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Hidden beauty of the north: a description of *Eubranchus scintillans* sp.n. (Gastropoda: Nudibranchia) from the Barents Sea and North-East Atlantic

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ABSTRACT: This paper represents results of an integrative analysis of specimens identified as *Eubranchus exiguus* (Alder et Hancock, 1848), a common nudibranch species inhabiting hydroid colonies in Northeast Atlantic marine communities. Our study included a molecular analysis, based on mitochondrial COI and 16S rRNA and nuclear histone H3 and 18S rRNA molecular markers. Phylogenetic relationships among species of *Eubranchus* Forbes, 1838 were estimated by phylogenetic reconstruction using two algorithms — Bayesian inference and maximum likelihood approaches. The phylogenetic hypotheses were accompanied by species delimitation methods: Assemble Species by Automatic Partitioning (ASAP), Bayesian implementation of the Poisson Tree Processes (bPTP) and General Mixed Yule Coalescent (GMYC). We used COI data to reconstruct a haplotype network for population structure analysis. Morphological defining features were analyzed with a light microscopy and scanning electron microscopy (SEM). Colouration and external appearance of specimens were studied with photographs. Results of the integrative approach indicated the existence of a pseudocryptic new species described herein under the name *Eubranchus scintillans* sp.n. This new species was found sympatrically with *E. exiguus* in all studied regions (the Barents and the North Seas). Also, we provide a comprehensive comparison of the new species with *E. exiguus*, as well as *E. pallidus* (Alder et Hancock, 1842), *E. doriae* (Trinchese, 1874), *E. rupium* (Møller, 1842), *E. rustyus* (Er. Marcus, 1961) and *Capellinia fustifera* (Lovén, 1846).
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KEY WORDS: Arctic, pseudocryptic species, species delimitation, molecular phylogeny, integrative taxonomy, Mollusca.

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Скрытая красота севера: описание нового вида голожаберных моллюсков *Eubranchus scintillans* sp.n. (Gastropoda: Nudibranchia) из Баренцева моря и северо-восточной Атлантики

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РЕЗЮМЕ: В данной статье представлены результаты интегративного анализа предполагаемых особей вида *Eubranchus exiguus* (Alder et Hancock, 1848), который является обычным на колониях гидроидов в природных сообществах северо-восточной Атлантики. Исследование включало молекулярно-генетический анализ данных на основе митохондриальных (COI и16S рРНК) и ядерных (гистон H3 и 18S рРНК) молекулярных маркеров. Взаимоотношения внутри рода *Eubranchus* были установлены с помощью построения филогенетической реконструкции с использованием двух алгоритмов — анализ на основе Байесовской статистики и метод максимального правдоподобия. Филогенетические гипотезы были проверены с использованием таких методов разделения видов как ASAP (Assemble Species by Automatic Partitioning), bPTP (Bayesian implementation of the Poisson Tree Processes) и GMYC (General Mixed Yule Coalescent). Последовательности молекулярного маркера COI использовали также для построения сетей гаплотипов, которые позволяют оценить структуру популяций. Для исследования морфологии видов были использованы такие методы как световая микроскопия и электронная сканирующая микроскопия (СЭМ). Определение особенностей окраски особей и общие особенности внешнего строения проводили по прижизненным фотографиям особей. Результаты интегративного анализа указывают на существование нового псевдокриптического вида, которые описан здесь под названием *Eubranchus scintillans* sp.n. Он был обнаружен симпатрически с видом *E. exiguus* во всех исследованных регионах (Баренцево и Северное моря). Здесь также приводятся сравнение нового вида с морфологически сходными видами *E. pallidus* (Alder et Hancock, 1842), *E. doriae* (Trinchese, 1874), *E. rupium* (Møller, 1842), *E. rustyus* (Er. Marcus, 1961) и *Capellinia fustifera* (Lovén, 1846).
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КЛЮЧЕВЫЕ СЛОВА: Арктика, псевдокриптические виды, разделение видов, молекулярная филогенетика, интегративная таксономия, Mollusca.

Introduction

Boreal and Arctic nudibranchs often represent complexes of cryptic or pseudocryptic species (Kienberger *et al.*, 2016; Ekimova *et al.*, 2022a). Cryptic species can be distinguished only by molecular data, and morphological differences cannot be detected (Bickford *et al.*, 2007; León de, Nadler, 2010). In pseudocryptic species, the morphological differences are not initially evident, but are found in more detailed morphological analyses. However, such species may still be difficult to distinguish (Vanelslander *et al.*, 2009; Kawauchi, Giribet, 2014; Lindsay, Valdés, 2016). Such cases are important for evolutionary theory, biogeography, and conservation measures (Beheregaray, Caccone, 2007; Bickford *et al.*, 2007; Pfenninger, Schwenk, 2007; Trontelj, Fiser, 2009).

The genus *Eubranchus* Forbes, 1838 includes 44 known species (MolluscaBase, 2022), however new species have been discovered at a significant rate. In a recent survey of Indo-Pacific nudibranch diversity, 28 potential new species were detected but have not been described yet (Gosliner *et al.*, 2015). The overall taxonomy of the genus *Eubranchus* remains poorly understood. Several attempts to revise this genus have been made in recent years. Cella *et al.* (2016) transferred the genus *Eubranchus* from a separate family (Eubranchidae) to the family Fionidae which was supported by presence of the common synapomorphies: acleioproctic anus, the shape of the head (circular with rounded extensions of the anterior part of the foot), and the penis usually with a penial gland. Later, an attempt was made to restore the original family Eubranchidae (Korshunova *et al.*, 2017) and to support the distinctness of the genus *Amphorina* Quatrefages, 1844, which was previously defined as valid within this family (Martynov, 1998; Korshunova *et al.*, 2020). Moreover, two new species of the latter genus were described (Korshunova *et al.*, 2020). However, some researchers highlighted poor phylogenetic resolution at the generic level and therefore did not support the validity of the genus *Amphorina* and the two newly described species because the genus is paraphyletic and new species do not form two monophyletic groups (Ekimova *et al.*, 2021). Poor taxon sampling and high levels of undescribed biodiversity within the genus *Eubranchus* make it more difficult to resolve the phylogenetic relationships and further studies of this group are required.

The nudibranch *Eubranchus exiguus* (Alder et Hancock, 1848) possesses a wide range in the Atlantic Ocean, including western parts of the Barents Sea (Martynov *et al.*, 2006), Scandinavia (Løyning, 1922; Løyning, 1927; Hadfield, 1963), the southern North Sea (Swennen, 1961), the British Isles (Alder, Hancock, 1845–1855, 1848; Garstang, 1890; Nichols, 1898; Colgan, 1914; Marine Biological Association, 1957; Miller, 1961), the English Channel and the Atlantic coast of France (Vayssière, 1913; Cornet, Marche-Marchad, 1951), and the Mediterranean Sea (Vayssière, 1913). This species is a common component of hydrozoan communities of the Barents Sea and Northeastern Atlantic (Lambert, 1991), as well as the species *Eubranchus rupium* (Møller, 1842), which has similar general appearance. Preliminary studies of *Eubranchus exiguus* in the Barents Sea indicated some variation of the external morphology of specimens. Based on experience with other boreal cladobranch species (Ekimova *et al.*, 2015, 2022a; Korshunova *et al.*, 2020; Sørensen *et al.*, 2020; Martinsson *et al.*, 2021) these differences may be interpreted either as intraspecific variation or as interspecific differences. Different researchers argued that it is necessary to be very careful with morphological analysis. This is because similar specimens may belong to different species (i.e. *Aeolidia filomenae* Kienberger *et al.*, 2016 and *Ae. papillosa* (Linnaeus, 1761) (Kienberger *et al.*, 2016); or *Dendronotus lacteus* (W. Thompson, 1840) and *D. europaeus* Korshunova *et al.*, 2017 (Korshunova *et al.*, 2017), however intraspecific variation also may be significant, i.e. *Coryphella verrucosa* (M. Sars, 1829) (see Ekimova *et al.*, 2022a) and *Felimida binza* (Ev. Marcus et Er. Marcus, 1963) (see Padula *et al.*, 2016)). If this is only intraspecific variation it is often influenced by various climatic and paleogeographical events, such as cycles of glaciation or the opening and closure of marine pathways (Clarke, Crame, 2010; Ekimova *et al.*, 2019; Laakkonen *et al.*, 2021). Such events promote allopatric speciation, due to the creation of physical barriers. But at the same time some species may have high morphological plasticity due to different ecological pressures on distant populations, thus

representing significant morphological differences. The main goal of our study was to investigate *Eubranchus exiguus* morphological variation using integrative approach, combining molecular and morphological data.

Material and methods

COLLECTION DATA. Sixteen specimens for this study were collected in the Barents Sea, Teriberka Bay in 2020. All samples were collected by SCUBA diving at depths of 4–20 m on rocky substrates. All specimens were photographed and fixed in 96% ethanol for morphological and molecular studies. This material is deposited in subsidiary of Zoological Museum of Lomonosov Moscow State University (ZMMU WS; DNA samples with Ter2020). Also, the COI sequences and digital photographs of 10 specimens of *Eubranchus exiguus* from the collections of the Zoological Museum, University of Bergen were provided by Dr. Manuel A. Malaquias (ZMBN vouchers). The new species was registered in ZooBank [lsid of the publication is: urn:lsid:zoobank.org:pub:54DC1363-2261-4DDF-A0CE-815F5CAF5FA9]. Details of sampling localities, depths and voucher numbers for each specimen are given in Table S1.

TAXON SAMPLING AND FOLLOWED TAXONOMICAL SCHEME. For phylogenetic reconstruction, datasets for the genus *Eubranchus* obtained in previous studies were incorporated in the analyses (Cella *et al.*, 2016; Korshunova *et al.*, 2020; Ekimova *et al.*, 2021), also some sequences from GenBank and BOLD were added to the dataset (Table S2; for sequences with ZMBN vouchers numbers from BOLD are provided). Representatives of the families Dendronotidae, Flabellinidae, Janolidae, Tritoniidae and Onchidoriddidae were used as outgroups. In our work we use the system suggested by Cella *et al.* (2016) and Ekimova *et al.* (2021): the genus *Eubranchus* belongs to the family Fionidae, *Amphorina* is a subjective synonym of *Eubranchus*.

DNA EXTRACTION, AMPLIFICATION, SEQUENCING. DNA was extracted from a small piece of soft tissue (approx. 1 mm^3) using a PALL™ AcroPrep 96-well purification plates by PALL Corp., following a protocol by Ivanova *et al.* (2006). The DNA samples were used as a template for amplification of partial cytochrome c oxidase subunit I (COI) (estimated length ~658 bp), 16S rRNA (~450 bp), histone H3 (~350 bp). Additionally, to confirm identity of putative species, the partial 18S rRNA was amplified. Due to the low quality of 18S sequences by a pair of primers 1F/5R and 18 Sa 2.0/9R, only 18S fragments sequences obtained with 3F/18Sbi primers were included in the analysis (~950 bp). Amplification programs and primers used for amplification and sequencing are shown in Table 1. Polymerase chain reaction were carried out in a 25-μL reaction volume (5 μL of 5× HS Taq Red buffer by Eurogen Lab, 0.5 μL of HS Taq polymerase by Eurogen Lab, 0,5 μL of dNTP (50 μM stock), 0.3 μL of each primer (10 μM stock), 1 μL of genomic DNA and 17.4 μL of sterile water). Both strands of each amplicon were sequenced with the BigDye Terminator v3.1 sequencing kit by Applied Biosystems and NovaDye Terminator sequencing kit by GeneQest. Sequencing reactions were analyzed by capillary electrophoresis on ABI 3500 Genetic Analyser (Thermo Fisher, USA) or "Nanophore-05" (Syntol, Russia) at the N.K. Koltsov Institute of Developmental Biology Core Centrum (Moscow, Russia). All new sequences were deposited to GenBank public database (Table S2). Raw reads for each gene were assembled and checked for improper base-calling (those sites were further modified) using Geneious-Pro 10.0.9 (Biomatters, Auckland, New Zealand).

DATA PROCESSING AND PHYLOGENETIC ANALYSES. Original data and publicly available sequences were aligned with the MUSCLE (Edgar, 2004) algorithm implemented in MEGA 7.0.26 (Kumar *et al.*, 2016). Protein-coding sequences were translated into amino acids to eliminate potential pseudogene reads by verifying CDS reading frame integrity. No pseudogenes were detected. The resulting alignments were of 597 bp for COI, 416 bp for 16S, 327 bp for H3 and 948 bp for 18S. 16S rRNA has a spatial structure and is characterized by the presence of highly polymorphic regions. These sequences were processed in the GBlocks program for indel-rich region (Dereeper *et al.*, 2008) and the resulting alignment was trimmed to 409 bp for 16S. Phylogenetic analysis was conducted for all datasets concatenated and for the COI and 18S genes individually. Sequences were concatenated by a Biopython script (Chaban *et al.*, 2019). The best fitting nucleotide evolution model was tested in the MEGA 7.0.26 (Kumar *et al.*, 2016) toolkit based on the Bayesian information criterion (BIC) for each partition. The best-fitting model for 16S and COI partitions was GTR + G + I, and for the both H3 and 18S it was K2 + I. Phylogenetic reconstructions were performed by Bayesian estimation of posterior probability and Maximum likelihood phylogenetic inference. The Bayesian analysis was performed in MrBayes 3.2.6 (Ronquist, Huelsenbeck, 2003) applying evolutionary models for partitions separately. Maximum likelihood-based phylogeny 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 individu-

Table 1. Amplification and sequencing primers and PCR conditions.
Таблица 1. Праймеры для реакций амплификации и секвенирования и условия ПЦР.

Marker	Primers	PCR conditions	References
Cytochrome c oxidase subunit I	**LCO1490** GGTCAACAAATCATAAAGATATTGG **HCO2198** TAAACTTCAGGGTGACCAAAAAATCA	5 min — 95 °C, 35x [15 s — 95 °C, 30 s — 45 °C, 1 min — 72 °C], 7 min — 72 °C	Folmer *et al.*, 1994
16S rRNA	**16Sar–L** CGCCTGTTTATCAAAAACAT **16S BRh** CCGGTCTGAACTCAGATCACGT	5 min — 95 °C, 35x [15 s — 95 °C, 30 s — 52 °C, 1 min — 72 °C], 7 min — 72 °C	Palumbi *et al.*, 1991
Histone H3	**H3AF** ATGGCTCGTACCAAGCAGACVGC **H3AR** ATATCCTTRGGCATRATRGTGAC	5 min — 95 °C, 35x [15 s — 95 °C, 30 s — 50 °C, 1 min — 72 °C], 7 min — 72 °C	Colgan *et al.*, 1998
18S rRNA	**1F** TACCTGGTTGATCCTGCCAGTAG **5R** CTTGGCAAATGCTTTCGC **3F** GTTCGATTCCGGAGAGGGA **18Sbi** GAGTCTCGTTCGTTATCGGA **18Sa2.0** ATGGTTGCAAAGCTGAAAC **9R** GATCCTTCCGCAGGTTCACCTAC	5 min — 95 °C, 35x [30 s — 95 °C, 30 s — 50 °C, 1 min — 72 °C], 7 min — 72 °C	Giribet *et al.*, 1996; Whiting *et al.*, 1997

ally. 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.

SPECIES DELIMITATION ANALYSES. The COI alignment was used for computational species delimitations methods. P-distances were calculated using MEGA 7.0.26 software package (Kumar *et al.*, 2016). To confirm the status of clades recovered in our phylogenetic reconstruction as putative species we used the Assemble Species by Automatic Partitioning (ASAP) method (Puillandre *et al.*, 2021) to detect breaks in the distribution of intra- and interspecific distances without any prior species hypothesis, referred to as the "barcode gap" (Hebert *et al.*, 2003). The ASAP analysis was run on the online version of the program (https://bioinfo. mnhn.fr/abi/public/asap/asapweb.html) with three proposed models — Jukes-Cantor (JC69), Kimura (K80) and Simple distance. It was complemented by Bayesian implementation of the Poisson Tree Processes (bPTP) (Zhang *et al.*, 2013). The test was run using the bPTP Server http://species.h-its.org/ptp/ with settings set as default with COI-based Maximum likelihood tree as an input. Additionally, we performed a GMYC test (Pons *et al.*, 2006) as implemented by Fujisawa & Barraclough (2013). The COI-based ultrametric tree was calculated using BEAST 2.6.6 (Bouckaert *et al.*, 2019) with 10^7 generations and then analyzed in the R environment (package *splits*), following instructions by Fujisawa & Barraclough (2013). Finally, to confirm distinct status of putative species, recovered in delimitation analyses based on mtDNA, the 18S alignment was inspected for phylogenetically important species-specific substitutions

HAPLOTYPE NETWORKS. Population study was based on the COI sequences, including original

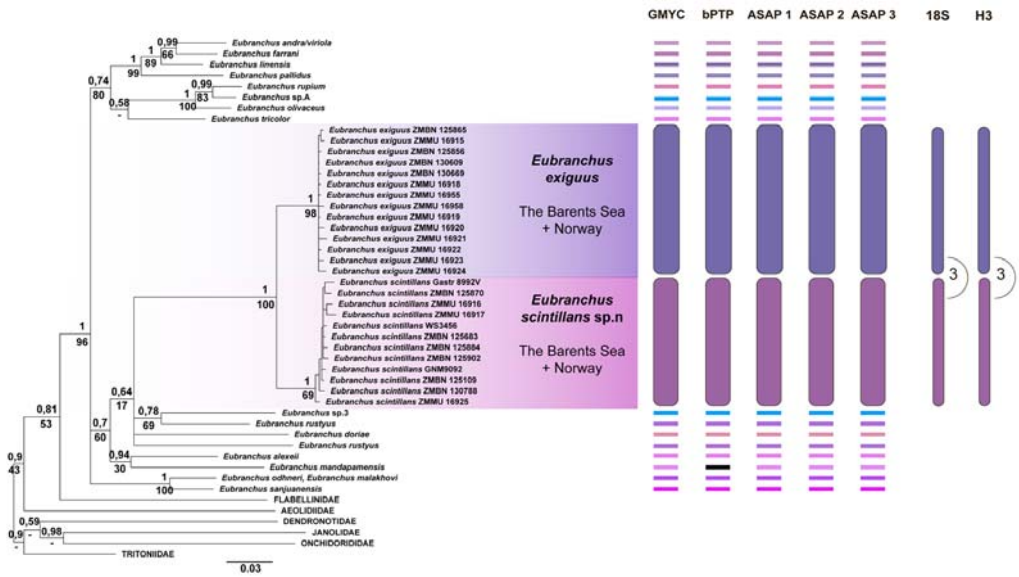

Fig. 1. Molecular phylogenetic reconstruction of the genus *Eubranchus* based on the concatenated dataset (COI + 16S + H3), Bayesian inference, combined with species delimitation. Species-level clades and outgroups are collapsed to a single branch, except the target species. *Eubranchus andra* and *E. viriola* do not form two separated monophyletic units, as *E. malakhovi* and *E. odhneri* and are shown as members of a single clade. Numbers above branches indicate posterior probabilities (PP) from Bayesian Inference, numbers below branches — bootstrap support from Maximum Likelihood (BS). Designations: ASAP 1 — Assemble Species by Automatic Partitioning with Jukes-Cantor model, score = 2.5; ASAP 2 — Assemble Species by Automatic Partitioning with Kimura-2P model, score = 2.5; ASAP 2 — Assemble Species by Automatic Partitioning with somple-destances model, score = 3.0; bPTP — Bayesian implementation of the Poisson Tree Processes; GMYC — General Mixed Yule Coalescent; 18S and H3 — number of substitutions in 18S and H3 molecular markers. Black squares mark unsupported clades.

Рис. 1. Реконструкция молекулярно–филогенетических отношений рода *Eubranchus*, построенная по последовательностям трех молекулярных маркеров (COI + 16S + H3) с применением Байесовского анализа, совмещенная с результатами делимитационного анализа. Клады видового уровня сколлапсированы до одной особи на вид. *Eubranchus andra* и *Eubranchus viriola* не образуют двух монофилетичных групп, как и *E. malakhovi* and *E. odhneri,* поэтому они показаны в составе единой клады. Цифры над ветвями обозначают апостериорные вероятности (PP) Байесовского анализа, цифры под ветвями — бутстреп–поддержки (BS) метода Максимального правдоподобия. Обозначения: ASAP 1 — выделение видов автоматическим разделением (ASAP) с использованием модели Джукса-Кантора, score = 2.5; ASAP 2 — выделение видов автоматическим разделением (ASAP) с использованием 2-параметрической модели Кимуры, score = 2.5; ASAP 3 — выделение видов автоматическим разделением (ASAP) с использованием модели простых попарных дистанций, score = 3.0; bPTP — Байесовская имплементация пуассоновского процессирования дерева; GMYC — генерализированный смешанный анализ коалесцентной модели Юля; 18S и H3 — число замен в нуклеотидных последовательностях молекулярных маркерах 18S и H3 между видами. Черными прямоугольниками отмечены не поддержанные клады.

sequences and those retrieved from the GenBank (Table S2). Haplotype networks were constructed using PopART software (Leigh, Bryant, 2015) with the TCS network method (Clement *et al.*, 2002). The output networks were annotated in Adobe Illustrator CC 2014.

MORPHOLOGICAL STUDIES. The external morphology was inspected under a stereomicroscope Olympus SZ51. For the internal morphology (jaws, radula and reproductive system) seven specimens (total for both species) were studied to test possible intraspecific variation. The buccal mass of each specimen was extracted in proteinase K solution for 2 hours at 60 °C to dissolve soft tissues and then processed in 5% sodium hypochlorite solution in water for 1–3 minutes. The radula and the jaws

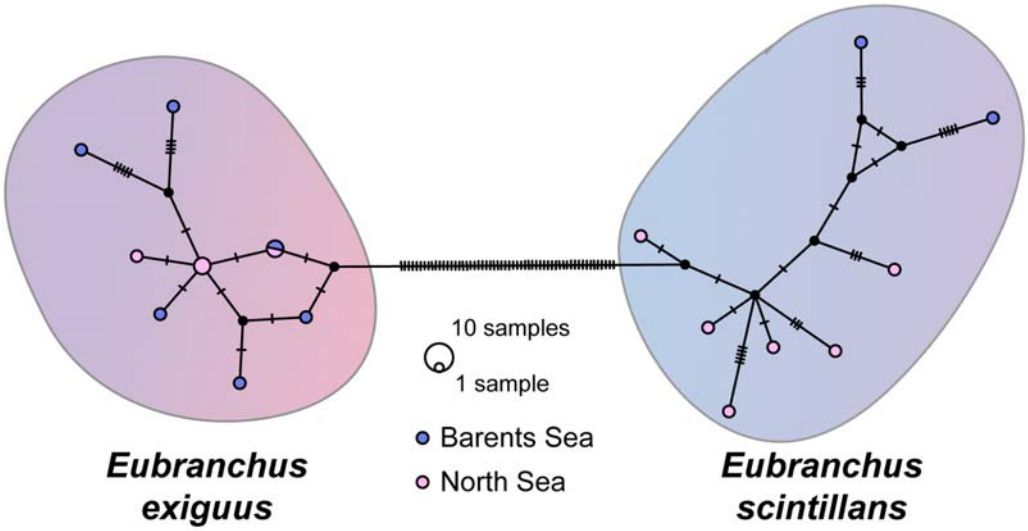

Fig. 2. COI haplotype network produced with TCS method in PopART. Colour of circles refers to the geographic origin of each haplotype. The relative size of circles is proportional to the number of sequences of that same haplotype

Рис. 2. Сеть гаплотипов, построенная по маркеру COI с помощью метода TCS в пакете программы PopART. Цвет кругов обозначает принадлежность гаплотипа к определенной географической области. Относительный размер кругов пропорционален количеству нуклеотидных последовательностей, соответствующих одному гаплотипу.

were rinsed in distilled water, air-dried, mounted on an aluminum stub, and sputter-coated with gold. Features of the jaws of each specimen were analyzed by optical microscopy with Zeiss Axioplan 2 with digital camera AxioCam HRM, and by scanning electron microscopy (SEM) with TESCAN MIRA3 LMH and JEOL JSM 6380 scanning electron microscopes. Radulae were examined and photographed using SEM, with same microscopes, and the reproductive systems were examined under a stereomicroscope.

Results

MOLECULAR PHYLOGENETIC ANALYSIS. The concatenated tree (16S, COI and H3) provided good resolution for most clades (Fig. 1), while trees based on the single-gene analyses were poorly resolved (Data S1). The topology of the concatenated trees generated with Bayesian inference (BI) and maximum likelihood (ML) analyses were congruent in most cases, except basal relationships within the genus *Eubranchus*. The genus *Eubranchus* was recovered as monophyletic with high statistical support (PP = 1; BS = 96). *Eubranchus exiguus* form two distinctive clades with high statistical support by Bayesian inference (PP = 1 and PP = 1), however the maximum likelihood tree shows low support for one of them (BS = 98 and BS = 69). The basal relationships within the genus were poorly resolved. *E. andra* Korshunova *et al.*, 2020 and *E. viriola* Korshunova *et al.*, 2020 do not form two monophyletic groups, they are mixed in the same clade. *E. malakhovi* is paraphyletic and nest in *E. odhneri.* They form a monophyletic group together.

SPECIES DELIMITATION. Two monophyletic groups recovered in the phylogenetic analysis were identified as candidate species in the species delimitation analyses (Fig. 1). Minimum interspecific distance was 13.94% and maximum intraspecific distances were 1.99% for *Eubranchus exiguus* and 2.43% for and *Eubranchus scintillans* sp.n. The ASAP analysis include species delimitation with three different nucleotide evolution's models — Kimura 2-parameter, Jukes-Cantor and simple-distances. The lowest ASAP score (3,0) was showed with K2P model for 49 groups for entire alignment. All ASAP analyses showed the validity of candidate species. The bPTP and the GMYC analyses recovered same groups as putative candidate species. Also, the 18S and H3 datasets show difference of two candidate species by three substitutions (positions 367, 567 and 596; 30, 99 and 258, accordingly). Thus, we suggest that specimens represent a new distinct species, and its formal taxonomical description is provided below. Species delimitations analy-

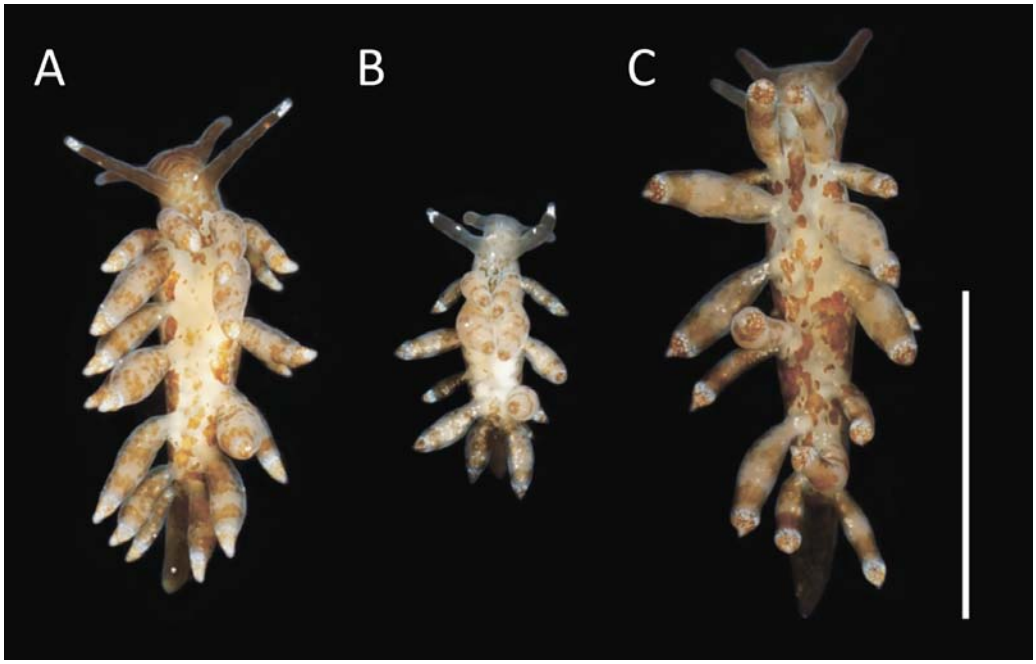

Fig. 3. Living specimens of *Eubranchus exiguus*. A — ZMMU WS 16915; B — ZMMU WS 16958; C — ZMMU WS 16923. Photo credits: Tatiana Antokhina. Scale bars: 5 mm.
Рис. 3. *Eubranchus exiguus*, прижизненные фотографии. A — ZMMU WS 16915; B — ZMMU WS 16958; C — ZMMU WS 16923. Фото: Татьяна Антохина. Масштаб: 5 мм.

sis also do not confirm *E. andra* and *E. viriola* as valid species. Same result we get for *E. malakhovi* is paraphyletic and *E. odhneri.*

POPULATION STRUCTURE. In the COI based TCS haplotype network (Fig. 2), studied specimens formed two haplogroups separated by 56 substitutions. Both species are sympatric and syntopic in the Barents and the North Seas.

Systematics

Order Nudibranchia
Suborder Cladobranchia
Superfamily Fionoidea
Family Fionidae *sensu* Cella *et al*., 2016
Eubranchus exiguus Alder et Hancock, 1848
Figs 3, 4, 5A, 5B, 6A.

Eolis exigua Alder, Hancock, 1848: 192
Aeolis exigua Meyer, Moebius, 1865: 35
Nudibranchus exiguus Martynov, 1998: 765

TYPE MATERIAL: 8 syntypes, BMNH 1858.5. 28.173 and few syntypes at the Hancock Museum (Martynov, 2006).

TYPE LOCALITY. Fowey Harbour and Falmouth, Cornwall, UK (Alder, Hancock, 1848).

MATERIAL EXAMINED: 4 specimens (ZMMU WS 16915, ZMMU WS 16918, ZMMU WS 16955, ZMMU WS 16958), Barents Sea, Teriberka Bay, 69°11′03.2″N 35°09′41.9″E, 4–8 m in depths, coll. T. Antokhina; 3 specimens (ZMMU WS 16919, ZMMU WS 16920, ZMMU WS 16921), Barents Sea, Teriberka Bay, 69°11′15.8″N 35°08′ 10.8″E, 5–16 m in depths, 16.08.2020, coll. T. Antokhina; 3 specimens (ZMMU WS 16922, ZMMU WS 16923, ZMMU WS 16924), Barents Sea, Teriberka Bay, 69°11′24.4″N 35°08′13.3″E, 5–18 m in depths, 16.08.2020, coll. T. Antokhina; 1 specimen (ZMBN 125856), Haugesund, Legern, Rogaland, North Sea, Norway, 59°30′41.0″N 5°14′32.0″E, 7 m in depths, 5.07.2018, coll. Haugesund team 2018, molecular data and photos were studied; 1 specimen (ZMBN 130609), Mandal, Guleskjær, Vest-Agder, North Sea, Norway, 57°59′54.0″N 7°26′41.0″E, 23. 05.2019, coll. Mandal Team 2019, molecular data and photos were studied; 1 specimen (ZMBN 130669), Mandal, Rennespynten, Vest-Agder, North Sea, Norway, 57°35′35.2″N 7°20′02.4″E, 10 m in depths, 25.05.2019, coll. Mandal Team 2019, molecular data and photos were studied; 1 specimen (ZMBN 125865), Haugesund, Rovaer Flatholmen, Rogaland, North Sea, Norway, 59°38′39.0″N 5°24′

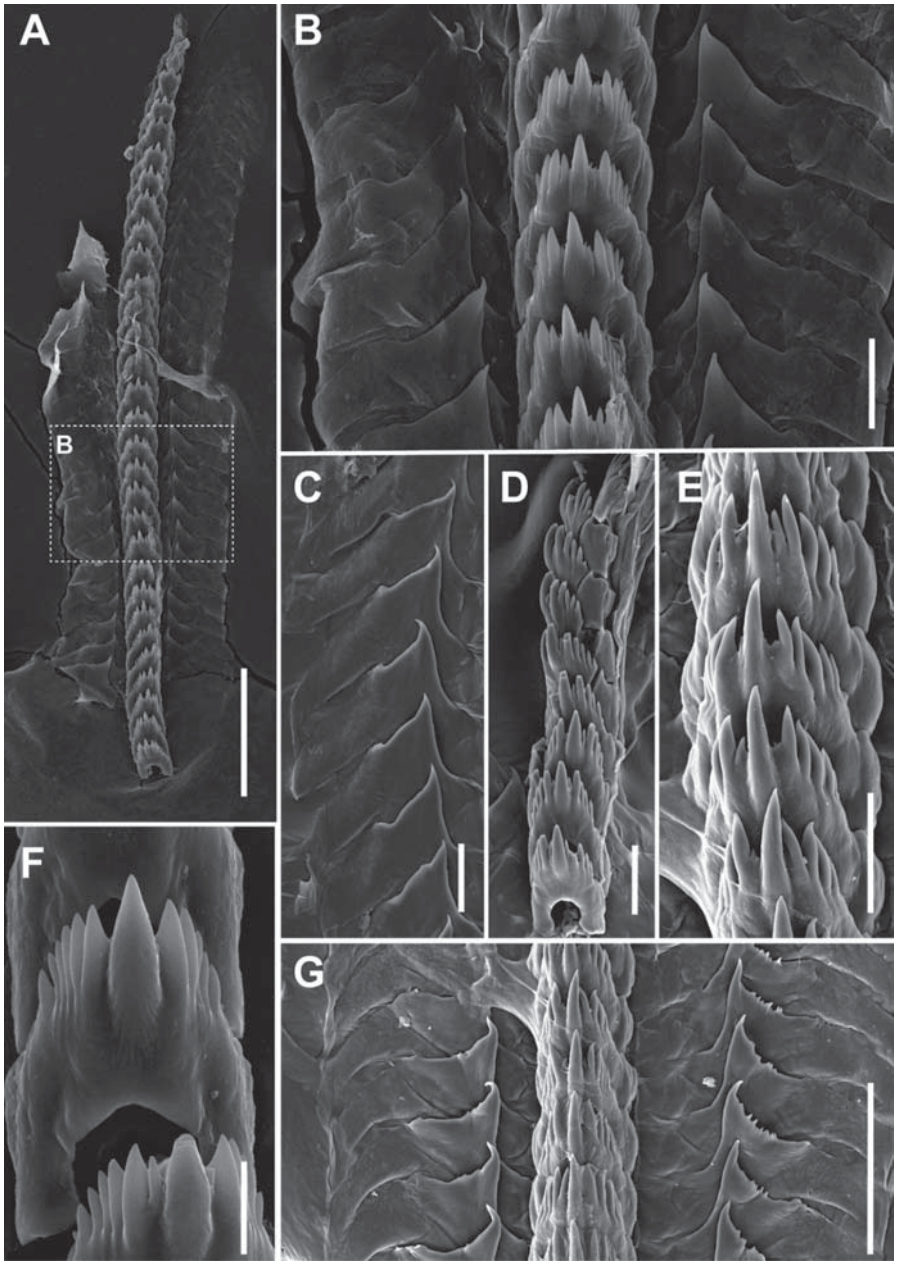

Fig. 4. Buccal armature in *Eubranchus exiguus*, SEM. A — ZMMU WS 16922, radula; B — ZMMU WS 16922 rachidian and lateral teeth, middle radular portion; C — ZMMU WS 16955, lateral teeth; D — ZMMU WS 16915, rachidian teeth, top and side view; E — ZMMU WS 16915, rachidian teeth; F — ZMMU WS 16955, lateral teeth; G — ZMMU WS 16915, rachidian teeth, top and side view. Scale bars: A — 100 μm; E — 50 μm; B, C, D, E — 20 μm; F — 10 μm.

Рис. 4. Радулярный аппарат *Eubranchus exiguus*, СЭМ. A — ZMMU WS 16922, радула; B — ZMMU WS 16922, центральные и латеральные зубы, средняя часть радулы; C — ZMMU WS 16955, латеральные зубы; D — ZMMU WS 16915, центральные зубы, вид сверху и сбоку; E — ZMMU WS 16915, центральные зубы; ZMMU WS 16955, латеральные зубы; G — ZMMU WS 16915, центральные зубы, вид сверху и сбоку. Масштаб: A — 100 μm; E — 50 μm; B, C, D, E — 20 μm; F — 10 μm.

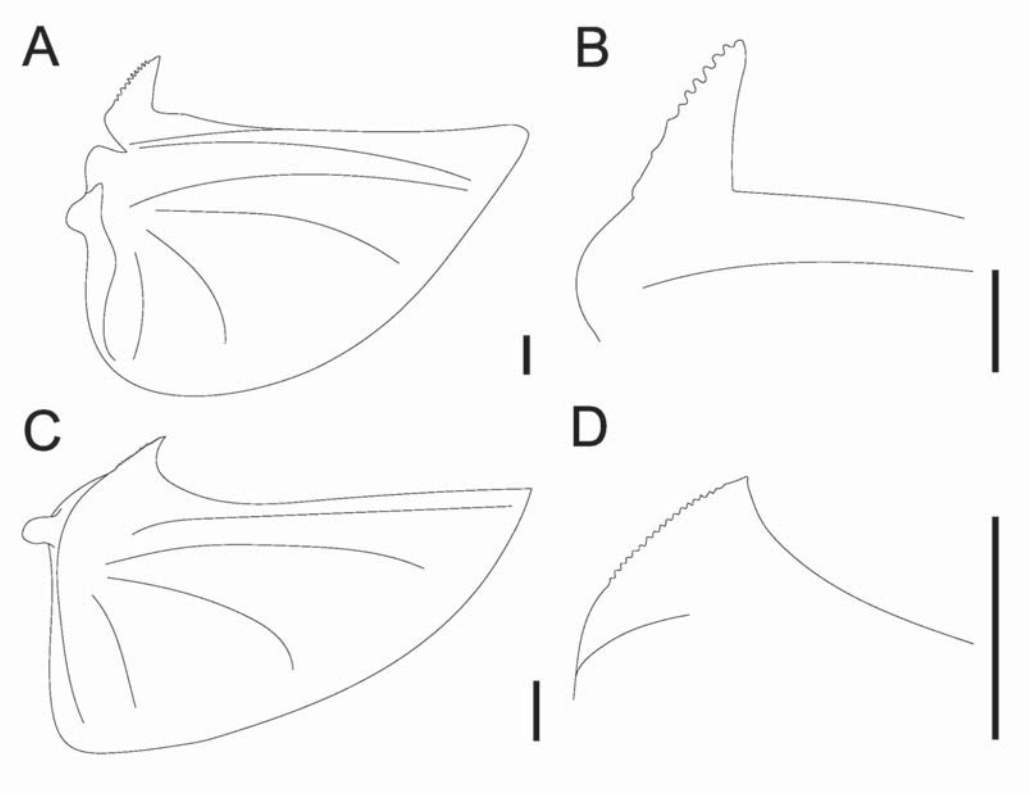

Fig. 5. Details of buccal armature, schematic drawings. A — *Eubranchus scintillans* sp.n., right jaw plate; B — *Eubranchus scintillans* sp.n., details of masticatory border denticulation; C — *Eubranchus exiguus*, right jaw plate; D — *Eubranchus exiguus*, details of masticatory border denticulation; Scale bars: A, B, C, D — 50 μm.

Рис. 5. Схематические изображения челюстей. A — *Eubranchus scintillans* sp.n., правая челюстная пластинка; B — *Eubranchus scintillans* sp.n., детали зазубренности жевательного отростка; C — *Eubranchus exiguus*, правая челюстная пластинка; D — *Eubranchus exiguus*, детали зазубренности жевательного отростка. Масштаб: A, B, C, D — 50 μm.

12.0″E, 8 m in depths, 6.07.2018, coll. Haugesund team 2018, molecular data and photos were studied.

EXTERNAL MORPHOLOGY. Length up to 7 mm (Fig. 3). Body elongate, narrow. Cerata arranged in 5–6 groups with 1–2 cerata per group. Cerata finger-shaped, pointed at top, some bearing small thickening in upper part. Largest ceras commonly in middle body part. Rhinophores elongated, smooth. Oral tentacles elongated, smooth. Rhinophores two times longer than oral tentacles. Reproductive opening located laterally under first ceratal row at right. Anal opening acleioproctic

COLOURATION. Background colour whitish or yellowish, semitransparent (Fig. 3). Dorsal side of body covered by numerous spots of irregular shape. Their colour varies from pale yellowish brown to dark brown. Head, tentacles, rhinophores are covered by spots. They may located very densely along dorsum. Cerata with distinct ring formed by densely packed brown patches, commonly two or three, but may be hardly distinguished (Fig. 7). Several specimens have small white opaque pigmentation on dorsum. White opaque bands locate on rhinophores tips, form rings under cnidosac areas.

ANATOMY. Triserial radula, radular formula 30–38 × 1.1.1 (Fig. 4). Rachidian tooth with numerous denticles. Central cusp separated from lateral denticles by 1–2 smaller denticles or a small gap. Lateral denticles number vary from 4 to 6. Lateral teeth wide, with rectangular base, triangular cusp. In most specimens smooth, but rarely bearing small denticles on outer edge of cusp. In single studied specimen lateral teeth on right side bear many denticles (Fig. 4G). Paired jaws triangle-shaped, thin, delicate, masticatory border of jaw with small distinct denticles (Figs 5A, B, S1A, B).

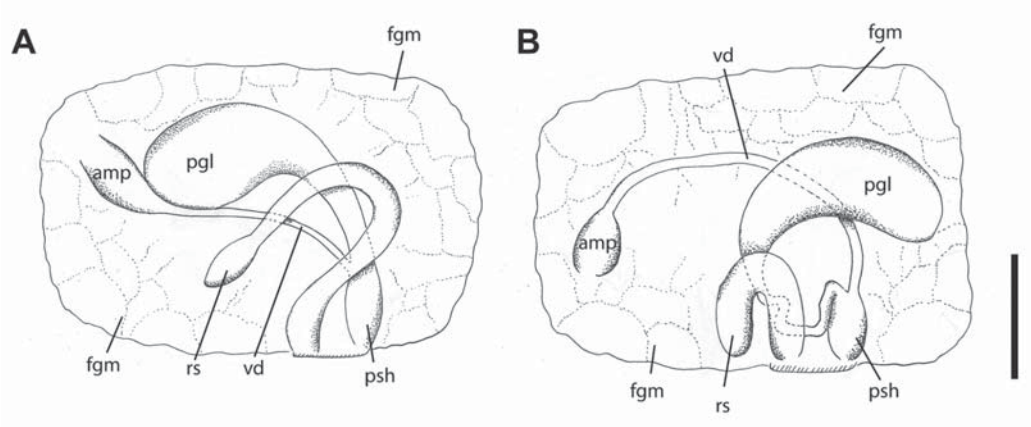

Fig. 6. Details of reproductive system anatomy, schematic drawings. A — *Eubranchus exiguus*; B — *Eubranchus scintillans* sp.n. Abbreviation: amp — ampulla; fgm — female gland mass; pgl —penial gland; psh — penial sheath; rs — receptaculum seminis; vd — vas deferens. Scale bars: 500 μm.
Рис. 6. Схематические изображения половых систем. A — *Eubranchus exiguus*; B — *Eubranchus scintillans* sp.n. Обозначения: amp — ампулла; fgm — комплекс женских желез; pgl — пениальная железа; psh — карман пениса; rs — семяприемник; vd — семяпровод. Масштаб: 500 μm.

Reproductive system diaulic (Fig. 6A). Ampulla small, elongated. Vas deferens thin, not curved. Penis is hook-shaped, pointed at end (Fig. S2A, B). Penial gland present, its duct wide, short, almost immediately entering penis sheath. Receptaculum seminis bent (~90°) in middle part, expanded to top. Vagina wide. Its wide base connects to the receptaculum seminis.

GEOGRAPHIC RANGE. Barents Sea (this study; Martynov *et al.*, 2006), Norway and North Sea (this study; Løyning, 1922; Løyning, 1927; Hadfield, 1963; Swennen, 1961; Martynov, 1998), British Isles (Alder, Hancock, 1848; Alder, Hancock, 1845–1855; Garstang, 1890; Nichols, 1898; Colgan, 1914; Marine Biological Association, 1957; Miller, 1961), English Channel and Atlantic coast of France (Vayssière, 1913; Cornet, Marche-Marchad, 1951), Mediterranean Sea (Vayssière, 1913).

BIOLOGY. This species was reported to feed on the hydrozoan species *Laomedea flexuosa* Alder, 1857, *Obelia geniculata* (Linnaeus, 1758), *Bougainvillia muscus* (Allman, 1863), *Abietinaria abietina* (Linnaeus, 1758), *Halecium halecinum* (Linnaeus, 1758), *Hydrallmania falcata* (Linnaeus, 1758) (Todd, 1981). It feeds by penetrating the hydrothecae with thinnest perisarc coverings, then it suctions soft tissues using buccal mass. However, the precise mechanism has not been clarified (Lambert, 1991).

Egg mass is a semicircle, up to 3.5 mm in length, it contains around 150 eggs. Reproduction period persists throughout all seasons (Løyning, 1922; Swennen, 1961).

REMARKS. *Eubranchus exiguus* is a well-defined species, based on both molecular and morphological data. Our specimens perfectly match the original description of *E. exiguus* (Alder, Hancock, 1848) and subsequent monograph (Alder, Hancock, 1845–1855): two pigmented rings on each ceras, distinctive spots form on dorsal part of the body, simple digestive gland diverticula in cerata. It differs from its sister new species by the body colouration, the structure of the digestive gland diverticula and minor differences in reproductive system and structures of jaws (for details see Remarks section in *Eubranchus scintillans* sp.n. description).

Eubranchus scintillans **sp.n.**
urn:lsid:zoobank.org:act:2E57412D-E58A-4179-8706-3C9EEC85AE2C
Figs 5C, 5D, 6B, 7, 8.

TYPE MATERIAL: Holotype ZMBN 125683, Eigersund, Egersund, Egersund havn, Rogaland, North Sea, Norway, 58°26′57.4″N 5°59′26.9″E, 5 m in depths, 20.04.2017, coll. Erling Svensen. Paratypes: ZMBN 125870, Haugesund, Lauvnesvika, Rogaland, North Sea, Norway, 59°36′59.4″N 5°31′09.8″E, 14 m in depths, 7.07.2018, coll. Haugesund team 2018; ZMBN 130788, Frogn, Digerud, Digerud Brygge, Akershus, North Sea, Norway, 60°22′07.3″N 5°10′28.3″E, 20 m in depths, 5.04. 2019, coll. Bjørnar Nygård, molecular data and photos were studied; 2 specimens (ZMMU WS 16916, ZMMU WS 16917), Barents Sea, Teriberka Bay, 69°11′03.2″N 35°09′41.9″E, 4–8 m in depths, 15.08.2020, coll. T. Antokhina; 1 sample (ZMMU

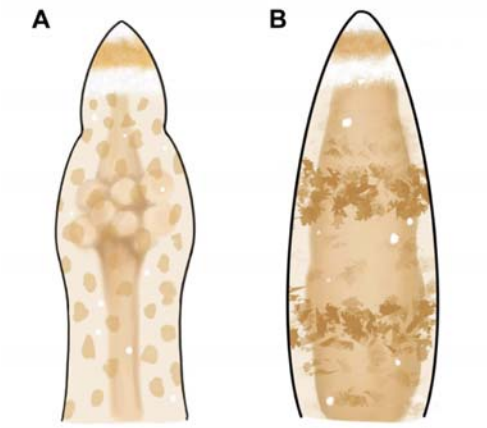

Fig. 7. Scheme of the color pattern of cerata. A — *Eubranchus scintillans* sp.n.; B — *E. exiguus*.
Рис. 7. Схема окраски церат. A — *Eubranchus scintillans* sp.n.; B — *E. exiguus*.

WS 16925), Barents Sea, Teriberka Bay, 69°11′ 01.4″N 35°09′32.8″E, 10–16 m in depths, 20.08. 2020, coll. T. Antokhina.

TYPE LOCALITY. Eigersund, Egersund, Egersund havn, Rogaland, North Sea, Norway, 58°26′ 57.4″N 5°59′26.9″E, 5 m in depths.

ADDITIONAL MATERIAL EXAMINED: 1 sample (ZMBN 125109), Drotningsvik, Bergen, Hordaland, North Sea, Norway, 59°43′30.7″N 10°35′17.3″E, 12.05.2018, coll. Drøbak team 2018, molecular data and photos were studied; 1 sample (ZMBN 125884), Haugesund, Tonjer, Tonjer lighthouse, Rogaland, North Sea, Norway, 59°33′40.0"N 5°20′04.0"E, 5 m in depths, 8.07.2018, coll. Haugesund team 2018, molecular data and photos were studied; 1 sample (ZMBN 125902), Haugesund, Trollholmen, Rogaland, North Sea, Norway, 59°24′ 40.8″N 5°15′05.1″E, 5 m in depths, 9.07.2018, coll. Haugesund team 2018, molecular data and photos were studied.

ETYMOLOGY. From the Latin *scintillans* (= sparkling) referring to the peculiar colouration of the cerata, which look like a small jar of glitter and the overall colouration resembling a flame of sparklers.

EXTERNAL MORPHOLOGY. Length up to 7 mm (Fig. 8). Body elongate, narrow. Head rounded. Cerata arranged in 5–6 groups with 1–3 cerata per group. Digestive gland diverticula with single knobby bulge in upper part. Cerata expanded in upper third part in same areas as digestive gland bulging. Rhinophores elongated, smooth. Oral tentacles elongated, smooth. Rhinophores two times longer than oral tentacles. Reproductive opening located laterally under first ceratal row at right. Anal opening acleioproctic.

COLOURATION. Background colour transparent yellowish. Dorsal body part with irregular spots of reddish-brown colour (Fig. 8). Lateral body part with irregular spots of reddish-brown colour. Lateral cerata surfaces with circular spots. Their colour semitransparent from light to dark brown. Rhinophores and oral tentacles semitransparent brown or yellowish, always with dark brown pigment ring in upper part. White opaque spots on rhinophores tips. White opaque spots form rings under cnidosac areas (Figs 7, 8).

ANATOMY. Triserial radula, radular formula 16–19 × 1.1.1 (Fig. 9). Rachidian tooth with numerous denticles. Lateral denticles number vary from 3 to 6. Central denticle usually is the same size of lateral denticles. Lateral teeth smooth, two times wider than rachidian tooth. Paired jaws triangle-shaped, thin, delicate, masticatory border of jaw with small denticles (Figs 5C, D, S1C, D).

Reproductive system diaulic (Fig. 6B). Ampulla small, spherical. Vas deferens thin, not curved. The penis tube-shaped, rounded at end (Fig. S2C, D). Penial gland present, its duct long, forming several convolutions. Receptaculum seminis elongated tube-form, bend in half. Vagina wide, connecting to receptaculum seminis at its wide base.

GEOGRAPHIC RANGE. Barents Sea, North-East Atlantic (North Sea).

BIOLOGY. Ecological features of the new species have not been studied and require further research. Specimens were found on hydroids colonies, at the same place with *Eubranchus exiguus*, but species identification for hydroids was not conducted. They occur at depths from 4 to 20 m.

REMARKS. This species differs from its closest relative *Eubranchus exiguus* by its body colouration, cerata morphology and features of the reproductive system. The key difference is the absence of organized pigment rings on the cerata: the spots of *E. scintillans* sp.n. are located either in a uniform layer throughout the cerata or scattered randomly (Fig. 7). Another important feature of the new species is the digestive gland diverticula morphology: *E. scintillans* sp.n. has a knobby bulge on the digestive gland diverticula, and this leads to an extension at the top of each ceras. In *E. exiguus* the digestive gland is simple and the cerata are finger-shaped. One more difference is the structure of jaws. *E. exiguus* has more elongate jaw plates than *E. scintillans* sp.n. Also, they differ by structure of masticatory boarder (Fig. 5). *E. scintillans* sp.n. has well separate from jaw plate masticatory boarder with narrow base and bears large individual teeth, while masticatory boarder of *E. exiguus* has wide base, attached to the jaw by it and bears small, almost indistinctive teeth. Several

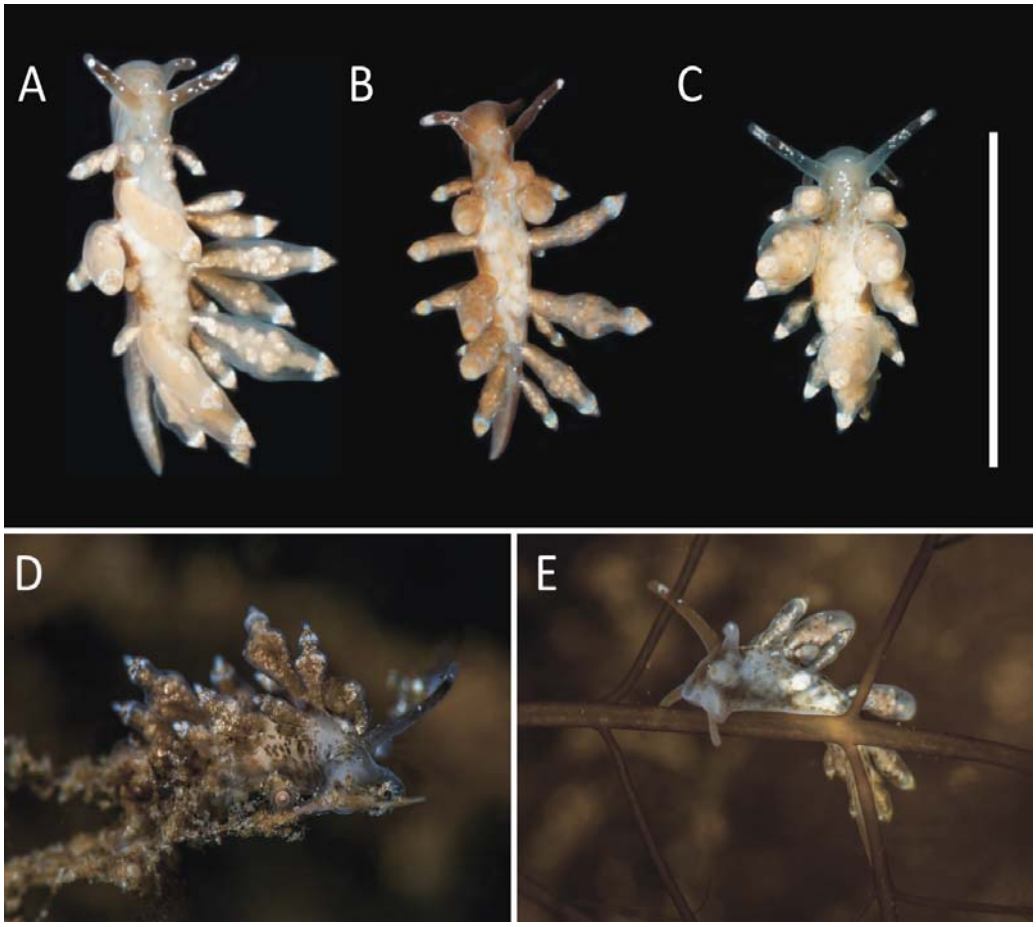

Fig. 8. Living specimens of *Eubranchus scintillans* sp.n. A — ZMMU WS 16916; B — ZMMU WS 16917; C — ZMMU WS 16925; D — living specimen in natural environment on unidentified hydroid colony; E — living specimen in natural environment on unidentified hydroid colony. Photo credits: A–C — Tatiana Antokhina; D–E — Manuel Malaquias. Scale bars: 5 mm.
Рис. 8. *Eubranchus scintillans* sp.n., прижизненные фотографии. A — ZMMU WS 16916; B — ZMMU WS 16917_261; C — ZMMU WS 16925; D — живой представитель в естественной среде обитания на колонии гидроидов; E — живая особь в естественной среде обитания на колонии гидроидов; фото: A–C — Татьяна Антохоина; D–E — Мануэль Малакиас. Масштаб: 5 мм.

minor differences may be found in reproductive system structure. These two species differ by receptaculum seminis form and the shape of the penial gland duct (Fig. 6). *E. scintillans* sp.n. has an extended penial gland duct, that forms several loops, whereas the *E. exiguus* penial gland duct is wide and short. The receptaculum seminis of *E. scintillans* sp.n. shorter than that of *E. exiguus* and bent at an 180° angle. The radula does not have any species-specific distinctive traits, and it is very similar to that of *E. exiguus* (Figs 4, 9). *E. exiguus* has small space between median denticle and lateral denticles in rachidian teeth, usually with small denticles within it. *E. scintillans* sp.n. also may have small gaps with denticles as in *E. exiguus,* but not in every specimen. Several other *Eubranchus* species show similarities to *E. scintillans* sp.n. The general appearance, colouration and digestive gland structure of the new species is similar to that in *E. pallidus* (Alder et Hancock, 1842), *E. doriae* (Trinchese, 1874) and *Capellinia fustifera* (Lovén, 1846). *Eubranchus pallidus* has significantly more swollen cerata, pigmented spots on the dorsal part of the body and the surface of the cerata are smaller and brighter, with a reddish

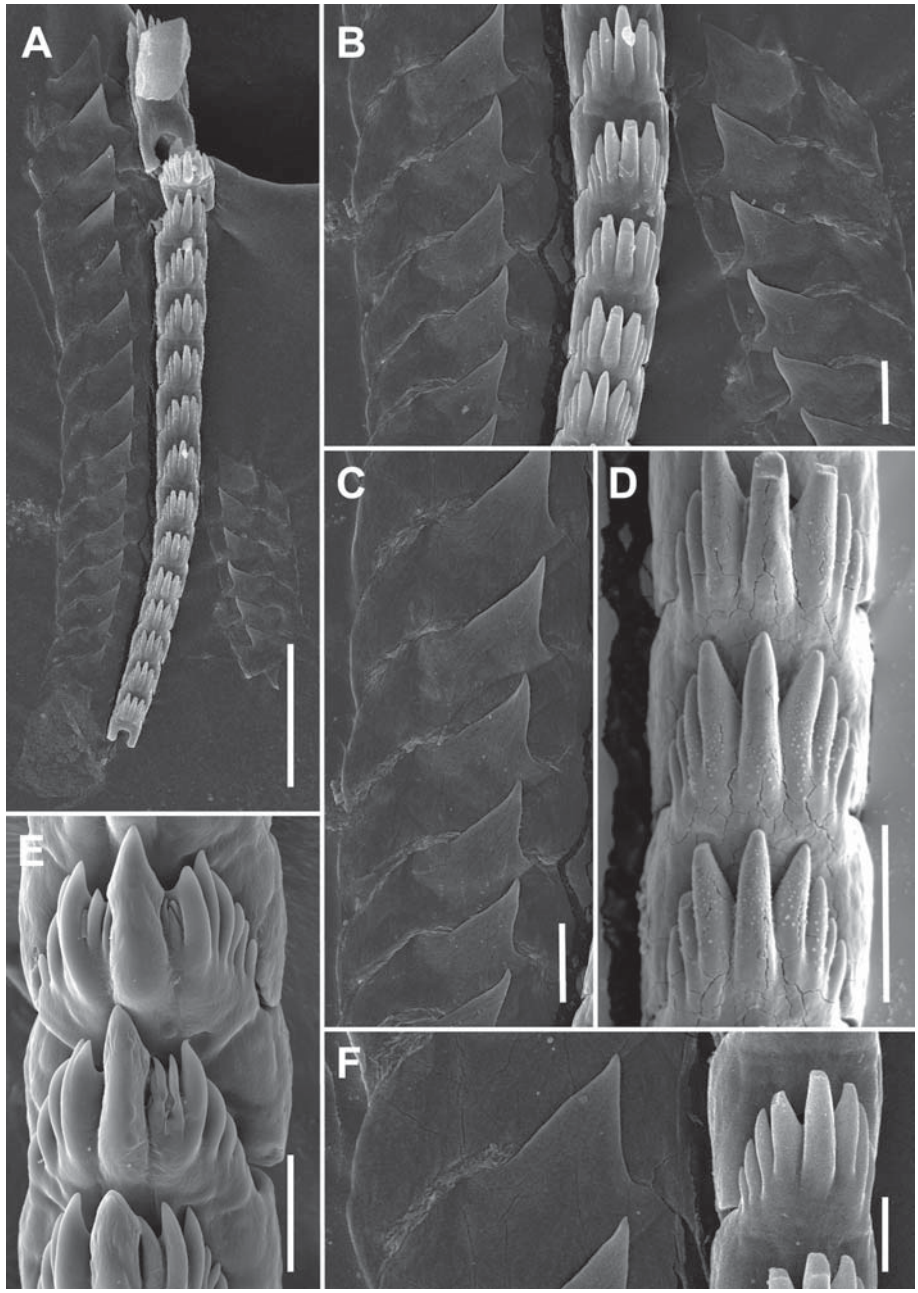

Fig. 9. Buccal armature in *Eubranchus scintillans* sp.n., SEM. A — ZMMU WS 16925, radula; B — ZMMU WS 16925, rachidian and lateral teeth, middle radular portion; C — ZMMU WS 16925, lateral teeth; D — ZMMU WS 16925, rachidian teeth; E — ZMMU WS 16916, rachidian teeth; F — ZMMU WS 16925, rachidian and lateral teeth, upper radular portion. Scale bars: A — 50 μm; B, C, D, E, F — 10 μm.
Рис. 9. Радулярный аппарат *Eubranchus scintillans* sp.n., СЭМ. A — ZMMU WS 16925, радула; B — ZMMU WS 16925, центральные и латеральные зубы, средняя часть радулы; C — ZMMU WS 16925, латеральные зубы; D — ZMMU WS 16925, центральные зубы; E — ZMMU WS 16925, центральные зубы; F — ZMMU WS 16925, центральные и латеральные зубы, верхняя часть радулы. Масштаб: A — 50 μm; B, C, D, E, F — 10 μm.

colour; the digestive gland diverticula in *E. pallidus* do not form any nodular local bulges. Also, there are some differences in the structure of reproductive system. *E. pallidus* has prostatic vas deferens (Edmunds, Kress, 1969). The differences with the species *Capellinia fustifera* and *Eubranchus doriae* are also in the cerata structure. These two species have two bulges in the digestive gland diverticula in the cerata, and there are additional radial extensions from each bulge. Another species that is similar to *E. scintillans* sp.n. in colouration is *E. rupium*, but it differs from the new species both in features of the external morphology and details of the internal structure. The most apparent external differences were found in the colour pattern of the two species. *E. rupium* has smaller spots of reddish colour, and the digestive gland diverticula in the cerata do not form any nodular bulges. The internal structure differences include both in the features of the structure of the reproductive system and the structure of the radular apparatus. In *E. rupium*, the central denticle of the rachidian tooth is recessed relative to the lateral teeth (Martynov *et al.*, 2006). The reproductive systems differ by the shape of penial gland, ampulla, receptaculum seminis and places where vas deference merge with penial sheath (Edmunds, Kress, 1969). *E. scintillans* sp.n. has bean-shaped penial gland, rounded ampulla, bended in half receptaculum seminis and its vas deference merge with penial sheath in its basal part. *E. rupium* has tubular straight penial gland, bean-shaped ampulla and receptaculum seminis and its vas deference merge with penial sheath in its apical part. Also, for *E. rupium* the presence of a penial stylet is documented (Martynov, 1999; Martynov *et al.*, 2006). The main differences between discussed species assembled in the Table S3.

Discussion

Our results show that *Eubranchus exiguus* and *E. scintillans* sp.n. are two distinct species. The new species *E. scintillans* sp.n. occurs sympatrically with *E. exiguus* in both studied regions: the Barents and the North seas. They are well distinguishable morphologically, however, *E. scintillans* sp.n. apparently has been considered as a colour form of *E. exiguus* for a long time. In example, in the paper of Cella *et al.* (2016) sequences appearing under the name *E. exiguus* are in fact *E. scintillans* sp.n. All methods of analysis consider *E. scintillans* sp.n. as the valid species. In the molecular analysis, the new species received low statistical support in our ML phylogenetic reconstruction, however all species delimitation and BI are congruent with our species hypothesis. Two nuclear molecular markers were studied — H3 and 18S rRNA. For H3 we have a small sample size (5 specimens of *E. exiguus* and 2 — of *E. scintillans* sp.n.), but three phylogenetically significant substitutions were found. In nudibranchs molluscs the nuclear histone H3 may be very conservative and identical in closely related species (Gonzalez *et al.*, 2013; Pola *et al.*, 2012; Ekimova *et al.*, 2015), however, sometimes in other nudibranch groups it can vary greatly, like in the genus *Coryphellina* (Ekimova, 2022a, b). Differences in H3 clearly indicates absence of possible interbreeding between the two species. Also, stable differences in 18S across sympatric specimens confirm this fact. The ML bootstrap support appears too low because of missing data for Norwegian specimens in the concatenated dataset, for which only COI is available.

The discovery of *Eubranchus scintillans* sp.n. indicates that the North Atlantic nudibranch biodiversity in general and the genus *Eubranchus* in particular requires a further study. This case represents another example of pseudocryptic speciation, in which well-studied species (presumably) can obscure some undescribed diversity. Also, our phylogenetic reconstruction (Fig. 1) shows no statistical support for basal clades of the genus *Eubranchus*, while individual species form monophyletic groups with high statistical support. We have only two exceptions: (1) *E. andra* and *E. viriola* and (2) *E. malakhovi* and *E. odhneri*. In the first case there are not any differences in molecular markers sequences between them (Data S1). As a result, they are mixed in the same clade in all phylogenetic reconstructions, including the paper with their initial description (Korshunova *et al.*, 2020; Ekimova *et al.*, 2021). In the second case, non-monophyly of *E. malakhovi* and *E. odhneri* may be caused by absence of 18S sequences in our concatenated dataset (in contrast to the analysis made by Ekimova *et al.*, 2021). Also, the genetic distance between species is small (2.48–3.38%), because of short period of time since their divergence event (~1.5 Mya) (Ekimova *et al.*, 2021). This situation is not unique. Some cases of amphiboreal species complex also face with species delimitation problems, for example, *Coryphella gracilis* (Alder et Hancock, 1844) and *C. amabilis* (Hirano et Kuzirian, 1991) (Ekimova *et al.*,

2022a). Each of them requires an individual approach.

The failure to recover the basal relationships is due to the small number of available sequences for different species from this genus (only for 19 from 53 valid species different sequences are available) and significant numbers of yet undescribed species (for example, Gosliner *et al.*, 2018). Further research is needed to solve phylogenetic relationships within the genus, and a revision of the genus *Eubranchus* is needed.

Compliance with ethical standards

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

Supplementary data. The following materials are available online.

Table S1. Collection data for novel specimens used in this study.

Table S2. Specimens from GenBank and sequences obtained for this study used for molecular analyses. Voucher numbers, collection localities and GenBank accession numbers are given. Sequences obtained for this study are highlighted in bold.

Table S3. Comparative characters of *Eubranchus scintillans* sp.n, *E. exiguus*, *E. rupium*, *E. pallidus*, *E. doriae* and *Capellinia fustifera*.

Data S1. Unedited Maximum likelihood phylogenetic trees based on a single marker (COI, 16S, H3, 18S) and unedited Maximum likelihood and Bayesian inference phylogenetic trees based on concatenated dataset (COI, 16S, H3).

Data S2. Unedited results of species delimitation methods (ASAP, bPTP and GMYC).

Figure S1. Details of buccal armature, light microscopy. A — *Eubranchus exiguus*, ZMMU WS 16918, right jaw plate; B — *Eubranchus exiguus*, ZMMU WS 16918, details of masticatory border denticulation; C — *Eubranchus scintillans* sp.n., ZMMU WS 16916, right jaw plate; D — *Eubranchus scintillans* sp.n., ZMMU WS 16916, details of masticatory border denticulation.

Scale bars: C — 200 μm, A, D — 100 μm; B — 50 μm.

Figure S2. Copulatory apparatus, light microscopy. A — *Eubranchus exiguus*, ZMMU WS 16915; B — *Eubranchus exiguus*, ZMMU WS 16915, apical part; C — *Eubranchus scintillans* sp.n., ZMMU WS 16916; D — *Eubranchus scintillans* sp.n., ZMMU WS 16916, apical part.

Scale bars: C — 200 μm, A — 100 μm; B, D — 50 μm.

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