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A new species of the genus *Eubranchus* (Gastropoda: Nudibranchia) from Vietnamese coastal waters

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ABSTRACT. A new species belonging to the Fionidae (*sensu* Cella *et al.*, 2016), *Eubranchus* ***flexus*** sp. nov. is described based on specimens collected in Nha Trang, Vietnam, South China Sea, Indo-West Pacific, from the upper sublittoral depth. An integrative analysis was conducted, including a molecular phylogenetic analysis based on three markers (COI, 16S, H3), and an analysis of the external and internal morphology using light and scanning electron microscopy. The distinctiveness of *Eubranchus* ***flexus*** sp. nov. is well established both morphologically and genetically. Phylogenetically *Eubranchus* ***flexus*** sp. nov. represents a derived branch in the phylogeny of *Eubranchus*, but its relationships with other *Eubranchus* species are unresolved likely due to low taxon sampling. *Eubranchus* ***flexus*** sp. nov. is the first representative of the genus *Eubranchus* for the nudibranch fauna of Vietnam and for now its range is restricted only to the type locality.

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Новый вид рода *Eubranchus* (Gastropoda: Nudibranchia) из прибрежных вод Вьетнама

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РЕЗЮМЕ. Описан новый вид семейства Fionidae (sensu Cella *et al.*, 2016), *Eubranchus* ***flexus*** sp. nov., обитающий в Южно-Китайском море, в районе Нячанга, Вьетнам. Данный вид был обнаружен в верхней сублиторали на небольших глубинах. Для его описания был проведен интегративный анализ, включающий молекулярно-филогенетический анализ по трем маркерам (COI, 16S, H3), а также анализ внешней и внутренней морфологии с использованием световой и сканирующей электронной микроскопии. Новый вид *Eubranchus* ***flexus*** sp. nov. значительно отличается от других видов рода *Eubranchus* морфологически и генетически. Филогенетически данный вид представляет отдельную ветвь, однако отношения внутри рода не разрешены в связи с нехваткой материала по другим видам. Новый вид *Eubranchus* ***flexus*** sp. nov. является первым представителем рода *Eubranchus* для фауны Вьетнама и на настоящий момент был обнаружен только в типовом местонахождении.

Introduction

Nudibranchs are shell-less gastropod molluscs (sea slugs) that display a high degree of variation in external morphology and coloration and are widely distributed in all seas and oceans. Although there are numerous nudibranch groups distributed in temperate and subpolar regions [Ekimova *et al.*, 2019; 2022a; Korshunova *et al.*, 2020a, b] the highest diversity occurs in tropical waters [Turner, Wilson, 2008; Gosliner, Fahey, 2011; Knutson, Gosliner, 2022; Soong *et al.*, 2022], especially in the Indo-West Pacific and the Coral Triangle [Gosliner *et al.*, 2018]. To date, more than 2000 of described and undescribed sea slugs have been reported in the Indo-West Pacific [Gosliner *et al.*, 2018]. At the same time, nudibranch biodiversity is different across various regions: while more than 1200 sea slugs are known from Philippines, only 646 were found in Papua New Guinea and 258 in Tanzania. This may be a result of different collection efforts and sampling efficiency varying across regions, or this may demonstrate an actual unequal distribution of sea slugs in different localities of the Indo-West Pacific [Gosliner *et al.*, 2018]. The fauna of coastal waters of Vietnam remains one of the most poorly studied in terms of the sea slug biodiversity [Martynov, Korshunova, 2012]. Until 2012 only 80 species were found, with a single review on Vietnamese sea slugs [Risbec, 1956] and some additional notes available in the literature [Loi, 1967; Sachidhanandam *et al.*, 2000]. The most recent revision of the sea slug fauna of Vietnam [Martynov, Korshunova, 2012] reported on 150 species with 116 species found for the first time for Vietnamese waters; also, for each species a comprehensive species description was given along with photos of the living animals. However, the latter study lacks molecular data, and further studies demonstrated that some of the recorded species may represent species complexes, for example example *Myja longicornis* Bergh, 1896 [Martynov *et al.*, 2019], and *Coryphellina rubrolineata* O'Donoghue, 1929 [Ekimova *et al.*, 2022b]. Some nudibranch species have been described from Vietnam, and several of them likely represent endemics, for example *Phestilla chaetopterana* (Ekimova, Deart et Schepetov, 2017) showing symbiotic relationships with marine tubeworm *Chaetopterus* [Ekimova *et al.*, 2019]. All this highlights an importance of further studies of local biodiversity in Vietnam coastal waters.

In this paper we describe a new species of the genus *Eubranchus* from Vietnamese coastal waters, based on the integrative approach combining morphological and molecular data. This new species is the first representative of the genus *Eubranchus* discovered in Vietnam.

Material and methods

Material

Two specimens with egg mass and the host hydrozoan colony were collected in 2017 at one site: Vietnam, Nha Trang, Hon Tre Island, Dam Bay, 12°11.688'N, 109°17.386'E by scuba diving at 3 m in depth. The holotype and paratype are deposited in the collection of Zoological Museum of Lomonosov Moscow State University, White Sea Branch (ZMMU WS).

Molecular methods

For molecular analysis we studied both sampled specimens and the egg-mass. Molecular methods included obtaining of the four molecular markers commonly used in *Eubranchus* systematics: cytochrome *c* oxidase subunit I, 16S rRNA and histone H3 [Cella *et al.*, 2016; Korshunova *et al.*, 2020c; Ekimova *et al.*, 2021; Grishina *et al.*, 2022]. DNA extraction, amplification, and sequencing followed methods described in Ekimova *et al.* [2019, 2020]. All newly obtained sequences were submitted to NCBI GenBank (Table S1). Raw reads for each gene were assembled and checked for ambiguities and low-quality data in Geneious R10 10.0.9 (Biomatters, Auckland, New Zealand). Edited sequences were verified for contamination using the BLAST-n algorithm run over the GenBank nr/nt database [Altschul *et al.*, 1990]. For phylogenetic reconstruction, datasets obtained in previous comprehensive study on the family Fionidae were used for the analysis [Cella *et al.*, 2016; Korshunova *et al.*, 2020c; Ekimova *et al.*, 2021; Grishina *et al.*, in press]. Indel-rich regions of the 16S alignment were identified and removed in Gblocks 0.91b [Talavera, Castresana, 2007] with the least stringent settings. Sequences were concatenated by a simple biopython script following Chaban *et al.* [2019]. Original data and publicly available sequences were aligned with the MUSCLE [Edgar, 2004] algorithm implemented in MEGA 7.0.26 [Kumar *et al.*, 2016]. Phylogenetic reconstruction was conducted for the concatenated multi-gene partitioned datasets. The Bayesian analysis was performed in MrBayes 3.2.6 [Ronquist, Huelsenbeck, 2003] applying evolutionary models for partitions separately. The best-fit nucleotide evolution models were tested in the MEGA 7.0.26 [Kumar *et al.*, 2016] based on the Bayesian Information Criterion (BIC) for each partition. Maximum likelihood-based phylogenetic inference was performed in RAxML v.8.2.12 [Stamatakis, 2014] with automatically estimated pseudoreplicates number defined by autoMRE algorithm [Pattengale *et al.*, 2010] under the GTRCAT model of nucleotide evolution, applied to partitions individually. Resulting phylogenetic tree graphs were rendered in FigTree 1.4.4 and then annotated in Adobe Illustrator CC 2014. According to Ekimova *et al.* [2021] the posterior probabilities from Bayesian Inferences (PP) higher than 0.99 and bootstrap support from the Maximum Likelihood (BS) higher than 90% were designated as "high"; PP from 0.95 to 0.98 and BS from 75 to 89% indicate moderate support; PP from 0.9 to 0.94 and BS from 60 to 74% indicate low support; branches that received lower support were interpreted as unsupported.

Morphological studies

One collected specimen was used for the examination of external morphology under a stereomicroscope. The internal morphology of this specimen was also examined, including the digestive and reproductive systems. The buccal mass of this specimen was submerged in 5% sodium hypochlorite water solution for 1-3 minutes. The radula and the jaws were rinsed in distilled water, air-dried, mounted on an aluminium stub, and sputter-coated with gold for

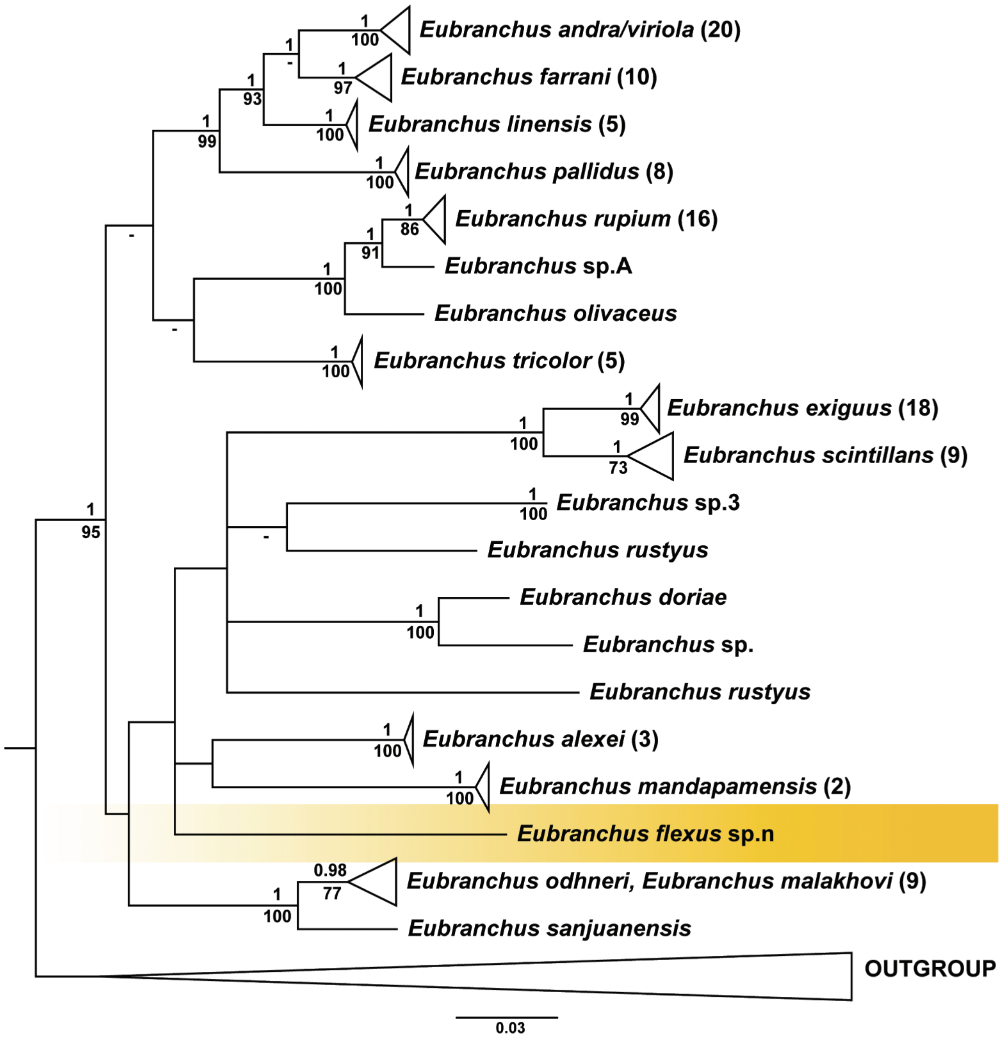

FIG. 1. Maximum likelihood phylogenetic tree of the genus *Eubranchus* based on the concatenated dataset COI+16S+H3. Numbers above branches indicate the posterior probabilities from Bayesian Inference, numbers below branches - bootstrap values from Maximum likelihood. The number of samples included in the analysis is indicated in brackets.

РИС. 1. Молекулярно-филогенетическое дерево для рода *Eubranchus,* построенное на основании комбинированного выравнивания (COI+16S+H3) методом максимального правдоподобия. Значения над ветвями обозначают апостериорные вероятности. Значения под ветвями обозначают поддержки бутстрепа. Число образцов, вошедших в анализ, указано в скобках.

visualization under a JEOL JSM 6380 scanning electron microscope (SEM). Features of the jaws were examined by optical stereomicroscopy and SEM. For study of the reproductive system the specimens was dissected from the dorsum along the midline and examined under a stereomicroscope.

Results

Phylogenetic analysis

The concatenated analyses of three markers (COI+16S+H3) recovered well-resolved and highly supported trees at the species level using both BI and ML (Fig. 1), while deep relationships were unsupported in most cases. The topology of BI and ML tree were similar in most cases. The new species represented a distinct separate branch from all other *Eubranchus* species. However, its relationship with other species was not resolved. The new species grouped with two clades: (1) *Eubranchus alexei* (Martynov, 1998) and the *E. mandapamensis* (K. P. Rao, 1968) clade (not supported in both analyses); (2) *E. exiguus* (Alder et Hancock, 1848); *E. scintillans* Grishina, Schepetov et Ekimova, in press; *Eubranchus* sp. 3*, E. doriae* (Trinchese, 1874); *Eubranchus* sp. and two separated *'E. rustyus'* specimens from GenBank; this clade also did not receive a high support.

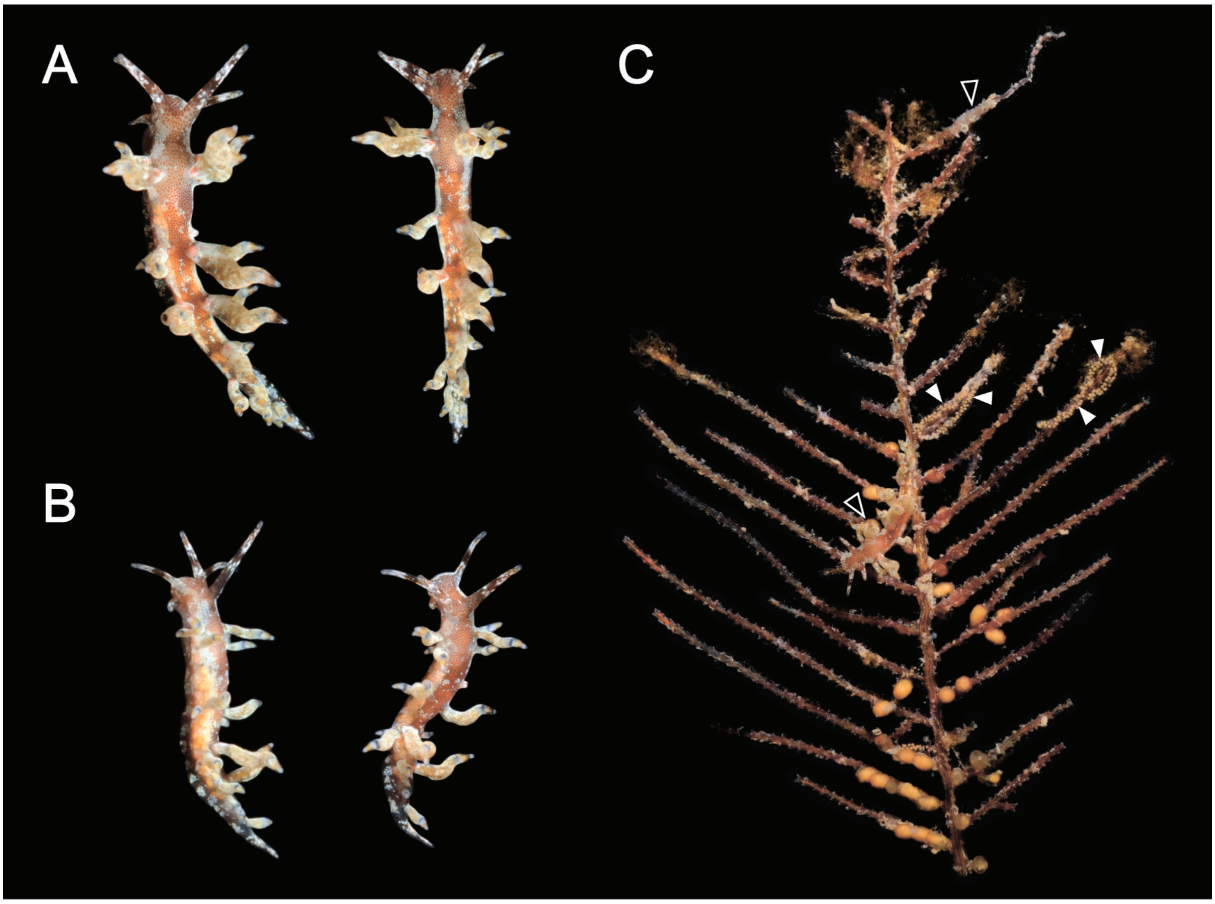

FIG. 2. Living specimens of *Eubranchus* ***flexus*** sp. nov. **A.** Holotype ZMMU WS19111, dorsal view. **B.** Paratype ZMMU WS19112, dorsal view, specimens was damaged during collection. Size of the fixed specimens is around 4 mm. **C.** *Eubranchus* ***flexus*** sp. nov. specimens with egg mass and the host hydrozoan colony; white arrows point to egg masses, black arrows with a white outline point to specimens.

РИС. 2. Прижизненные фотографии *Eubranchus* ***flexus*** sp. nov. **A.** Голотип ZMMU WS19111 вид с дорсальной стороны. **B.** Паратип ZMMU WS19112. Размер зафиксированных образцов составляет около 4 мм. **C.** Особи *Eubranchus* ***flexus*** sp. nov. на гидроиде; белые стрелки указывают на кладки яиц, черные стрелки с белым контуром указывают на особей моллюсков.

Taxonomic description

Order Nudibranchia de Blainville, 1814
Suborder Cladobranchia William et Morton, 1984
Superfamily Fionoidea Gray, 1857
Family Fionidae Gray, 1857
Genus *Eubranchus* Forbes, 1838

Eubranchus ***flexus*** sp. nov.
(Figs 2–4)

ZooBank registration: urn:lsid:zoobank.org:act:8F3D392E-EFC3-48C7-A04E-89D41C125453

Type material. Holotype ZMMU WS 19111, Vietnam, Nha Trang, Hon Tre Island, Dam Bay, 12°11.688’N. 109°17.386’E, 3 m in depths, 27.10.2016, coll. – T. Antokhina. One paratype (from the type locality): ZMMU WS 19112.

Type locality. Vietnam, Nha Trang, Hon Tre Island, Dam Bay, 3 m depth, 12°11.688’N, 109°17.386’E.

Description. *External morphology* (Fig. 2 A, B). Length 6–17 mm. Body elongate, narrow. Foot narrow, significantly wider anteriorly. Head large, bearing oral tentacles and rhinophores. Rhinophores elongated, smooth. Oral tentacles elongated, smooth. Rhinophores of same length as oral tentacles. Cerata arranged in 5–6 groups with 1–3 cerata per group. Cerata located opposite to each other. Cerata smooth, winging, curved, pointed distally. Reproductive opening located laterally under first ceratal row at right. Anal opening acleioproctic.

Coloration (Fig. 2 A, B). Background color translucent whitish. Rhinophores, oral tentacles, head, dorsal and dorsolateral sides of body, except areas between groups of cerata, covered with densely arranged reddish-brown small, rounded spots. Midline of body and areas with no brownish spots with small clusters of opaque white and yellowish pigment. Cerata bases transparent. Clusters of white and yellowish opaque spots of irregular shape located along distal parts of foot on each side. Cerata semitransparent, milky-white, covered with large semitransparent brownish spots of irregular shape (up to 2/3 of cerata length), and with ramdomly scattered opaque white and yellowish pigment. Cnidosac area with opaque white pigment on ceratal tip and subapical reddish-brown pigmental ring. Rhinophores and oral tentacles semitransparent, with intense opaque-white pigmental aggregations of irregular shape and yellowish apical ring and brownish ring in middle part.

Internal morphology (Figs 3, 4). Paired jaws elongate, thin, delicate, masticatory border of jaw with small denticles. Radula triserial, radular formula 58 × 1.1.1. Rachidian tooth with 3 denticles on each side. Central cusp separated from lateral denticles by small gap. Lateral teeth wide, 2–3 times wider than rachidian tooth, with rectangular base and triangular cusp placed close to rachidian tooth. Lateral teeth with small triangular denticle on outer side of cusp. Reproductive system diaulic. Receptaculum seminis spheric. Vagina wide. Ampulla elongated, narrow, tubular. Vas deferens short and wide. Penial gland present, elongated, with short duct.

Distribution. South China Sea. This species is known only from the type locality.

Ecology. (Fig. 2C). Specimens were found on hydrozoan colonies of the family Sertulariidae with their egg masses. The egg mass is deposited on hydrozoan as an irregular loop, beige in color.

Remarks. Both morphological and molecular analyses support the distinctiveness of *Eubranchus* ***flexus*** sp. nov. from other species in the genus (Fig. 1). Morphologically, there is no other described species of the genus *Eubranchus*, demonstrating similar coloration, external appearance and radular characters (see Discussion for details). *Eubranchus* ***flexus*** sp. nov. is a single representative of the genus *Eubranchus* that described from Vietnam.

Etymology. From Latin "*flexus*" (bending, winding, curvilinear), referring to specific shape of cerata in this species.

Discussion

The systematics of the genus *Eubranchus* remains poorly understood. Among 54 described species molecular data have been obtained only for 20 of them. Also, there is a vast undescribed biodiversity, especially in tropical waters [Bouchet, 2006; Gosliner *et al.*, 2018]. For example, in the last review on nudibranch biodiversity of the coral triangle [Gosliner *et al.*, 2018] only four described species were identified [*Eubranchus inabai* Baba, 1964*, E. ocellatus* (Alder et Hancock, 1864)*, E. mandapamensis* (K. P. Rao, 1968) and *E. virginalis* (Baba, 1949)] and 28 undescribed species were noted. The discovery of the first representative of the genus *Eubranchus* in the Vietnam coastal waters indicates that the rate of undescribed biodiversity in the Indo-West Pacific is even higher.

Undescribed biodiversity impedes ecological, taxonomic, and phylogenetic studies of the genus *Eubranchus.* For now, within Eubranchidae *sensu* Korshunova et al., 2017 four genera are regarded as valid: *Eubranchus, Amphorina, Leostyletus* and *Capellinia* [MolluscaBase, 2022], however *Leostyletus* lacks molecular data, and distinctiveness of *Amphorina* and *Capellinia* are not supported by some studies [Ekimova *et al.*, 2021; Grishina *et al.*, 2022]. At the same time, the evolutionary distances between different *Eubranchus sensu* Cella *et al.*, 2016 are very high in contrast to other Fionidae, which may indicate the presence of unnamed taxa of a higher rank within *Eubranchus.* Therefore, an integrative revision is needed for further studies of the genus, with a critical analysis of phylogenetically important characters. The coloration, radular and jaw morphology, and the reproductive system anatomy are traditionally used for identification and taxonomical studies of Nudibranchia. While some *Eubranchus* species may have a distinct coloration pattern (for example this is a good distinctive trait for *Eubranchus* ***flexus*** sp. nov.), some species show chromatic variation patterns [*e.g. Eubranchus farrani* (Alder et Hancock, 1844) species complex]. In the case of the reproductive system several attempts have been made to develop *Eubranchus* taxonomy based on the penial gland location, the presence of a distinct prostate, and the morphology of copulatory organs [Martynov, 1998]. At the same time, some of proposed genera (*Amphorina, Nudibranchus, Capellinia sensu* Martynov, 1998) are not recovered as monophyletic in molecular phylogenies [Cella *et al.*, 2016; Ekimova *et al.*, 2021; Grishina *et al.*, in press] and it is likely that some of these characters may have evolved independently in different clades, and are not useful as phylogenetically important traits. Another internal character is the morphology of the radular teeth. *Eubranchus* ***flexus*** sp. nov. has an extremely wide lateral teeth (their width exceeds more than twice the width of the medial tooth). This character is not common for all *Eubranchus* species, however, it is found in *Eubranchus conicla* (Marcus, 1958); *Eubranchus convenientis* Ortea et Caballer, 2002; *Eubranchus cucullus* Behrens, 1985; *Eubranchus glacialis* (Thiele, 1912); *Eubranchus leopoldoi* Caballer, Ortea et Espinosa, 2001; *Eubranchus mannarensis* K. P. Rao, 1968; *Eubranchus occidentalis* MacFarland, 1966; *Eubranchus odhneri* (Derjugin et Gurjanova, 1926); *Eubranchus prietoi* Llera et Ortea, 1981; *Eubranchus steinbecki* Behrens, 1987; *Eubranchus tanzanensis* Edmunds, 1969; *Eubranchus telesforoi* Ortea, Caballer et Bacallado, 2002; *Eubranchus toledanoi* Ortea et Caballer, 2002; *Eubranchus vascoi* Ortea, Caballer et Moro, 2002.

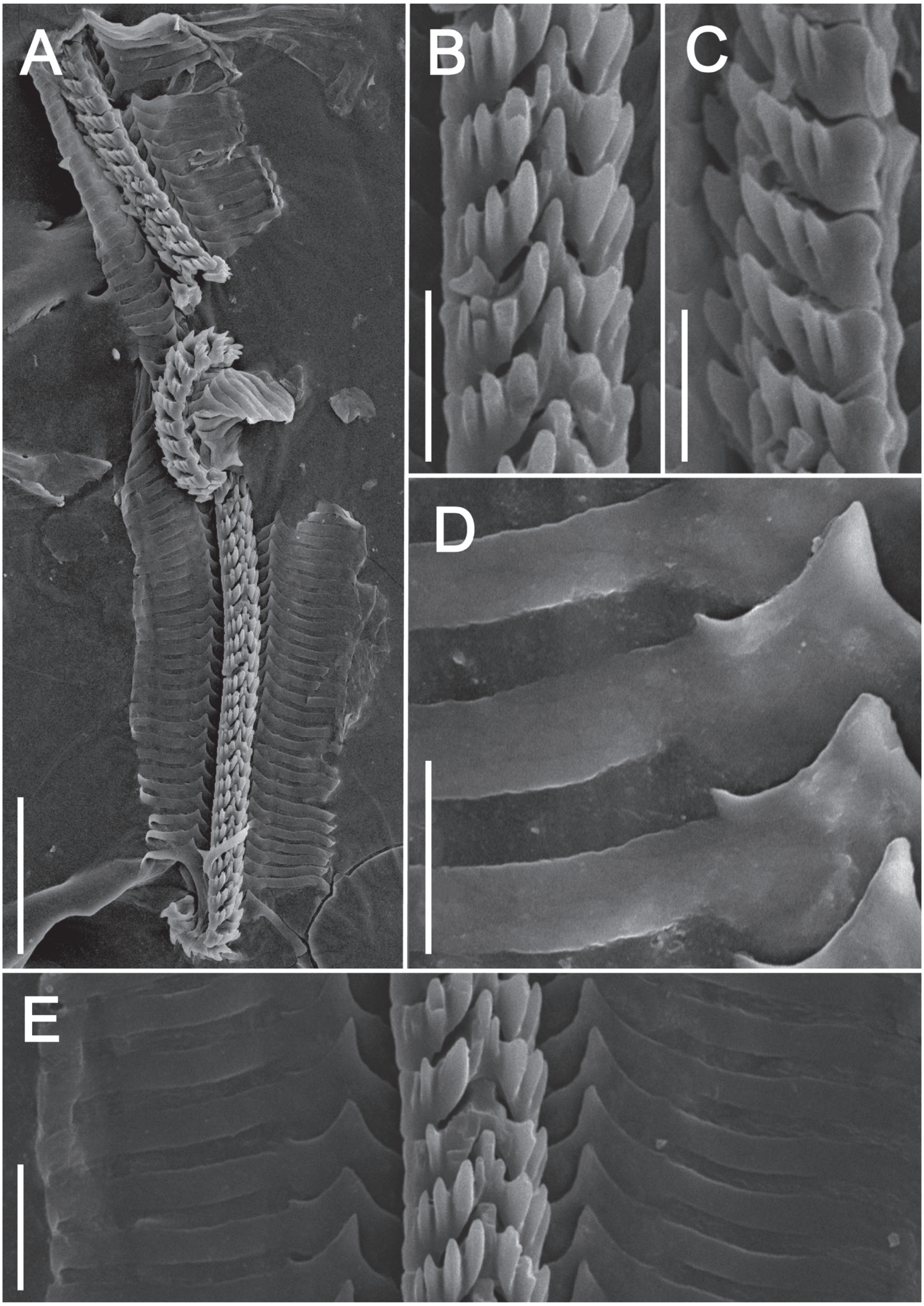

FIG. 3. Buccal armature in *Eubranchus* ***flexus*** sp. nov., paratype ZMMU WS19112. **A.** Radula. **B**. Rachidian teeth. **C**. Paratype ZMMU WS19112, rachidian teeth, side view. **D.** Denticles on lateral teeth. **E.** Rachidian and lateral teeth. Scale bars: A – 100 µm. B, C, E – 20 µm. D – 10 µm.

РИС. 3. Глоточное вооружение *Eubranchus* ***flexus*** sp. nov., паратип ZMMU WS19112. **A.** Радула. **B.** Центральный зуб. **C.** Центральный зуб, вид сбоку. **D.** Зубчики латеральных зубов. **E.** Центральные и латеральные зубы. Масштабные линейки: A – 100 µm. B, C, E – 20 µm. D – 10 µm.

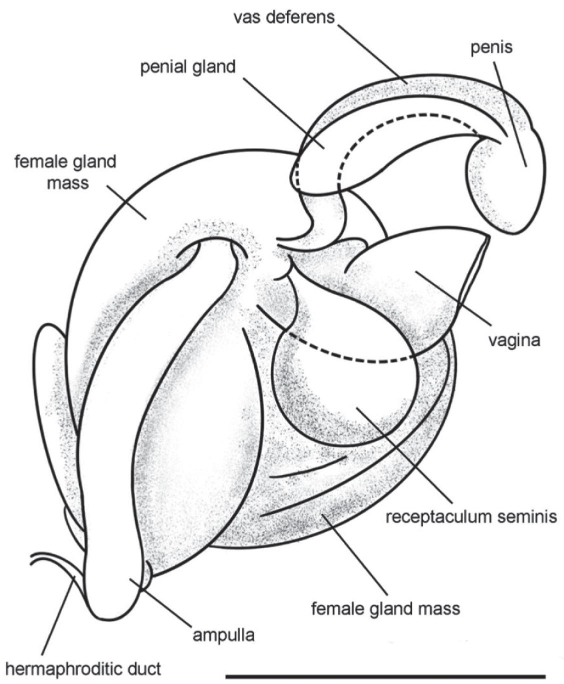

FIG. 4 The reproductive system of *Eubranchus* ***flexus*** sp. nov. (paratype ZMMU WS 19112). Scale bar: 500 μm.

РИС. 4. Половая система *Eubranchus* ***flexus*** sp. nov. (паратип ZMMU WS 19112). Масштабная линейка: 500 μm.

Among these species, molecular data are present only for *E. odhneri,* however the phylogenetic relationships between this species and *E.* ***flexus*** sp. nov. are not resolved, likely due to limited taxon sampling. Besides that, *E. malakhovi* and *E. odhneri* species are non-monopheletic. However, it can be explained by absence of 18S sequences in our concatenated dataset (in contrast to the analysis made by [Ekimova et al., 2021]). It was previously suggested that the radular morphology may have a phylogenetic signal within *Eubranchus* [Mikhlina *et al.*, 2020]. Testing this hypothesis should be a goal for further studies.

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SUPPLEMENTARY MATERIAL

Table S1. List of specimens used in this study. Voucher numbers, collection locality and GenBank accession numbers are given. Sequences obtained for this study are highlighted in bold.

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