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Molecular and morpho-anatomical assessment of the family Doridiidae (Mollusca, Nudibranchia) in the Mediterranean and North-East Atlantic

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Abstract. In the present study, a revision of the phylogeny and taxonomy of the family Doridiidae is carried out focusing on the genus *Doris* Linnaeus, 1758. The type species *D. verrucosa* Linnaeus, 1758 and a blueish and yellow morphotype of *D. ocelligera* collected in different localities in the Mediterranean Sea and the North-East Atlantic were sequenced, as well as *D. bertheloti* and the elusive *D. marmorata* for the first time. The genetic markers include the cytochrome *c* oxidase subunit I, 16S rRNA, and histone 3. The phylogenetic results suggest that the genus *Doris* is paraphyletic, and *D. ocelligera* morphotypes separate into two species, as confirmed with species delimitation tests. To complement the phylogenetic evidence with morphoanatomical data, the dissection of two specimens of each morphotype is conducted. Significant differences in morphological traits such as body shape, colouration patterns, and mantle tubercles come to light, together with anatomical differences in the relative shape and size of the radular teeth and reproductive structures. Considering the modern and old descriptions of *D. ocelligera*, it is finally concluded that the blueish morphotype belongs to *D. ocelligera*. In contrast, the yellow morphotype responds to the actual synonym *Aldisa berghi* (Vayssière, 1901), which is resurrected here as *Doris berghi* comb. rest. Considering the broad phylogeny of the family, some systematic notes at the genus level are here provided.

Keywords. *Doris ocelligera*, hidden speciation, sympatric species, restored combination, taxonomy and systematics.

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Introduction

Considering the current climate crisis, biodiversity and its preservation have become one of the most relevant and urgent fields of action. Molluscs constitute the second most diverse animal phylum and, within it, heterobranchs are one of the most species-rich groups of gastropod molluscs (Dinapoli & Klussmann-Kolb 2010). These embody a varied and charismatic group of marine, limnic, and terrestrial snails and slugs with a large amount of ecological and morphological adaptations to all environments (Moles & Giribet 2021). Marine heterobranchs, known traditionally as opisthobranchs, have a worldwide diversity of approximately 9000 species (WoRMS Editorial Board 2023) and are especially diverse in temperate and warm waters (Valentine & Jablonski 2015). Every year, systematic molecular studies are published and contribute to a better understanding of the relationship between heterobranchs and their diversity. One of the origins of this expansion in knowledge is cryptic speciation, which consists of the existence of two or more different species with apparent minimal morphological variation but with significant genetic differences. This together with the need to survey underexplored areas is the main reason why the existing biodiversity can be underestimated (Korshunova *et al.* 2019; Moles & Giribet 2021; Araujo *et al.* 2022). Many cases of cryptic speciation are pseudo-cryptic indeed, as distinct, discrete, morphological characters come to light once investigated thoroughly. This led to the discovery of hidden diversity and the resurrection of species.

The Mediterranean Sea is a biodiversity hot spot because of its biogeographical conditions (Bianchi & Morri 2000), but its biodiversity is still underestimated. Molluscs are no exception and nudibranchs, which are a group of marine gastropod molluscs characterized by their soft body and the lack of external shells, are especially underestimated (Garzia *et al.* 2021). Nudibranchs are extremely adaptive and diverse animals with a very specific diet and, therefore, could serve as bioindicators of coastal well-being (Kurnianda *et al.* 2020). A high alpha diversity indicates that the ecosystem is rich in terms of overall community biodiversity and thus relatively little disturbed. Even though it is a very diverse and studied group, there is much unknown biodiversity. In this context, the need for more taxonomic studies arises to discover new species, which are usually cryptic and difficult to identify underwater only through morphological characters.

The suborder Doridina Odhner, 1934 is one of the larger groups of nudibranchs in which cryptic speciation is frequent (Hallas *et al.* 2017). Morphological and molecular analyses have shown it to be monophyletic and currently consists of 19 families. This suborder is divided into five superfamilies according to morphological variation in gills and feeding structures (Wollscheid-Lengeling *et al.* 2001; Valdés 2002, 2004). Species that possess the ability to retract their gills and present a radula make up the superfamily Doridoidea Rafinesque, 1815 (Valdés 2002). Within it, the family Dorididae Rafinesque, 1815 is composed of seven accepted genera, one of them being *Doris* Linnaeus, 1758, which consists of 54 species and is distributed worldwide. The type species for this genus is *D. verrucosa* Linnaeus, 1758, inhabiting the Mediterranean Sea and the Atlantic Ocean. A species with a controversial taxonomic history is *Doris ocelligera* (Bergh, 1881). The original name of the species was *Staurodoris ocelligera*, first described by Bergh in 1881 (type locality: Trieste, Italy). Even so, it was afterwards transferred to the genus *Doris* when the genus *Staurodoris* Bergh, 1878 was suppressed by ICZN Opinion 1980 (ICZN 2001). A few years before, in 1826, Risso described *D. lutea*, and later, in 1901, Vayssière described *Aldisa berghi* (type locality: Gulf of Marseille, France) – although at present, both are considered synonyms of *D. ocelligera* (Schmekel & Portmann 1982). These synonymies were based on morphological descriptions, but no molecular evidence was provided.

Doris ocelligera is an oval-shaped nudibranch ranging from 0.25 to 1 cm in length. Its mantle is yellow and orange-coloured or green-blueish depending on the specimen, and it gets darker in the centre of the dorsum, where the viscera are found (Vayssière 1901). Dorsally, *D. ocelligera* is also covered with tubercles characterized by a dark apex. As for the rhinophores and gills, they are pale yellowish

coloured and translucid (Ortea *et al.* 2014). This nudibranch lives in rocky environments, under stones, between algae or *Posidonia oceanica* Delile rhizomes, and it feeds on demosponges that belong to the genus *Halichondria* Fleming, 1828, *Haliclona* Grant, 1841, and *Hymeniacidon* Bowerbank, 1858 (McDonald & Nybakken 1997; Ortea *et al*. 2014).

To investigate the colour variation observed within *D. ocelligera*, we here provide a comparative molecular and morpho-anatomical study of several specimens from the Mediterranean and the North-East Atlantic. Additionally, we widen the phylogenetic framework of the family by including other species of *Doris*. Starting from recollected specimens from SCUBA diving, this study aims to carry out a genetic comparison between individuals who are morphologically alike, evaluate their systematics, and identify possible cases of cryptic or hidden speciation.

Material and methods

Taxon sampling

The four different species collected for this study are *D. ocelligera*, *D. bertheloti* (d'Orbigny, 1839), *D. marmorata* (Risso, 1818), and *D. verrucosa.* Each of the specimens was collected by SCUBA diving in shallow areas down to 12 m depth in Gran Canaria, Lanzarote, Galicia, Sant Feliu de Guíxols, Blanes, Begur, and France (Fig. 1). The specimens were photographed alive underwater with a Nikon D500 coupled with 60-, 90-, and 105-mm macro lenses. After collection, the specimens were fixed in 95% EtOH. Ten of them were deposited at the Bavarian State Collection of Zoology in Munich (ZSM, Germany), and the remaining two were deposited at the Museum of Comparative Zoology (MCZ, Harvard University, USA). Permits to collect samples from the Mediterranean were issued by

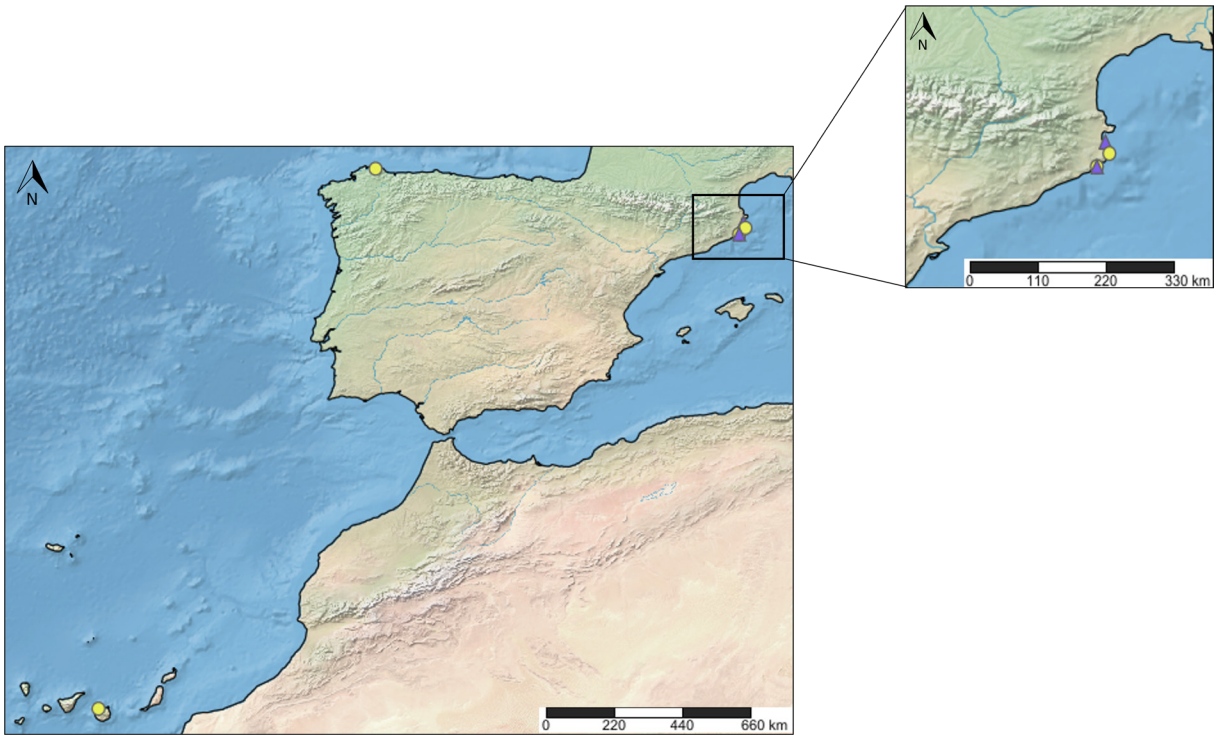

Fig. 1. Map of the Iberian Peninsula and North-West Africa showing the sampling localities where specimens of *Doris ocelligera* (Bergh, 1881) were collected. The purple triangles show the locations where the blueish morphotype specimens were taken, whereas the yellow circles show the locations where the yellow morphotype specimens were collected. Map generated at https://www.simplemappr.net

the local Catalan (permits SF/0589/2018, SF/0495/2019, and DG051201-333/2022) and Canary Islands governments (SGBTM/BDM/AUTSPP/13/2023).

DNA extraction, amplification, and sequencing

The DNA was extracted from a small piece of the foot of each specimen using the Speedtools Tissue DNA Extraction kit (Biotools) and following the manufacturer's protocol. Three markers were amplified for each sample, two of them were the mitochondrial genes cytochrome *c* oxidase subunit I (COI) using the primers LCO1490 and HCO2198 (Folmer *et al.* 1994) and the 16S rRNA using the primers 16S ar-L and 16S br-H (Palumbi *et al.* 1991). As for the third marker, the nuclear gene histone-3 (H3) was amplified with the primer pair H3AD5'3' and H3BD5'3' (Colgan *et al.* 1998). Polymerase chain reactions (PCR) were carried out in 20 µL volume reactions with 9 µL of Sigma dH_2O, 8 µL of REDExtract-N-Amp™ PCR ReadyMix (Sigma Aldrich, St. Louis, MO, USA), 0.5 µL of each primer, and 2 µL of genomic DNA of each sample. PCR conditions for the COI marker included an initial 5 min Hot Start step at 94–95°C, 35–40 cycles of 30 s at 95°C for denaturation, 35 s at 46–54°C for annealing, and 30–45 s at 68–72°C for extension, with a 5 min final extension at 72°C. The conditions for the 16S marker included an initial denaturation at 95°C for 15 min, 40 cycles of 30 s at 94°C, 90 s at 49–50°C for annealing, and 90 s at 72°C for extension with a final extension step of 10 min at 72°C. For the H3 marker, the conditions were the same as for COI except for an annealing temperature of 54°C. Successful amplifications were sequenced by Macrogen, Inc. (Madrid, Spain) after an ExoSAP-IT™ Express PCR Product Clean-up Reagent purification. All sequences were deposited to GenBank at NCBI (Supp. file 1: Table S1).

Phylogenetic analyses

Contamination was assessed against the GenBank nucleotide database, using the BLAST algorithm (Altschul 1997). All sequences were confirmed as belonging to species of the genus *Doris* or related genera. The visualization, edition, and assembly of the sequences were carried out in Geneious Pro ver. 8.1.8 (https://www.geneious.com). Forward and reverse sequences for each specimen were assembled and primer ends were trimmed. MAFFT was used for the multiple alignments (Katoh & Standley 2013), using the G-INS-i algorithm for coding genes (COI and H3) with global homology and L-INS-i for 16S, which contains conserved domains and long gaps. Codon translations were carried out to test for contamination or potential errors. Missing data were coded as 'N'.

A total of 46 taxa were used in the phylogenetic analyses and the outgroup comprised species of the genus *Aphelodoris* Bergh, 1879. These phylogenetic analyses were accomplished using the maximum likelihood (ML) and the Bayesian inference (BI) approaches and were run on the CIPRES Science Gateway ver. 3.3 (http://www.phylo.org/). ML analyses were performed using IQ-TREE ver. 2.1.2. and were conducted for both single-gene and concatenated alignments. The best model selection was carried out automatically with ModelFinder (Kalyaanamoorthy *et al.* 2017) and more specifically using the TESTMERGE option, which possibly merges partitions to reduce over-parameterization and increase model fit. Branch support was estimated via ultrafast bootstrap (bs) with 1500 replicates (Hoang *et al.* 2018). BI was conducted using MrBayes ver. 3.2.7a (Ronquist *et al.* 2012) with BEAGLE (Ayres *et al.* 2019). The Markov chain Monte Carlo (MCMC) simulations technique was used to approximate the posterior probability (pp) distribution of trees, which is the probability of a tree conditioned on the observations. The nucleotide substitution model selected for each partition of the concatenated alignment was GTR+G+I (Tavaré 1986), and four parallel runs of four coupled MCMC chains were run for 20 million generations with a sampling and check frequency of 1000 and 20 000 generations respectively, discarding the first 25% trees as burn-in. Trees were visualized in FigTree ver. 1.4.4 and edited using Adobe Illustrator 2020.

Species delimitation tests (SDT) were performed on the aligned COI and 16S datasets for the species of the genus *Doris* using the Assemble Species by Automatic Partitioning (ASAP; Puillandre *et al*. 2021)

test. ASAP was run in the web interface https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html with default parameters and the substitution model Kimura (K80) ts/tv 2.0 to compute the distances.

Morphological and anatomical analyses

The total length (*L*) of the preserved specimens was measured with a Vernier calliper with the aid of fine forceps, and an exhaustive description of the external morphology was conducted. After that, dissections of two specimens of each morphotype: blueish and yellow, were carried out with the aid of fine forceps. The digestive and reproductive systems were carefully observed, described, and separated. To extract the radula, the buccal bulb was dissected. The organic matter was dissolved by immersing it in a KOH 10% solution for approximately one hour followed by three rinses with distillate water, and a final one with 96% EtOH. The radula was mounted on metallic stubs with bioadhesive carbon sticky tabs and coated with carbon for scanning electron microscopy (SEM) with a Field Emission Scanning Electron Microscope JSM-7100F at the UB Scientific and Technological Centres (CCiT-UB). Spicule slides, both from the mantle and stomach content, were prepared by dissociation in chlorine, and rinsed with distillate water and 96% EtOH. All plates including the photographs of the specimens alive, the SEM micrographs, and the schematic drawings of the reproductive system were edited with Adobe Photoshop 2020.

Results

Phylogenetic results

The final sequence dataset for the 46 specimens included 1449 bp, i.e., 659 bp for COI, 462 characters for 16S, and 328 bp for H3. Out of the 46 specimens, 12 were sequenced in this study and the rest were obtained from GenBank (see Supp. file 1: Table S1). The substitution models selected according to ModelFinder were TVM+F+I+G4 (Posada 2003; Le *et al.* 2012) for the three codon positions of COI and 16S, and K2P+I (Kimura 1980) for H3, accounting for the three codon positions. ML and BI analyses yielded slightly different results for the concatenated alignment (Fig. 2; Supp. file 1: Figs S1–S4). However, both showed a clear phylogenetic differentiation in genera – which were all found monophyletic except for *Doris* – with high branch support for *Aphelodoris* (ML not supported, pp = 0.97), *Austrodoris* Odhner, 1926 (bs = 98, pp = 1), *Conualevia* Collier & Farmer, 1964 (pp = 0.95), *Homoiodoris* Bergh, 1882 (bs = 99, pp = 0.98), *Doriopsis* Pease, 1860 (ML not supported, pp = 0.96), and *Archidoris* Bergh, 1878 (bs = 91, BI not supported).

Regarding our results, we believe that the specimen *Doris* sp. CCS-201 and *Aphelodoris* sp.1 CCS-2010 may have their names shifted in the GenBank database. As our phylogenetic results illustrate, with high support, the specimen of *Doris* is the sister group of *Aphelodoris*, and the specimen of *Aphelodoris* was recovered as an ingroup of *Archidoris* (bs = 95, pp = 1). Also based on our results, the specimen Doridiidae sp. 1467385 belongs to the genus *Doriopsis* (bs = 79, pp = 1). Even though the genera *Austrodoris* and *Archidoris* are currently unaccepted and encompassed under the genus *Doris*, our results show a differentiation with high branch support value.

The genus *Doris* appears to be paraphyletic. On the one hand, there is a branch with the highest support values that includes the type species *D. verrucosa*, *D. ocelligera*, and the resurrected species *Doris berghi* comb. rest. (see Systematic descriptions). With this evidence, we can state that both truly belong to the genus *Doris*. On the other hand, there are two more branches with a low support value (ML and BI not supported): (1) includes the specimens belonging to the species *D. marmorata* and *D. bertheloti*, and (2) includes the specimens of the recently described *D. adrianae* Urgorri & Señarís, 2021 (Urgorri *et al.* 2021). In this study, there is not enough molecular evidence to declare if *D. marmorata*, *D. bertheloti*, and *D. adrianae* may belong to the genus *Doris* or not.

In outline, the phylogenetic results show a separation with maximum branch support in three different clades within one of the branches of the genus *Doris*. The first clade corresponds to the type species *D. verrucosa*, the second one refers to *D. ocelligera*, and the third one to *D. berghi* comb. rest. This third clade includes four sequences from our specimens and three downloaded from GenBank that were identified as *D. ocelligera* and *D. verrucosa*. These clades are corroborated by ASAP results (Fig. 3), which showed nine and five best partitions or species hypotheses for COI and 16S, respectively. Both present coherent nucleotide distances between species of about 5–10%. Thus, the two groups of specimens originally considered as *D. ocelligera* are here confirmed as two molecularly separated species.

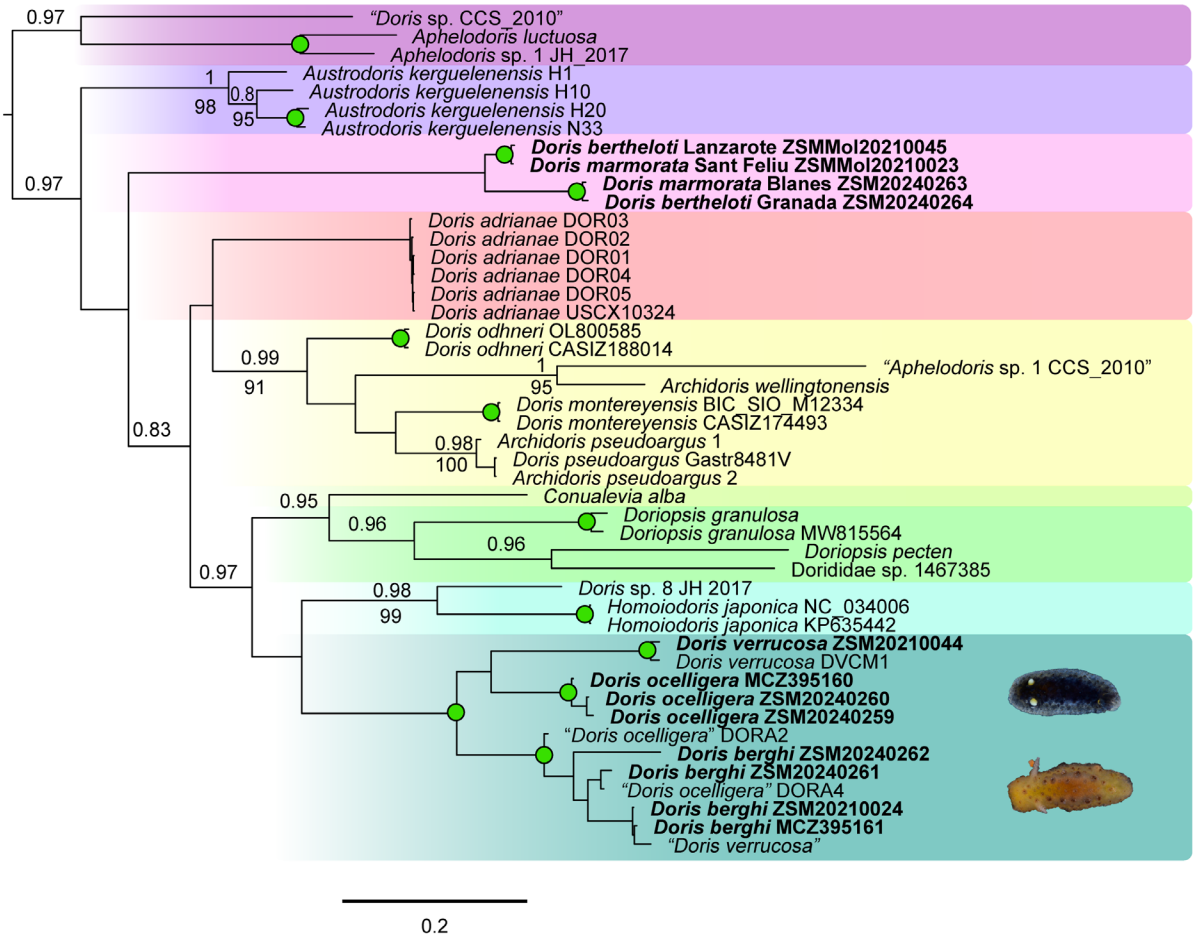

Fig. 2. Bayesian phylogenetic tree of Dorididae Rafinesque, 1815 based on the concatenated alignment of COI, 16S, and H3 markers. Posterior probabilities and bootstrap support values are shown above and below branches, respectively. The outgroup used to root the tree was the genus *Aphelodoris* Bergh, 1879. Green dots indicate nodes with maximum branch support in both analyses. The scale bar indicates substitutions per site. Names of the sequences downloaded from GenBank have not been modified, yet taxa that may belong to a different identity are specified between quotation marks. Specimens sequenced in this study are highlighted in bold.

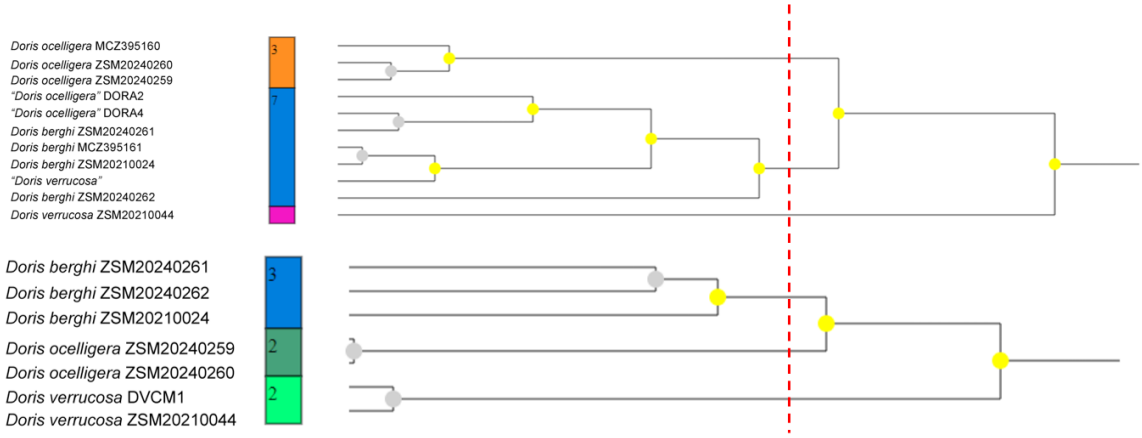

Fig. 3. Results from ASAP for both COI (top) and 16S (bottom) markers of the alignment of the species within the genus *Doris* Linnaeus, 1758 based on the phylogenetic tree results. Colour boxes refer to species hypotheses and the dash-line is the threshold (representing a 5–10% genetic divergence distance) in which species hypotheses were selected.

Systematic descriptions

Class Gastropoda Cuvier, 1795
Subclass Heterobranchia Burmeister, 1837
Order Nudibranchia Cuvier, 1817
Suborder Doridina Odhner, 1934
Family Dorididae Rafinesque, 1815

Genus ***Doris*** Linnaeus, 1758

Type species

Doris verrucosa Linnaeus, 1758.

Doris ocelligera (Bergh, 1881)
Figs 1–3, 4F–H, 5A–C, 6A

Staurodoris ocelligera Bergh, 1881: 95, pls 11–21.
Doris lutea Risso, 1826: 31.

Doris ocelligera – Pruvot-Fol 1954: 234. — Schmekel 1968a: 114. — Schmekel & Portmann 1982: 75–77, pls 20.2, 30.8 fig. 7.12. — Ortea *et al*. 2014: 64–65.

Type locality

Trieste, Italy, the Mediterranean Sea.

Diagnosis

Compressed body, blueish colouration of mantle, sometimes with yellowish mantle margin, tubercles darker. Rhinophores dark yellow. Gills composed of 10 pinnate leaflets. Cusp of outermost lateral teeth extending more than ⅔ of total length. Seminal receptacle spherical, orange. Bursa copulatrix bean-shaped, rose in colour.

Material examined

SPAIN – **Catalonia** • 2 specs (1 sequenced, 2 dissected: L = 1.2 cm, L = 0.6 cm); Girona, Sant Feliu de Guíxols, Coves Cala Maset; 41.8098° N, 3.0412° E; 1 m depth; 9 Aug. 2022; Xavier Salvador leg.; GenBank: OR286434; ZSM20240260 • 2 specs (1 sequenced); same data as for preceding; 10 Jul. 2022; Xavier Salvador leg.; GenBank: OR286433; ZSM20240259 • 1 spec. (sequenced); Girona, L'Escala; 42.1153° N, 3.1689° E; 12 m depth; 8 Mar. 2015; Xavier Salvador leg.; GenBank: OR286432; MCZ395160.

Description

External morphology (Fig. 4G). Body compressed, oval-shaped. Mantle blue to dark green when preserved, yellowish alive; margins and central part of dorsum paler, showing a whitish and violet colouration, respectively. Mantle edge extending, covering foot. Dorsal tubercles protruding, dark blue, homogeneously distributed, but less numerous in margins, and especially concentrated around rhinophores. Spicules seen in tegument by transparency, connecting tubercles. Rhinophores dark yellow, lamellated (6 lamellae); rhinophoral sheaths blue. Gills exposed, blueish-violet in colour, consisting of 10 pinnate leaflets, with a slightly translucid apex.

Radula (Fig. 5A–C). Radular formula 22–32 × 33–37.1.0.1.33. Teeth hook-shaped, cusp acute. First lateral tooth with a width base, short cusp. Inner and outermost lateral teeth thin, smooth. Outermost with a longer cusp, extending more than ⅔ of total length.

Digestive system. Salivary glands sausage-shaped, extending after pharynx to oesophagus. Stomach connects with a caecum and intestine, conformed by fine and translucid tissue. Digestive gland dark grey.

Reproductive system (Fig. 6A). Gonad covering digestive gland, representing three-quarters of viscera. Ampulla sausage-shaped, pink in colour, folded once, end connecting to gonad rather sharp. Vaginal duct short, thin, smooth. Penial sheath thick, conforming to distal part of vas deferens. Proximal prostatic part of vas deferens little folded (Schmekel & Portmann 1982). Seminal receptacle spherical, orange, connected with bursa copulatrix via a thin duct. Bursa copulatrix bean-shaped, rose, with darker end.

Ecology

Found above the sponge *Terpios gelatinosus* (Bowerbank, 1866), copulating with another specimen, smaller and blue. As this nudibranch grazes on the sponge, it sinks into it until totally camouflaged. No sponge spicules were found in the stomach or intestine of the dissected specimen studied. The egg mass is a spiral ribbon of light-yellow eggs, with just over 5000 eggs per cm of ribbon, with a mean diameter of 85 µm (Ortea *et al*. 2014). These eggs are already fully capable of development and swimming veliger hatch after 12 days at 16°C (Schmekel & Portmann 1982).

Distribution

Doris ocelligera is distributed from the North of the Iberian Peninsula to the Savage Islands, Madeira, the Canary Islands, and Cape Verde. It also lives in the Azores Islands (Azevedo & Gofas 1990) and the Western Mediterranean (Ortea *et al*. 2014). Our three samples, including four specimens in total, were collected on the Catalan coast: ZSM20240259 and ZSM20240260 in Sant Feliu de Guíxols, and MCZ395160 in L'Escala. No specimens belonging to *D. ocelligera* were found in the sampling sites of the Nort-East Atlantic (see Fig. 1).

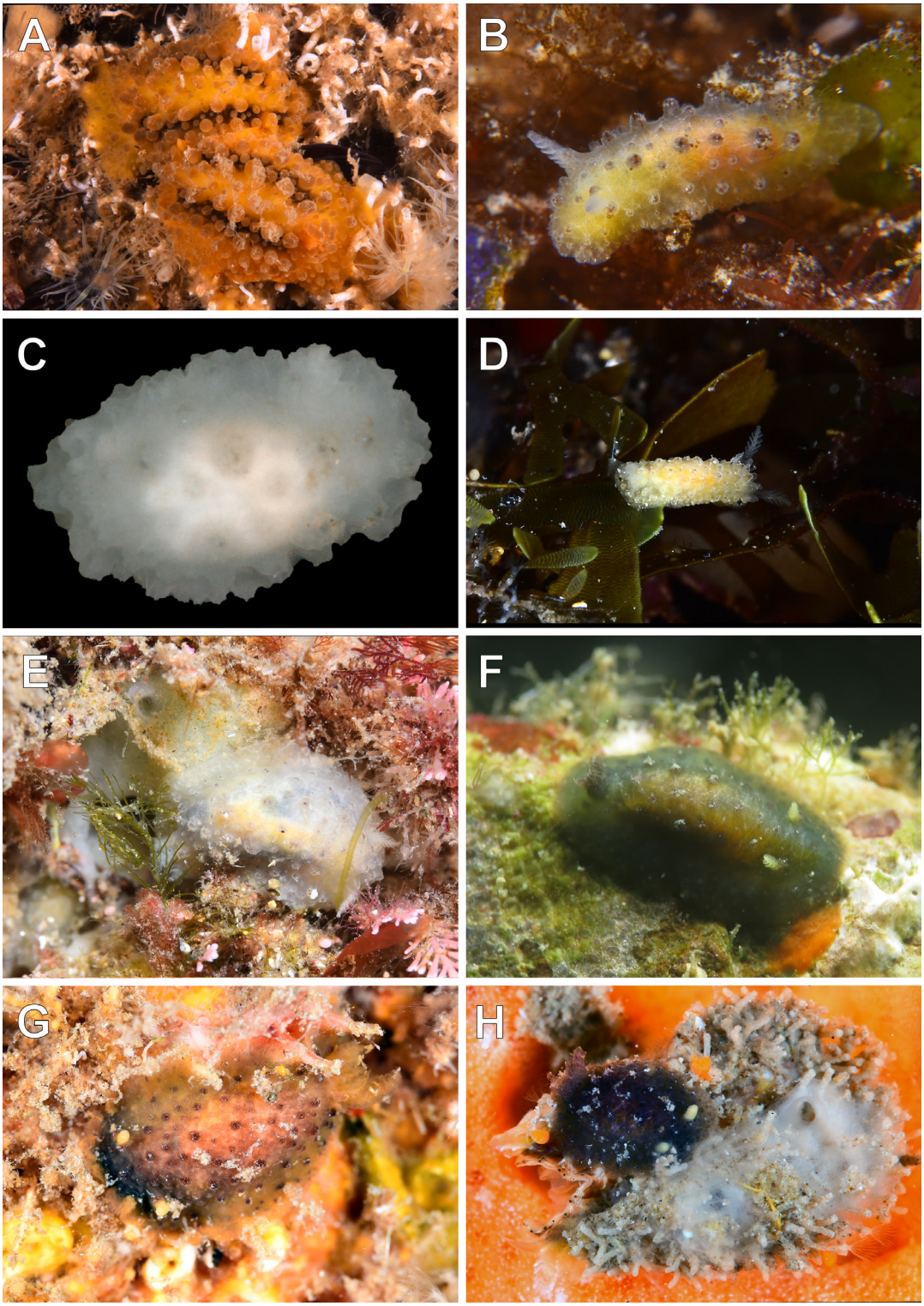

Fig. 4. A. *Doris verrucosa* Linnaeus, 1758 (ZSM20210044) from Thau, SE France. **B–E**. *Doris berghi* (Vayssière, 1901) comb. rest. **B**. Specimen from Sant Feliu de Guíxols, NE Spain (ZSMMol20210024). **C**. Specimen from Begur, NE Spain (MCZMal395161). **D**. Specimen from Gran Canaria (ZSM20240262). **E**. Specimen from Galicia, NW Spain (ZSM20240261). **F–H**. *Doris ocelligera* (Bergh, 1881). **F**. Specimen from l'Escala, NE Spain (MCZ395160). **G**. Specimen from Sant Feliu de Guíxols, NE Spain (ZSM20240260). **H**. Specimen from Sant Feliu de Guíxols, NE Spain (ZSM20240259).

Remarks

Living specimens, both the sequenced and the one copulating with it, presented different colourations. The one described and sequenced was dark yellow/brown, whereas the other (only sequenced) was blue. Once fixed in EtOH, both specimens turned into a blueish colouration. The description provided herein matches Bergh's (1881: 95) description of *D. ocelligera*: "Dorid, oval in shape, with yellow or orange mantle, with dark ochre to black dots on the top of the most developed tubercles of the notum. The colour of the posterior part is variable, from light blue to black or from black to yellow, and the apex of the eyes is dark. It also presents white rhinophores and blue gills". Therefore, the blueish morphotype is here attributed to *D. ocelligera*. Risso's (1826) description of *D. lutea* is flimsy, but he described a nudibranch characterized by its mantle's golden yellow colouration, thus potentially like *D. ocelligera*. Being a species of small size, *D. ocelligera* has been confused with juveniles of *D. verrucosa*. However, when compared, only the morphology of the outer lateral teeth of the radula resembles each other. The reproductive system of *D. ocelligera* is very different from that of *D. verrucosa*, which lacks a reduced prostate undifferentiated from the vas deferens and a seminal receptacle with a similar shape as the copulatory bursa and separated from it (Ortea *et al*. 2014). Both species differ in the ampulla, which is simply a folded tube in *D. ocelligera* while elongated and tubular in *D. verrucosa* (Lima & Simone 2015). The prostatic part of the vas deferens is less convoluted and shorter in *D. ocelligera* than in *D. verrucosa*. Also, the strong retractor muscle observed in the distal part of the vas deferens of *D. verrucosa* was not observed in *D. ocelligera* (Schmekel & Portmann 1982). Comparing the radular teeth of *D. ocelligera* (ZSM20240260) and *D. verrucosa* (ZSM20210044, Fig. 7A–B), noticeable differences come to light. While the first lateral teeth resemble each other, the inner and outermost lateral teeth exhibit slight differences. In *D. verrucosa*, the superior part of the innermost lateral teeth presents a thin and high arch before the beginning of the sharp and hook-shaped cusp, whereas in *D. ocelligera*, this arch is less noticeable. As for the outermost lateral teeth, those from *D. verrucosa* present a perfect hook shape with a shorter cusp, only extending ⅓ of the total length.

Taking all the characters into account, this species is clearly distinguished from *D. verrucosa* by the external colouration, the size and shape of the mantle tubercles, the morphology of the lateral teeth, the extension of the outermost lateral teeth cusp, and the shape and size of the reproductive structures.

Doris berghi (Vayssière, 1901) comb. rest.
Figs 2–3, 4B–E, 5D–F, 6B

Aldisa berghi Vayssière, 1901: 27, pl. 1 figs 26–27.

Type locality

Gulf of Marseille, France, the Mediterranean Sea.

Diagnosis

Body shape convex. Mantle yellow whitish, dorsal tubercles white, translucid. Rhinophores white. First lateral tooth hook-shaped; outermost lateral teeth with a cusp extending ½ of the total length. Seminal receptacle elongated, brown. Bursa copulatrix round, whitish or slightly orange.

Material examined

SPAIN – **Galicia** • 1 spec. (sequenced and dissected: L = 70 mm); Galicia, Playa Viveiro; 43.6740° N, 7.6004° W; 1 m depth; 1 Jul. 2022; Xavier Salvador leg.; GenBank: OR286435; ZSM20240261. – **Canary Islands** • 1 spec. (sequenced); Gran Canaria, Piscinas de Agaete; 28.1068° N, 15.7112° W; 2 m depth; 5 Jul. 2022; Xavier Salvador leg.; GenBank: OR286436; ZSM20240262. – **Catalonia** • 1 spec.

(sequenced and dissected: L = 0.4 cm); Girona, Sant Feliu de Guíxols, Coves Cala Maset; 41.7865° N, 3.0447° E; 3 m depth; 22 Feb. 2019; Xavier Salvador leg.; GenBank: OR286438; ZSMMol20210024.

Description

External morphology (Fig. 4E). Body compressed, convex in shape. Mantle white to yellow, darker at centre of dorsum, where viscera are found, translucid in margins. Mantle edge extending, covering foot. Dorsal tubercles white, with a brown-orange apex, more prominent and larger in a central band that goes from the rhinophores to the branchial plume, smaller tubercles with more intense apex colouration. Spicules translucid, interconnecting tubercles. Gills translucid. Rhinophores white, lamellated (6 lamellae), retracted into rhinophoral sheath once fixed.

Radula (Fig. 5D–F). Radular formula 24 × 40.1.0.1.40. First lateral tooth hook-shaped, thin. Consequent lateral teeth present a less noticeable hook shape, thick base, smooth and relatively short cusp. Outermost lateral teeth with a cusp extending ½ of total length.

Digestive system. Alike *D. ocelligera* (ZSM20240260).

Reproductive system (Fig. 6B). Gonad alike *D. ocelligera*. Ampulla pink with shady parts, round end connecting to gonad. Vaginal duct and penial sheath alike *D. ocelligera*, visible portion of vas deferens shorter. Seminal receptacle brown, elongated. Bursa copulatrix round, white, or slightly orange.

Ecology

Found in a rocky substrate. No sponge spicules were found in the stomach or intestine of the dissected specimens.

Distribution

Originally described from the Mediterranean part of France. Here, we found two specimens of *D. berghi* comb. rest. in the Nort-West part of the Mediterranean Sea, on the Catalan coast, and two more in the North-East part of the Atlantic Ocean, in Galicia and Gran Canaria. Therefore, the distribution seems to overlap with that of *D. ocelligera*.

Remarks

Here, we describe the restored combination *D. berghi* comb. rest., which coincides with Vayssière's (1901: 27) description of *Aldisa berghi*, considered as a synonym of *D. ocelligera* and described as: "Dorid presenting a golden yellow colouration. The dorsal side of the mantle presents white dots scattered over its entire extent. Tubercles of variable size, wart-like with a large brown spot at their top. The yellow colouration is more accentuated on the anterior surface and the lower surface of the foot. It presents pale yellow rhinophores and gill leaflets with a few white or greyish mottled spots". This species is clearly distinguished from *D. ocelligera* by the external pale-yellow colouration, the white rhinophores, the extension of the outermost lateral teeth cusp, and the shape and relative size of the copulatory bursa and seminal receptacle.

Doris marmorata Risso, 1818
Figs 2–3, 8

Doris marmorata Risso, 1818: 369.

Doris marmorata – Schmekel 1968b: 177, figs 5–8. — Schmekel & Portmann 1982: 72–75, pls 20.3, 30.10 figs 7–11.

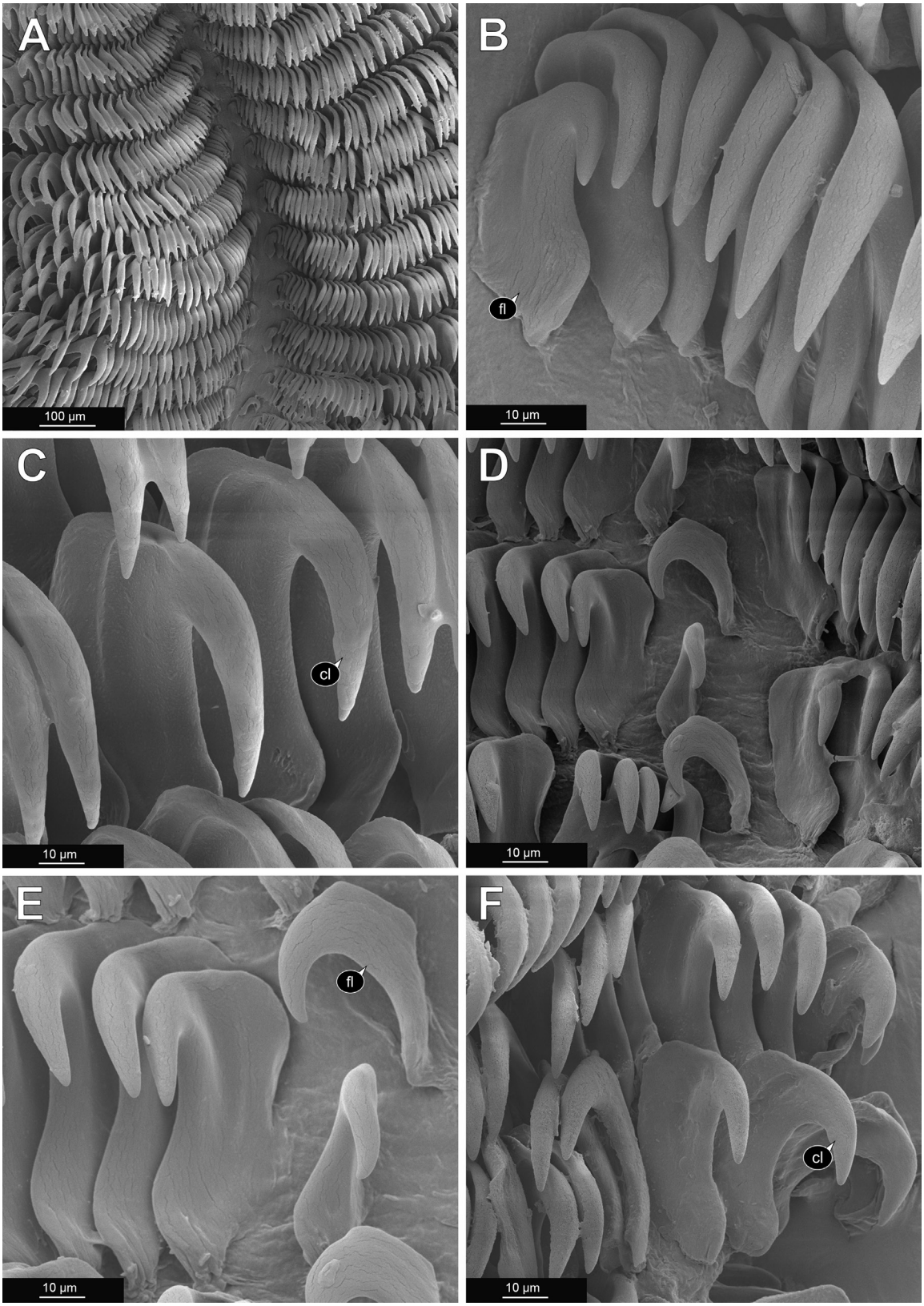

Fig. 5. Scanning electron micrographs of the radular teeth. **A–C**. *Doris ocelligera* (Bergh, 1881) (ZSM20240260). **D–F**. *Doris berghi* (Vayssière, 1901) comb. rest. (ZSM20240261). Showing first (fl) and subsequent (cl) lateral teeth.

Type locality

Nice, France, Mediterranean Sea.

Diagnosis

Body rounded, flat, light white. Mantle tubercles numerous, light orange in color. Rhinophores lamellar, orange distally. Gills white.

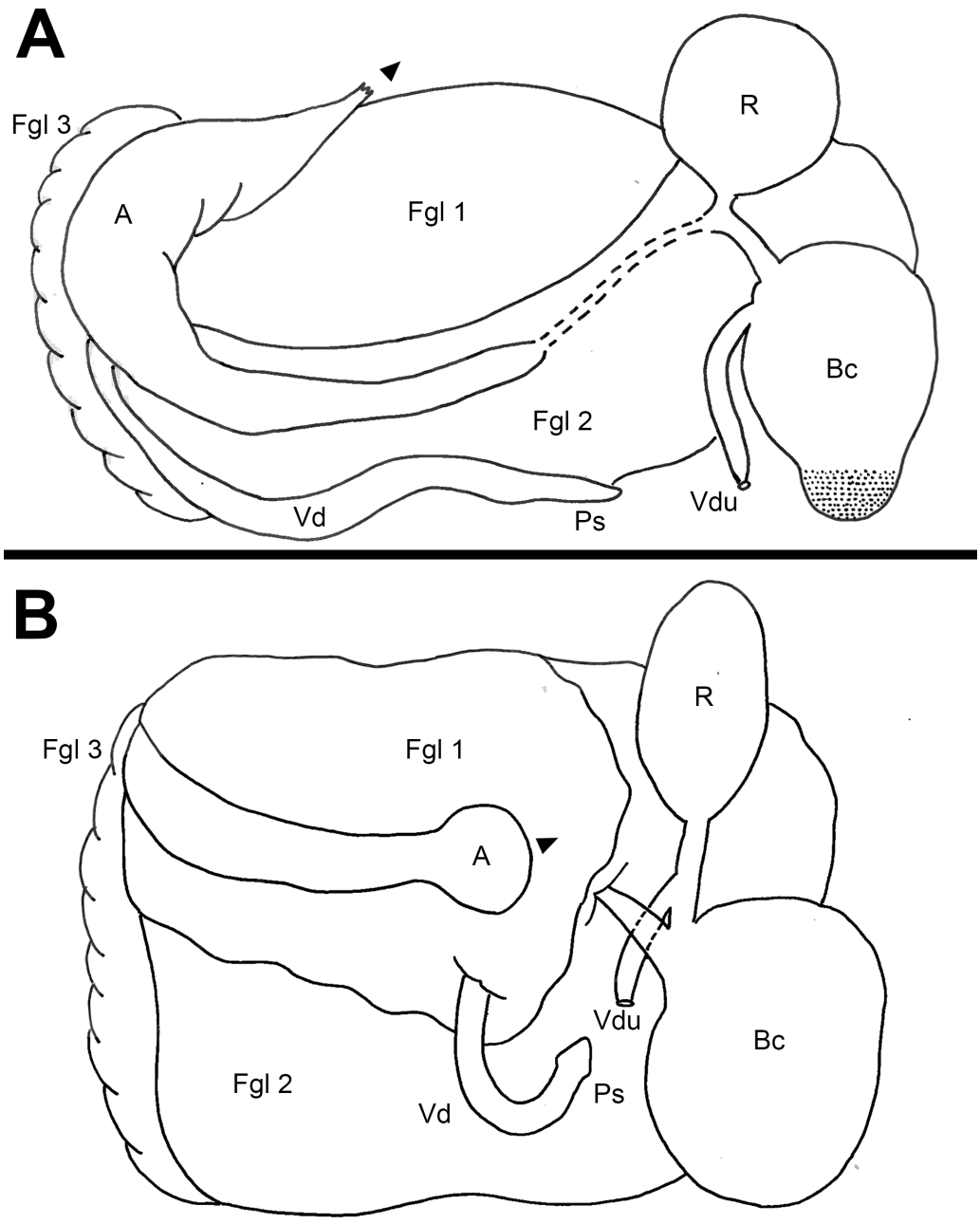

Fig. 6. Schematic drawing of the reproductive systems. **A**. *Doris ocelligera* (Bergh, 1881). **B**. *Doris berghi* (Vayssière, 1901) comb. rest. Abbreviations: A = ampulla; Bc = bursa copulatrix; Fgl = female glands; Ps = penial sheath; R = seminal receptacle; Vd = vas deferens; Vdu = vaginal duct. The black arrowheads represent the connection of the ampulla to the gonad.

Material examined

SPAIN – **Catalonia** • 1 spec. (sequenced: L = 0.4 cm); Girona, Blanes, Punta Santa Anna; 41.67° N, 2.80° E; 7 m depth; 5 Feb. 2016; Xavier Salvador leg.; GenBank: OR286430; ZSM20240263 • 1 spec. (sequenced: L = 1.5 cm); Girona, Sant Feliu de Guíxols, Coves Cala Maset; 41.7865° N, 3.0447° E; 2 m depth; 7 Feb. 2019; Xavier Salvador leg.; GenBank: OR286431; ZSMMol20210023.

Description

External morphology. Body rounded, flat, light white. Tubercles numerous, the larger specimen with top dark yellow to orange. Rhinophores lamellar, with middle until top part orange. Gills white, with little orange points in external part of branchial leaves.

Ecology

The specimen ZSM20240263 was observed during the day crawling over undetermined algae in a rocky wall. Specimen ZSMMol20210023 was recorded during a night dive actively moving over the algae.

Distribution

France (Mediterranean coast; Risso 1818), Italy (Between Capo Posillipo and Nisida; Schmekel & Portmann 1982), Northern Aegean Sea (Koutsoubas *et al*. 1993), and Catalonia (this study).

Remarks

In the description of Schmekel & Portmann (1982), the central notal region is surrounded by dark spots, but in our specimens, the central region is whiter than the rest of the notum and the dark spots are not visible. The orange tubercles and rhinophores are exactly alike. The maximum length of the specimens described is 12 mm and the maximum length of our specimens while alive was 15 mm. There, they describe that the rhinophore leaned forward when moving, a behaviour that was observed in specimen ZSMMol20210023. Overall, the morphological description of our specimens matches the description of the species.

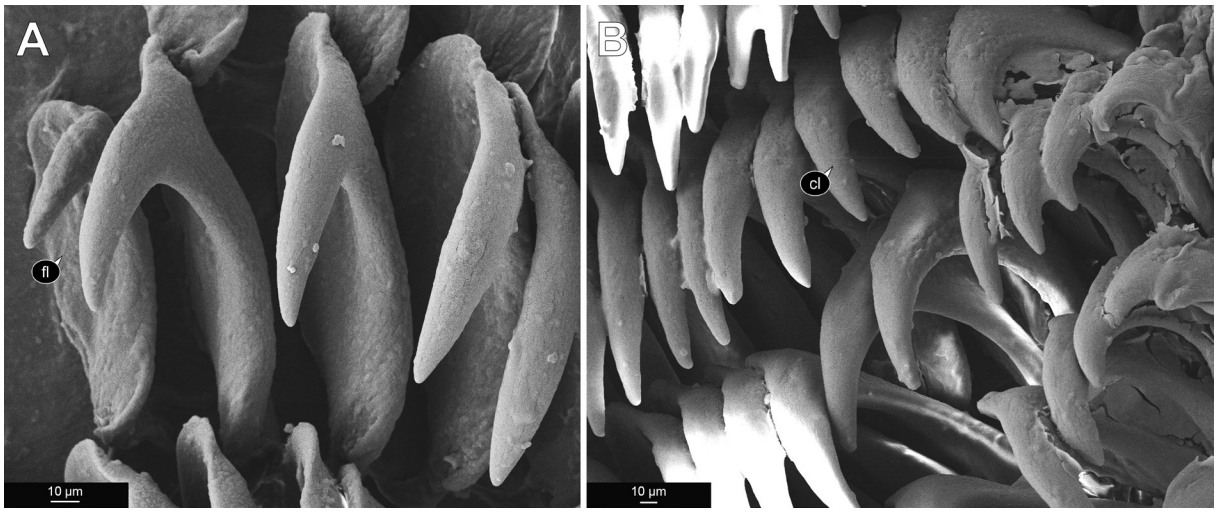

Fig. 7. A–B. Scanning electron micrographs of the radular teeth of *Doris verrucosa* Linnaeus, 1758, showing first (fl) and subsequent (cl) lateral teeth.

Discussion

The phylogeny of Doridiidae

In this study, we sequenced the COI, 16S, and H3 markers of seven specimens initially identified as different morphotypes of *Doris ocelligera*, two specimens of *D. marmorata*, two of *D. bertheloti* and a last one of the type species *D. verrucosa*. The final phylogenetic tree shows several species from

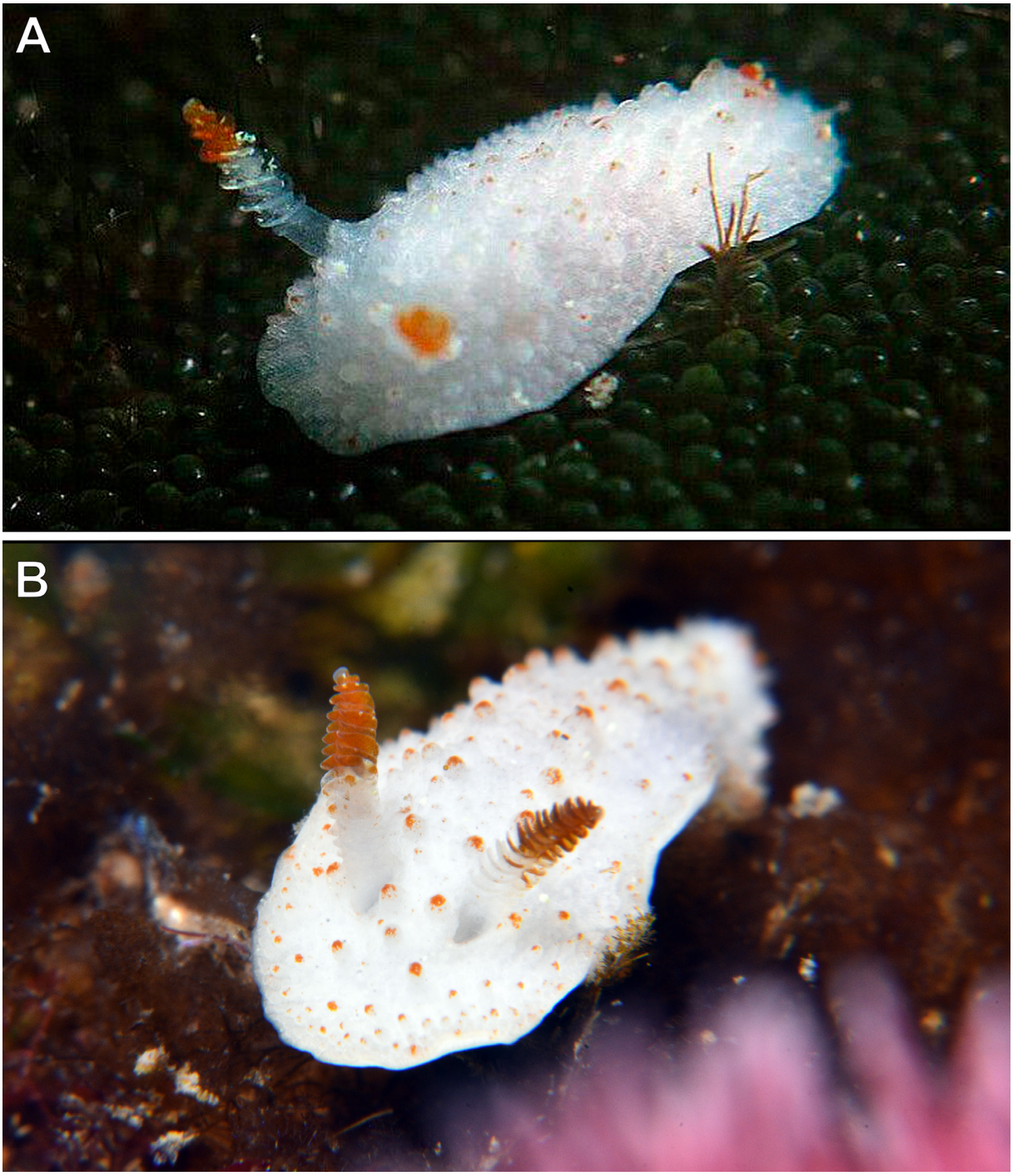

Fig. 8. *Doris marmorata* (Risso, 1818). **A**. Specimen from Blanes, NE Spain (ZSM20240263). **B**. Specimen from Sant Feliu de Guíxols, NE Spain (ZSMMol20210023).

potentially different genera within Doridiidae, all of them found monophyletic except for *Doris* (Fig. 2). One of these clades includes specimens referrable to the blueish morphotype of *D. ocelligera* and others referrable to the yellow one, found in the Mediterranean and along the Atlantic coasts. Both the phylogeny and the species delimitation tests show a genetic divergence between the two morphotypes, thus suggesting the resurrection of *D. berghi* comb. rest. We can state that these two species belong to the genus *Doris*, as they are related to the type species *D. verrucosa* with maximum branch support in both analyses. The sequenced specimens of both *D. marmorata* and *D. bertheloti* were found far away from the type area, so a necessary morphological reassessment may be needed to ensure the generic identity. Further research should be done to clarify the phylogenetic relationship between these two species, which seems inconclusive in our tree. *Doris marmorata* has only been found three times until now, in France (Risso 1818), Italy (Schmekel & Portmann 1982), and the Northern Aegean Sea (Koutsoubas *et al*. 1993). We provide a description based on two new specimens found along the Catalan Coast, constituting the first record of this species on the Iberian Coast and the fourth worldwide.

Two conflicting and suppressed genera are *Austrodoris* and *Archidoris*. Regarding *Austrodoris*, Odhner (1926) described the genus based on differentiation in the salivary glands, which were short, wide, and non-attached respectively to the situation in *Doris* and *Archidoris*. Later, there was great confusion between the use of the names *Archidoris* and *Austrodoris* that was resolved using the former for species from the Northern Hemisphere and the latter for species from the Southern Hemisphere (Valdés 2002). This ignored the anatomical characters of the specimens described when it came to terms of classification. Then, Wägele (1990) redescribed the genus *Austrodoris* and included all the species previously described as synonyms of *A. kerguelensis* (Bergh, 1884). Finally, based on different reproductive features, Valdés (2002) synonymized several genera names with *Doris*, including *Archidoris* and *Austrodoris* (Wilson *et al.* 2009). He also claimed that the monophyly of the traditional taxa synonymized with *Doris* is unlikely. However, these proposed synonyms can only be confirmed through species-level phylogenetic analyses. The phylogenetic analyses carried out in this study bring the possibility of denying the monophyletic character of the genus *Doris*. We also show a separation (pp = 0.97) between *Austrodoris* and *Archidoris* from *Doris*, with many recognized genera such as *Conualevia* or *Homoiodoris* closely related to the type species of *D. verrucosa*. Taking these data into account, it would seem necessary to use a broader dataset with more representative outgroup taxa to investigate at a genus level the systematics of Doridiidae and maybe reconsider the level of diversification that took place in this clade to resurrect some traditional taxa such as *Austrodoris* and *Archidoris*.

On the morphology and anatomy of the blue and yellow *Doris*

Bergh's (1881), Schmekel & Portmann's (1982), and Ortea's *et al.* (2014) descriptions agree with our description of *D. ocelligera* (Fig. 4F–H), suggesting that the synonym and original combination *Staurodoris ocelligera* corresponds to *D. ocelligera*. Vayssière's (1901) description of the synonym *Aldisa berghi* coincides with the external morphology of the species referred to as *D. berghi* comb. rest. in this study (Fig. 4B–E). Therefore, after the molecular evaluation of these morphotypes, we can support that the actual synonym *Aldisa berghi* belongs to a different species; resurrected herein as *Doris berghi* comb. rest. Regarding the overall external morphology of the two morphotypes of '*D. ocelligera*', noticeable differences are appreciated. The most apparent one is the mantle colouration: blueish for *D. ocelligera* and pale yellow for *D. berghi* comb. rest. As for the mantle tubercles, they are dark blue in *D. ocelligera*, but white and translucid – with a brownish apex – in *D. berghi*.

The radular formula of the specimen of *D. ocelligera* dissected in this study matches the formula provided by Ortea *et al.* (2014); only differing in two rows and two lateral teeth. Regarding the internal anatomy of the two species *D. ocelligera* and *D. berghi* comb. rest., significant differences are also observed and detailed herein. These differ in the number of rows, being 22–32 and 24 for *D. ocelligera* and *D. berghi*, respectively. The number of outer lateral teeth is 33–37 for *D. ocelligera* and 40 for *D. berghi*.

The first lateral tooth of *D. berghi* presents a thin base and a neat hook shape, which is different from the consequent lateral teeth; thicker and less curved. Contrarily, the laterals of *D. ocelligera* are more similar. Also, the outermost lateral teeth cusp is longer in *D. ocelligera* than in *D. berghi*, concretely about one-third. The differences found between the radular teeth of *D. ocelligera* and *D. verrucosa* (Figs 5A–C, 6A–B) are equivalent to the ones found between *D. ocelligera* and *D. berghi* (Fig. 5D–F), thus evidencing their distinction between two different species.

Regarding the reproductive system, both *D. ocelligera* and *D. berghi* comb. rest. show noticeable differences in the relative shape and size. The seminal receptacle is spherical in *D. ocelligera*, whereas in *D. berghi* it is rather saccular. As for the bursa copulatrix, in *D. ocelligera* it is bean-shaped, while it is rounded in *D. berghi*. The ampulla is similar in both species but in *D. berghi* the end connecting to the gonad is more rounded, whereas in *D. ocelligera* it is rather sharp. Still, we ought to bear in mind that the descriptions are based on the dissection of one specimen for each species, since the rest were juveniles and, therefore, the reproductive system was not fully developed. Nevertheless, our descriptions match the ones provided by Schmekel & Portmann (1982) and Ortea (2014).

Both *D. ocelligera* and *D. berghi* comb. rest. are found in the Northeast Atlantic and Mediterranean Sea. That is to say that the reproduction isolation and the resulting evolutive divergence of a new species from a survival ancestral could have been accomplished without any physical, geographic, or hydrodynamic barrier, while both species continued to inhabit the same geographic region. Considering the integrative taxonomical information provided, the originally thought different morphotypes are indeed sympatric species. The molecular, morphological, and anatomical revision of the species *D. ocelligera* has provided valuable insight into a pseudo-cryptic species complex in this region. Additional efforts in other species seem urgent to estimate the existing hidden biodiversity of Doridiidae.

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Fig. S1. Maximum likelihood tree for Doridiidae Rafinesque, 1815 based on the concatenated alignment of COI, 16S, and H3 markers (1449 bp). Bootstrap support values are shown on branches. The outgroup used to root the tree was *Aphelodoris* Bergh, 1879. Scale bar indicates substitutions per site.

Fig. S2. Maximum likelihood tree for Doridiidae Rafinesque, 1815 based on the COI marker (659 bp). Bootstrap support values are shown on branches. The outgroup used to root the tree was *Aphelodoris* Bergh, 1879. Scale bar indicates substations per site.

Fig. S3. Maximum likelihood tree for Doridiidae Rafinesque, 1815 based on the 16S marker (462 bp). Bootstrap support values are shown on branches. The outgroup used to root the tree was *Aphelodoris* Bergh, 1879. Scale bar indicates substations per site.

Fig. S4. Maximum likelihood tree for Doridiidae Rafinesque, 1815 based on the H3 marker (328 bp). Bootstrap support values are shown on branches. The outgroup used to root the tree was *Doris verrucosa* Linnaeus, 1758. Scale bar indicates substations per site.

Table S1. Material used in molecular phylogenetic analyses and species delimitation tests, with GenBank accession numbers and relevant references.