistent with species-level separation.

Conclusion

This study is the first taxonomic description of *B. humeralis* eggs and early life stages on the west coast of India, detailing egg morphology, early larval development, and molecular characterisation. *B. humeralis* eggs were demersal, pyriform, and surface-attached with filaments forming a single clutch, which probably indicates external fertilisation and the typical parental care strategy of Gobiids. Results emphasise both the limitations of accurately identifying morphologically similar gobiid eggs and the taxonomic resolution provided by DNA barcoding. Progressive morphological changes occur in larval stages from egg hatching to 144 HPH, and at 96 HPH, yolk absorption is completed. Cochin estuary, part of the Vembanad-Kol Wetland, one of Kerala's three Ramsar sites, is the largest estuary along the southwest coast of India. The presence of substratum-attached *B. humeralis* eggs and their occurrence in zooplankton samples from Cochin estuary indicate that this estuary provides ideal conditions for the breeding and nursing of this species. This study enhances understanding of the early life history of *B. humeralis* and the use of DNA barcoding to identify ambiguous and unknown ichthyoplankton.

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Author contributions

Conceptualisation: SMK, VLS; Methodology: MKS, VLS, KAS; Data Collection: SMK, VLS; Data Analysis: SMK, VLS, RRK; Writing Original Draft: VLS, SMK; Writing Review and Editing: RRK, PK; Supervision: RRK, PK

Data availability

The data are available and can be requested from the corresponding author. The generated sequence was submitted to NCBI GenBank with Accession Nos. OP872738, PZ256944, PZ256945.

Conflicts of interest

The authors declare that they have no financial or non-financial conflicts of interest that could have influenced the outcome or interpretation of the results.

Ethical statement

No ethical approval was required as the study did not involve activities requiring ethical clearance or the use of protected organisms/ human subjects/ collection of sensitive samples/ protected environments.

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