

Original Article

A global phylogeny of *Elysia* Risso, 1818 (Gastropoda: Heterobranchia): molecular systematic insights focusing on European taxa and description of a new species

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ABSTRACT

The genus *Elysia* comprises about one-third of the species richness in Sacoglossa. However, the species diversity in the genus remains poorly characterized in some areas like the north-eastern Atlantic and Mediterranean waters. To clarify the systematics of this genus and to characterize the species diversity in undersampled regions, we performed an integrative study based on a thorough literature review, molecular and morphological analyses, and species delimitation approaches. We conducted phylogenetic analyses of partial sequences of two mitochondrial genes (*COI* and *16S*) and two nuclear genes (*H3*, *28S*) using Bayesian inference and maximum likelihood methods, which confirmed the presence of five of the recognized European *Elysia* species: *Elysia viridis*, *E. timida*, *E. flava*, *E. margaritae*, and *E. rubeni*. Moreover, a new species (*Elysia azorica* sp. nov.) was identified in the Azores, and *E. gordanae*, currently considered a junior synonym of *E. margaritae*, was recovered as a distinct species. In addition, we consider *E. hetta* as a junior synonym of *E. gordanae*, and *E. translucens* as a *taxon inquirendum*. Finally, the tropical *E. evelinae* is recorded along European coasts for the first time. Our results demonstrate the value of integrative approaches in resolving taxonomic uncertainty surrounding polymorphism and unravelling potential cases of cryptic or pseudocryptic species complexes.

Keywords: integrative taxonomy; new species; phylogenetic systematics; Sacoglossa; species delimitation

INTRODUCTION

The genus *Elysia* Risso, 1818 is the most diverse lineage in the family Plakobranchidae with over 130 species, including more than 30 undescribed candidate species (Krug *et al.* 2015, 2016, Gosliner *et al.* 2018, Hirokane *et al.* 2022, MolluscaBase 2023a). Research on these sacoglossan sea slugs has focused on larval development mode, the ability of slugs to sequester functional chloroplasts (kleptoplasty) or chemical compounds with feeding-deterrent properties (kleptochimistry), *de novo* biosynthesis of sunscreen compounds to protect from UV radiation

(Torres *et al.* 2020), and the host specificity for diverse algae (e.g. Händeler *et al.* 2009, Rumpho *et al.* 2011, Krug *et al.* 2013, 2015, 2016, Christa *et al.* 2014a, Cruz *et al.* 2014, De Vries *et al.* 2014, Gavagnin *et al.* 2015, 2019, 2020, Rauch *et al.* 2017, 2018, Laetz and Wägele 2018, Ávila and Angulo-Preckler 2020).

However, in terms of systematics, *Elysia* remains a problematic group for several reasons. The hierarchical placement of species into proposed divisions (or synonymized genera) has been impeded by the lack of data on the internal anatomy of these animals, and few identified morphological synapomorphies

(Jensen 1992). Moreover, the intraspecific polymorphism within Mediterranean and Eastern Atlantic *Elysia* species that share an overall cryptic green colour has resulted in historical confusion about the identity and true number of species and genera in this region that persists to the present day (e.g. Pruvot-Fol 1946, Marcus 1980, Bouchet 1984, Thompson and Jaklin 1988).

In the last 10 years, molecular phylogenetic analyses focused at the genus level have included diverse *Elysia* species in efforts to reconstruct the evolution of larval development in Sacoglossa (Vendetti *et al.* 2012, Krug *et al.* 2015), to analyse photosynthetic activity levels and determine the origin of kleptoplasty (Händeler *et al.* 2009, Christa *et al.* 2014a, Rauch *et al.* 2017, 2018, Hirokane *et al.* 2022), and to conduct species delimitation studies in specific regions (Oladi *et al.* 2018) or address other evolutionary or systematic goals. However, most datasets were limited in the number of ingroup species sampled (Bass and Karl 2006, Krug *et al.* 2011, 2013). The comprehensive work of Krug *et al.* (2016) on the systematics of Caribbean *Elysia* species was a step forward in the revision of *Elysia* in that region. This study not only contributes to settling many pending taxonomic or nomenclatural issues, using morphological and developmental data to reinforce conclusions drawn from molecular data, but also pinpoints those in need of further revision.

Although the majority of species included in *Elysia* occur in tropical and subtropical regions, several are found in temperate regions, and a few have an unexpectedly wide distribution (Clark and Goetzfried 1978, Bouchet 1984, Clark and DeFreese 1987, Jensen 2007). There are 12 currently accepted species of *Elysia* on the Atlantic coast of Europe, including the four archipelagos from Macaronesia *sensu lato* (the Azores, Madeira, the Canary Islands, and Cape Verde) and the Mediterranean Sea: *E. viridis* Montagu, 1804; *E. timida* Risso, 1818; *E. ornata* Swainson, 1840; *E. grandifolia* Kelaart, 1858 (non-native species); *E. flava* Verrill, 1901; *Elysia cf. nealae* Ostergaard, 1955 (non-native species); *E. translucens* Pruvot-Fol 1957; *E. margaritae* Fez 1962; *E. hetta* Perrone 1990; *E. tomentosa* Jensen, 1997 (non-native species); *E. manriquei* Ortea and Moro, 2009; and *E. rubeni* Martín-Hervás *et al.* 2020 (Gofas *et al.* 2001, Cervera *et al.* 2004, Yokes and Rudman 2004, Borges *et al.* 2010, Pasternak and Galil 2012, Manousis *et al.* 2020, Trainito *et al.* 2022). In addition, there are two more *Elysia* spp. with a controversial status. *Elysia fezi* Vilella, 1968 is classified as a *taxon inquirendum* by MolluscaBase (2023b), since it has not been collected since its description, but Bouchet (1984) considers *E. fezi* as a valid species if its description is correct. *Elysia gordanae* Thompson & Jaklin 1988 has been considered a synonym of *E. margaritae* by Ortea *et al.* (2017) but without a meaningful justification. Besides the above species, several older names exist, most of which have been synonymized with other Mediterranean species, primarily *E. viridis* (see: Bouchet 1984), but a few have been overlooked in the literature.

The monophyly of *Elysia* has been well supported in prior phylogenetic hypotheses (e.g. Krug *et al.* 2013, 2015, 2016, Hirokane *et al.* 2022). However, previous work has undersampled European taxa. Our main goals are to reassess the phylogeny of *Elysia* in order to update previous hypotheses and to identify the evolutionary relationships of species sequenced here for the first time. We further tested the monophyly of *Elysia* spp. from the Atlantic and Mediterranean regions. The molecular

analyses were integrated with radular morphology and external appearance of live animals to assess intra- and interspecific variations. We thus revised the systematics and taxonomy of eastern Atlantic and Mediterranean *Elysia*, and place all supported species in a global phylogenetic framework.

MATERIALS AND METHODS

Sample collection

Elysia specimens used in the present study ($N = 142$) were collected by the authors and colleagues from several Atlantic and Mediterranean localities by scuba-diving and were preserved in 96–100% ethanol for molecular analyses; additional specimens were obtained from museum collections. Samples were provisionally identified as six putative species based on morphology and comparison with the literature using the taxonomic expertise of the authors, but only the final identification is shown in the Supporting Information, Table S1. Voucher specimens were used from, and deposited in, the collections of the Göteborgs Naturhistoriska Museum, Gothenburg, Sweden (GNM); the Museu Nacional de História Natural e da Ciência, Lisbon, Portugal (MB); and the Museo Nacional de Ciencias Naturales, Madrid, Spain (MNCN). The sequences of 111 additional specimens of *Elysia* from GenBank were used in phylogenetic analyses. The genera *Thuridilla* Bergh 1872, *Plakobranthus* van Hasselt, 1824, and *Bosellia* Trinchese, 1891 were included as outgroup taxa, rooting trees on *Bosellia* in accordance with prior analyses (Krug *et al.* 2016).

DNA extraction, amplification, and sequencing

DNA was extracted from foot tissue of specimens using the DNeasy Blood and Tissue Kit of Qiagen (Qiagen, Inc., Valencia, CA, USA), following the manufacturer's instructions with minor changes (100 μ L of AE buffer in the final extraction). Partial sequences of two mitochondrial genes, cytochrome *c* oxidase subunit I (*COI*) and the small ribosomal subunit rRNA (16S), and one nuclear gene, histone 3 (*H3*), were amplified via polymerase chain reaction (PCR) using the following universal primers: LCO1490 and HCO2198 for *COI* (Folmer *et al.* 1994); 16S ar-L and 16S br-H for 16S (Palumbi *et al.* 2002); and H3F and H3R for *H3* (Colgan *et al.* 2000). Modified *COI* primers were used (see: Berriman *et al.* 2018) for samples that did not yield bands using Folmer primers. For the 28S gene, three overlapping fragments were amplified and concatenated using the following pairs of primers: 28SC1 and 28SD2R (Vonnemann *et al.* 2005), 28SF2 and 28SR3, and 28SF3 and 28SR1 (Morgan *et al.* 2003). The master mix for the PCR was prepared with nuclease-free water up to 25 μ L volume reaction, 2.5 μ L of Qiagen buffer (10 \times), 2.5 μ L of dNTP (2 mM), 5 μ L of 'Q-solution' (5 \times), 1.5–3.5 μ L $MgCl_2$ (25 mM), 1 μ L of each forward and reverse primer (10 μ M), 0.25 μ L of Qiagen DNA polymerase (5 units/ μ L), and 2 μ L of DNA.

Amplification of *COI* was performed with an initial denaturation for 2 min at 95°C, followed by 35 cycles of 30 s at 94°C, 45 s annealing at 45°C and 45 s at 72°C, with a final extension of 5 min at 72°C. The 16S amplification began with an initial denaturation for 2 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s annealing at 50°C and 1 min at 72°C, with a final extension of

Table 1. Maximum intraspecific (bold) and minimum COI uncorrected p-distances (%) amongst Atlantic and Mediterranean *Elysia* species included in the phylogenetic analysis depicted in [Figure 3](#).

A— <i>Elysia</i> species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 <i>E. azorica</i> sp. nov.	–															
2 <i>E. buanoi</i>	16.3	–														
3 <i>E. canguzua</i>	14.1	17.0	1.3													
4 <i>E. chlorotica</i>	16.4	17.0	11.2	1.3												
5 <i>E. christinae</i>	17.9	15.5	15.2	17.6	–											
6 <i>E. cornigera</i>	16.6	16.3	15.3	18.9	19.7	2.5										
7 <i>E. crispata</i>	13.3	18.2	13.5	16.5	17.9	18.6	6.6									
8 <i>E. ellenae</i>	16.4	18.7	13.0	17.1	19.3	18.8	12.6	–								
9 <i>E. evelinae</i>	16.0	18.1	13.2	14.0	19.0	16.9	17.6	14.7	0.5							
10 <i>E. flava</i>	10.6	17.6	16.2	16.7	18.5	16.2	17.8	19.0	17.0	1.5						
11 <i>E. gordanae</i>	16.6	16.9	13.0	14.8	19.0	15.4	20.2	16.9	16.3	16.4	2.9					
12 <i>E. lobata</i>	16.1	18.0	16.7	18.2	18.0	16.2	17.4	18.4	17.2	15.3	17.2	–				
13 <i>E. marcusii</i>	13.5	16.1	12.7	16.5	16.0	14.4	14.8	14.4	14.6	15.1	15.7	13.9	3.7			
14 <i>E. margaritae</i>	17.4	17.4	14.4	16.4	16.9	17.3	19.0	16.1	16.4	16.4	14.2	16.7	15.4	0.3		
15 <i>E. orientalis</i>	16.1	16.9	12.1	14.8	17.8	16.3	16.7	16.1	13.7	15.3	15.8	17.4	14.4	16.4	–	
16 <i>E. ornata</i>	13.8	15.5	12.1	12.6	16.3	15.4	16.7	14.4	12.6	15.5	14.6	16.2	12.2	14.2	13.3	1.1
17 <i>E. papillosa</i>	15.7	15.6	14.2	16.4	16.1	16.8	17.4	17.3	14.3	16.0	13.9	16.0	14.4	14.7	14.4	15.0
18 <i>E. patina</i>	14.3	15.8	14.9	18.0	15.9	14.2	17.5	19.0	15.7	14.6	15.9	16.0	13.7	14.4	15.4	12.2
19 <i>E. pawliki</i>	15.5	17.6	14.2	16.6	17.5	15.1	18.1	18.1	16.1	14.4	17.2	16.7	15.8	14.5	16.6	15.2
20 <i>E. pratensis</i>	16.3	17.5	15.5	18.9	16.6	13.8	17.1	19.0	17.3	16.5	16.0	13.9	13.5	16.3	16.7	15.3
21 <i>E. rubeni</i>	19.0	19.3	15.4	16.0	18.5	18.1	18.1	16.9	17.6	18.2	15.7	18.1	17.0	16.9	16.9	17.3
22 <i>E. serca</i>	16.9	18.6	13.3	15.9	17.8	18.6	17.5	17.5	16.9	17.0	16.2	18.0	14.8	17.9	14.9	14.6
23 <i>E. subornata</i>	14.9	16.4	15.3	18.6	16.2	15.6	17.9	16.6	16.7	16.4	13.6	14.2	12.1	14.9	17.3	15.4
24 <i>E. aff. subornata</i>	15.8	17.1	13.2	17.0	17.5	14.7	17.7	17.3	16.4	15.8	14.2	13.6	11.9	14.9	17.3	13.7
25 <i>E. taino</i>	16.6	14.9	14.7	17.5	17.2	16.5	17.5	18.0	14.8	18.3	17.0	18.5	16.6	15.9	15.3	14.6
26 <i>E. timida</i>	16.6	17.1	15.5	18.7	18.3	9.30	19.5	17.0	17.0	17.1	16.1	16.7	15.8	16.0	17.4	15.3
27 <i>E. velutinus</i>	17.5	16.6	16.4	15.3	17.8	15.7	18.6	17.5	16.9	17.4	15.2	18.2	17.4	15.0	17.6	15.0
28 <i>E. viridis</i>	16.0	18.5	14.5	15.7	16.7	18.3	17.7	16.7	14.7	18.5	16.9	19.0	16.5	17.2	15.0	15.5
29 <i>E. zemi</i>	16.7	17.9	15.6	19.8	17.9	16.3	18.2	17.3	15.8	16.0	15.7	16.9	14.8	16.0	17.8	14.4
30 <i>E. zuleicae</i>	16.8	9.70	16.7	16.4	16.3	16.6	18.4	17.6	17.9	16.8	15.4	15.0	14.0	15.8	16.6	15.9
31 <i>Elysia</i> sp.6	15.2	16.1	13.5	15.4	15.8	14.6	19.0	18.0	15.9	17.0	14.9	17.6	14.6	13.7	15.8	13.4
A— <i>Elysia</i> species	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
1 <i>E. azorica</i> sp. nov.																
2 <i>E. buanoi</i>																
3 <i>E. canguzua</i>																
4 <i>E. chlorotica</i>																
5 <i>E. christinae</i>																
6 <i>E. cornigera</i>																
7 <i>E. crispata</i>																
8 <i>E. ellenae</i>																
9 <i>E. evelinae</i>																
10 <i>E. flava</i>																
11 <i>E. gordanae</i>																
12 <i>E. lobata</i>																
13 <i>E. marcusii</i>																
14 <i>E. margaritae</i>																
15 <i>E. orientalis</i>																
16 <i>E. ornata</i>																
17 <i>E. papillosa</i>	1.1															
18 <i>E. patina</i>	12.2	4.6														

Table 1. Continued

A— <i>Elysia</i> species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
19 <i>E. pawliki</i>	17.1	15.2	–													
20 <i>E. pratensis</i>	15.7	13.0	14.1	0.5												
21 <i>E. rubeni</i>	16.8	18.4	17.8	18.3	1.7											
22 <i>E. serca</i>	17.8	17.0	17.0	17.5	19.1	–										
23 <i>E. subornata</i>	15.4	12.8	14.9	9.30	17.2	18.6	1.4									
24 <i>E. aff. subornata</i>	15.6	12.9	14.7	9.20	19.0	17.5	9.30	–								
25 <i>E. taino</i>	9.30	13.0	17.5	17.0	18.3	17.8	18.0	16.4	0.9							
26 <i>E. timida</i>	15.6	15.4	16.6	13.3	19.0	19.8	14.4	14.3	16.4	5						
27 <i>E. velutinus</i>	16.2	16.9	17.0	16.2	18.2	19.6	15.9	15.3	15.6	15.6	3.6					
28 <i>E. viridis</i>	16.8	17.2	17.8	18.7	17.1	16.6	17.9	16.9	16.1	17.9	17.5	4.8				
29 <i>E. zemi</i>	15.1	15.1	15.0	15.8	17.8	18.1	14.1	14.9	18.0	16.0	17.7	18.3	–			
30 <i>E. zuleicae</i>	15.3	15.5	16.7	16.1	17.1	19.0	16.5	16.0	15.2	17.6	16.8	17.0	16.5	3.7		
31 <i>Elysia</i> sp.6	13.8	13.9	14.6	12.8	18.0	18.0	12.6	12.6	13.7	14.0	9.40	17.5	15.6	14.5	–	

7 min at 72°C. *H3* amplification was performed with an initial denaturation for 2 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s annealing at 45°C and 1 min at 72°C, with a final extension of 7 min at 72°C. Finally, the amplification of 28S gene was accomplished using the reaction conditions previously described by Krug *et al.* (2008). Successful PCR products were purified and sequenced by MacroGen Spain (Madrid, ES).

Sequence editing and alignment

The resulting forward and reverse reads were assembled, edited, and consensus sequences extracted using GENEIOUS v.10.0.9 (Kearse *et al.* 2012). MAFFT v.7.402 server (Katoh and Standley 2013) was employed to align the sequences using the L-INS-i iterative refinement algorithm via the CIPRES Portal Science Gateway (Miller *et al.* 2010). The alignments were manually refined using MacClade v.4.06 (Maddison and Maddison 2005). Protein-coding sequences were translated into amino acids for confirmation of alignment and the most variable regions from the 16S gene were removed using the less stringent criteria in GBlocks v.0.91b (Talavera and Castresana 2007). After primer removal, aligned sequence partitions were of 328 bp (*H3*), 658 bp (*COI*), 419 bp (16S), and 1393 bp (28S). The best-fit models of evolution were determined for each gene using the Akaike information criterion AIC (Akaike 1998) implemented in jModelTest 2.1.10 (Darriba *et al.* 2012). The evolutionary model selected for each of the four genes was the general time-reversible (GTR+I+ Γ).

Species delimitation analyses

The automatic barcode gap discovery (ABGD) method (Puillandre *et al.* 2012) was performed for the *COI* alignment using the online version of the software (available at <https://bioinfo.mnhn.fr/abi/public/abgd/>) with the default settings to generate a preliminary partition of sequences. To apply ABGD to specimens for which *COI* sequences were not available, 16S sequences were instead analysed. The general mixed Yule coalescent (GMYC) method (Fujisawa and Barraclough 2013) and the Bayesian Poisson tree processes (bPTP) analysis (Zhang *et al.*

2013) were conducted on the *COI* ingroup sequences through the bPTP web-server (<https://species.h-its.org>). GMYC was executed with the single threshold parameter and bPTP analysis was done using the best ML tree, running 300 000 MCMC generations, Thinning = 100 and Burn-in = 0.1. Minimum and maximum pairwise uncorrected *p*-distance values of *COI* were calculated among specimens sequenced in this study and their West Atlantic congeners using MEGA X (Kumar *et al.* 2018) (Table 1).

Phylogenetic analyses

Bayesian Inference (BI) and maximum likelihood (ML) analyses were conducted for two different datasets: a shorter dataset with three concatenated genes (*H3*, *COI*, and 16S, 1405 bp) and a longer dataset with four concatenated genes (*H3*, *COI*, 16S, and 28S, 2798 bp). The concatenated dataset with three genes included sequences from (i) specimens collected from European waters, (ii) museum specimens, and (iii) a representative of each of the remaining *Elysia* species from the Atlantic available in GenBank that was previously differentiated with species delimitation analyses. Phylogenetic analyses based on this dataset were used to identify our specimens, as well as to infer supported relationships between the Atlantic and Mediterranean species. In order to perform the four-gene concatenated phylogenetic tree we used only one exemplar per species, including all representatives of *Elysia* in GenBank, according to the results from species delimitation analyses (only shown ABGD results). Phylogenetic analyses were then used to detect the position of *Elysia* spp. from European waters (hereinafter referred to as European *Elysia* species, EES) in a reliable global phylogenetic framework.

BI analyses were performed for both concatenated datasets using the software package MrBayes v.3.2.6 (Ronquist *et al.* 2012) for 30 and 50 million generations, respectively, with two independent runs with four MCMC chains, a sampling frequency of 1000 generations and a 'burn-in' of 25%. Nodal support was estimated as posterior probabilities (PP), with values $\geq 90\%$ taken as significant (Huelsenbeck and Rannala 2004).

ML analyses were conducted using the software RAxML v.7.0.4 (Stamatakis 2006). To determine the nodal support in ML, a non-parametric bootstrap (BS) with 5000 pseudoreplicates was implemented under the GTR+I+ Γ model selected for the original dataset partitioned for each gene. Values $\geq 75\%$ were considered statistically significant. The trees obtained were visualized in FigTree v.1.4.2 (Rambaut 2012) and edited in Adobe Illustrator CC 2018.

Morphological examination

To compare external morphological characteristics among species, samples were examined in the laboratory and photographed alive to record colour patterns of the body, dorsal 'vessels' [actually sinuses according to Neusser et al. (2019)], and rhinophores. Internal organs were examined after the removal of dorsal tissue and drawn using a Leica Wild M8 dissecting microscope and a Nikon camera lucida connected to a Nikon compound microscope. Special attention was paid to the buccal mass to isolate the radula, and the reproductive system was carefully examined to determine if a chitinous stylet was present on the penis from each delimited candidate species. These structures were photographed using a Nikon DS-Fi1 digital camera to document the position of organs. Buccal masses were removed and placed in 10% NaOH solution for 72 h to dissolve excess tissue. The radulae were rinsed in distilled water and then rinsed with 96% ethanol prior to drying. Clean radulae were finally mounted on scanning electron microscope (SEM) stubs for sputter coating. Radulae were visualized using a Nova NanoSEM™ scanning electron microscope available at the SC-ICYT, University of Cadiz (Puerto Real, Spain).

RESULTS

Species delimitation and genetic distances

A total of 638 elysioid specimens were used in the species delimitation analysis (ABGD, GMYC, and bPTP), of which 114 were exclusively from Atlantic and Mediterranean waters, and successfully sequenced in this study, while 524 worldwide specimens were obtained from GenBank.

Analyses of the COI data by GMYC estimated 111 species in our dataset with a minimum of 107 and a maximum of 118 species, whereas ABGD (92 spp.; Supporting Information, Fig. S1) and bPTP (106 spp.) analyses recovered slightly fewer worldwide entities (Table 2). The species assignments were highly consistent when comparing methods, but GMYC and bPTP oversplit taxa relative to ABGD (Fig. 1). The ABGD analysis with 16S also recovered species *Elysia* sp. 30 and *Elysia* sp. 10, for which COI data were not available, yielding a total of 94 species

identified in our dataset. Thus, according to ABGD results a barcoding gap was supported between 6% and 7% for *Elysia* (Fig. 2).

The maximum intraspecific uncorrected *p*-distance was 6.6% for specimens of *Elysia crispata*. For other species from the Atlantic Ocean and the Mediterranean Sea it ranged from 0% to 5.0% (Table 1), consistent with the COI threshold established by ABGD (Table 2).

Regarding the EES (Fig. 3), the three methods supported a new candidate species from the Azores provisionally identified as *E. flava* (*Elysia azorica* sp. nov.), as well as seven previously recognized species: *E. evelinae* Er. Marcus 1957 here recorded for the first time in European waters, *E. flava*, *E. gordanae*, *E. margaritae*, *E. rubeni*, *E. timida*, and *E. viridis*. Of those, *E. flava* and *E. evelinae* are present on both sides of the Atlantic Ocean, including the Mediterranean.

Phylogenetic analysis

BI and ML phylogenetic analyses of three concatenated genes (*H3* + COI + 16S), including only the Atlantic and Mediterranean species of *Elysia*, returned similar topologies (Fig. 3); only the tree obtained by BI analysis is shown, which yielded generally high support at most nodes. All *Elysia* spp. formed a maximally supported clade (PP = 1, BS = 100) in a tree rooted on *Bosellia* (not shown) with *Thuridilla* and *Plakobranthus* as outgroups. The EES (indicated with bold branches) were distributed in four different subclades labelled from A to D (Fig. 3).

The amphi-Atlantic distribution of *E. timida* proposed by Carmona et al. (2011) is here recognized as a misidentification of the location from a specimen of *E. timida* from the Florida Keys (Carmona et al. 2011; Supporting Information, Table S1) that was actually collected in France (see: Bass 2006), making that species an EES.

From the alignment of the longer concatenated dataset (*H3* + COI + 16 + 28S) with worldwide representatives of *Elysia*, we obtained a phylogenetic hypothesis by BI (Fig. 1). The topology of the ML concatenated tree was entirely congruent with the BI results and thus the ML tree is not shown. All *Elysia* specimens formed a maximally supported clade (PP = 1, BS = 100) when rooted on *Bosellia* (not shown) with *Thuridilla* and *Plakobranthus* as outgroups. *Elysia* specimens from the eastern Atlantic and Mediterranean did not form a monophyletic group.

The sister-relationships between some EES from the three-gene analysis were slightly changed when all available *Elysia* spp. were included. For instance, the phylogenetic position of the species from Clade A (Fig. 3) was not the same in the four-genes analyses; the sister-pair *E. gordanae* and *E. margaritae* (PP = 0.96,

Table 2. Species delineation analyses in *Elysia* based on COI barcoding fragment of 638 specimens from both GenBank ($N = 524$) and obtained in this study ($N = 114$)

Analysis type	#Entities	Statistics
ABGD	92	Prior maximal distance (P): 0.0129; Threshold of interspecific divergence: 6–7%
GMYC Single	111	Likelihood of null model: 3615.68; Likelihood of GMYC model: 3719.09; Likelihood ratio: 206.83; Confidence interval: 107–118
bPTP	106	Acceptance rate: 0.2672; Merge: 250166; Split: 249834; Confidence interval: 105–145

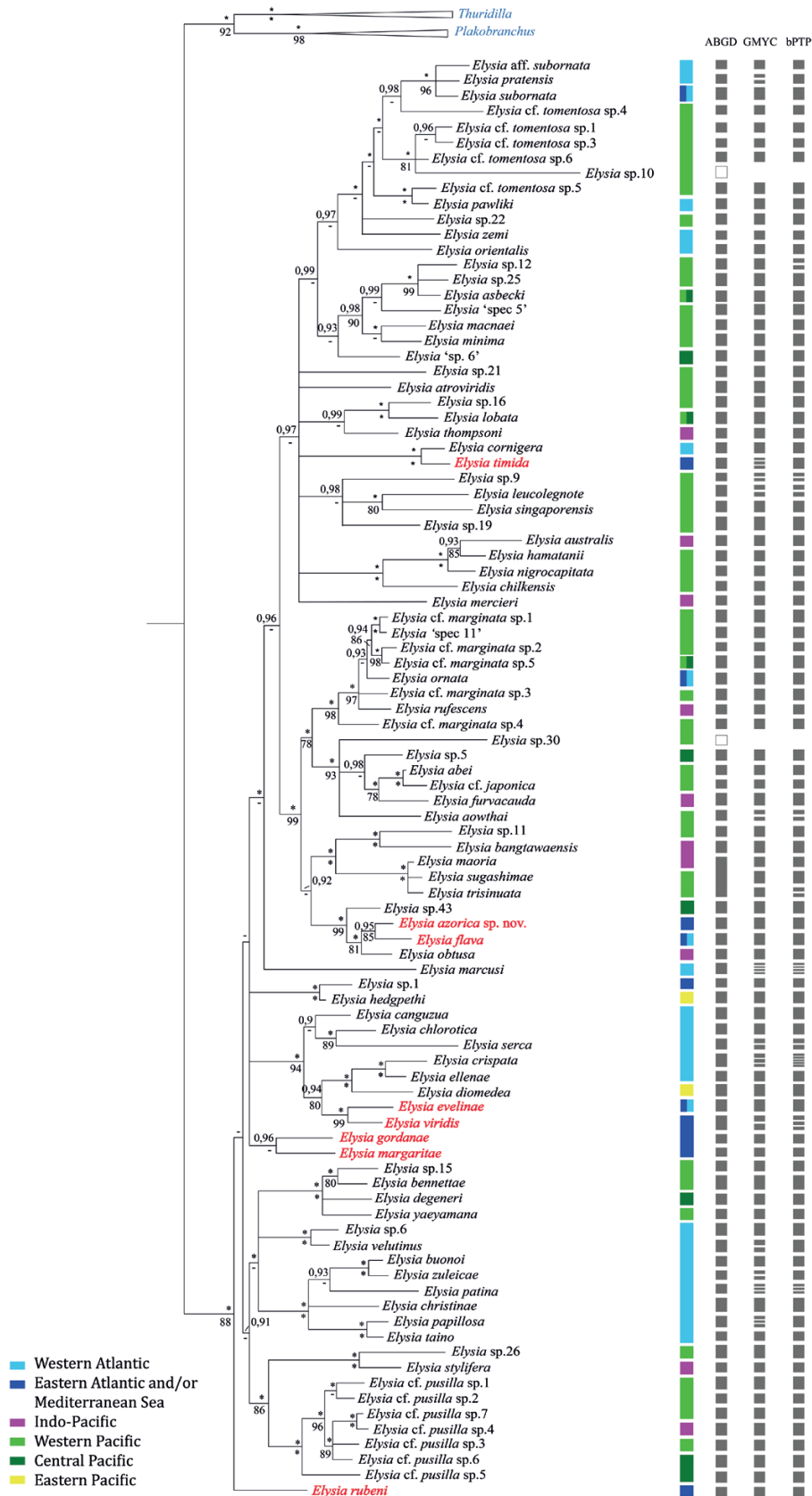


Figure 1. Molecular phylogeny of worldwide *Elysia* species rooted on genus *Bosellia* (not shown). Topology is based on Bayesian inference analysis of concatenated dataset (*H3* + *COI* + *16S* + *28S*). Only one specimen per species is shown according to species delimitation analyses. Bold red species names indicate the species sequenced in this study that are present in European waters. Significant support values are given as ML bootstrap percentages (BS, below branch) and BI posterior probabilities (PP, above branch). Asterisks indicate complete support (PP = 1.0, BS = 100%). Vertical grey bars indicate specimens grouped as species entities by ABGD, GMYC and bPTP methods based on the *COI* gene. Unfilled vertical bars indicate the specimens grouped as species based on the *16S* gene.

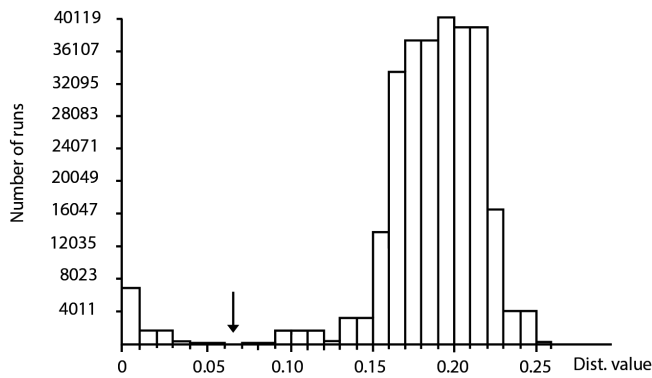


Figure 2. Histogram of pairwise genetic *COI* distances (Kimura parameter) among 638 sequences including all available National Center for Bioinformatics (NCBI) sequences for *Elysia*, showing barcoding gap; the arrow indicates the threshold that allows to distinguish intraspecific (left) and interspecific (right) distances for the *COI* region, based on ABGD analyses.

BS = not significant; Fig. 1) was closely related to the clade that includes *E. viridis* and *E. evelinae* (PP = 1, BS = 99) rather than being sister to *E. rubeni*, whose phylogenetic position in the four-gene analysis was unresolved (Fig. 1). In addition, *E. obtusa* Baba, 1938 was recovered as sister to the clade comprising *Elysia azorica* sp. nov. and *E. flava* (PP = 0.95, BS = 83; Fig. 1), instead of being sister to *E. ornata* as in the three-gene analysis (Fig. 3).

Morphological analysis

Differences in external colour patterns (Figs 4–6), as well as the morphology of radular teeth (Fig. 7) and penises, revealed diagnostic character differences among the delimited species. These variations were further supported by phylogenetic analyses (Figs 1, 3) in which supported clades corresponded to the delimited and morphologically distinct species. Consequently, a total of seven lineages of *Elysia* were identified as diagnosable species in the eastern Atlantic and Mediterranean.

Intraspecific morphological differences were observed among individuals of *E. gordanae* in the Mediterranean (Fig. 4A–F) and also in *E. viridis* specimens from the eastern Atlantic, including the Mediterranean (Fig. 5A–F). *Elysia gordanae* is characterized by a translucent greenish skin with a sprinkling of superficial iridescent red, white, and blue specks, which sometimes give it an orange rather than greenish ground colour (Fig. 4F). Some specimens can appear translucent with white spots scattered on the inner margin of the parapodia (Fig. 4B). The parapodial edge is lined by a series of white papillae that in some cases are interspersed by a bright green line (Fig. 4A, B, E). The head has a translucent patch between the eyes that expands to the rhinophores in the form of small spots to the colourless tips. *Elysia viridis* is a highly polymorphic species (see Fig. 5A–F), which can present from a vivid apple-green or bottle-green to a bright brownish colour with a speckling of tiny glistening red, yellow, blue, and green spots. In some specimens, white patches may surround eyes. The parapodia can be undulated or lobed when the animal is contracted, with whitish or reddish patches along the edges. Thus, the parapodial size and shape are very plastic in some *Elysia* species. Intraspecific differences in external morphology were not observed among specimens of other

species, such as *E. margaritae* (Fig. 4G, H), *E. evelinae* (Fig. 5G, H), *E. azorica* sp. nov. (Fig. 6A), *E. flava* (Fig. 6B), *E. rubeni* (Fig. 6C), and *E. timida* (Fig. 6D). However, it would be necessary to examine more representatives of such species to analyse potential external differences and to evaluate some characters as the presence of dorsal ‘vessels’, since in some specimens there were no evident these anatomical structures.

Internally, specimens collected from different hosts in this study had radular differences. In *E. viridis* (Fig. 7C, D), the radular teeth vary intraspecifically depending on diet, as previously noted (Jensen 1993, Rauch et al. 2018), and also in *E. gordanae* (Fig. 7G, H). In addition, the shape of the penis is not an internal reliable trait for species identification since it may be contracted, but the presence of a stylet is a key trait to consider in *Elysia* (Krug et al. 2016). No EES had a visible penial stylet.

Detailed information for comparison among the Atlantic and Mediterranean green-coloured *Elysia* species is summarized in Martín-Hervás et al. 2020 (Table 2). Based on our morphological studies, molecular results and species delimitation analyses, we present a re-description of the poorly characterized taxon *E. margaritae* and a description of the new species *Elysia azorica* sp. nov.

Systematics

Superorder Sacoglossa von Ihering, 1876

Superfamily Plakobranchoidea Gray, 1840

Family Plakobranchidae Gray, 1840

Genus *Elysia* Risso, 1818

Elysia margaritae Fez 1962

(Figs 4G, H, 7I, J)

Type material: *Elysia margaritae*—untraceable.

Material examined: MNCN15.05/90816, one specimen, dissected, 5 mm preserved length, Santa María al Bagno, Lecce, Italy, (40° 7′ 14.1″ N, 17° 59′ 56.68″ E), April 2018; MNCN15.05/90817, one specimen, dissected, 7.5 mm preserved length, Torre Lapillo, Lecce, Italy, (40° 16′ 51.10″ N, 17° 50′ 26.63″ E), May 2015; MNCN15.05/94854, one specimen, dissected, 15 mm length alive, Otranto, Lecce, Italy, (40° 9′ 11.45″ N, 18° 29′ 31.14″ E), May 2021. All specimens were collected by Fabio Vitale in infralittoral zone from 1 to 15 m depth.

External morphology:

Adults with maximum length of 15 mm (present study). Overall coloration greenish, lighter on dorsum and plantar side, with small light-blue spots irregularly arranged on outer and inner sides of parapodia and on the head. Some scattered white dots may also be present on head and sides of parapodia. Body relatively long and wide. Small eyes located behind the rhinophores. Rhinophores relatively long, rolled with a truncated apex and uniform with body colour.

Dorsal renopericardial prominence rounded with two ‘vessels’ radiating from each side, branching irregularly on the inner

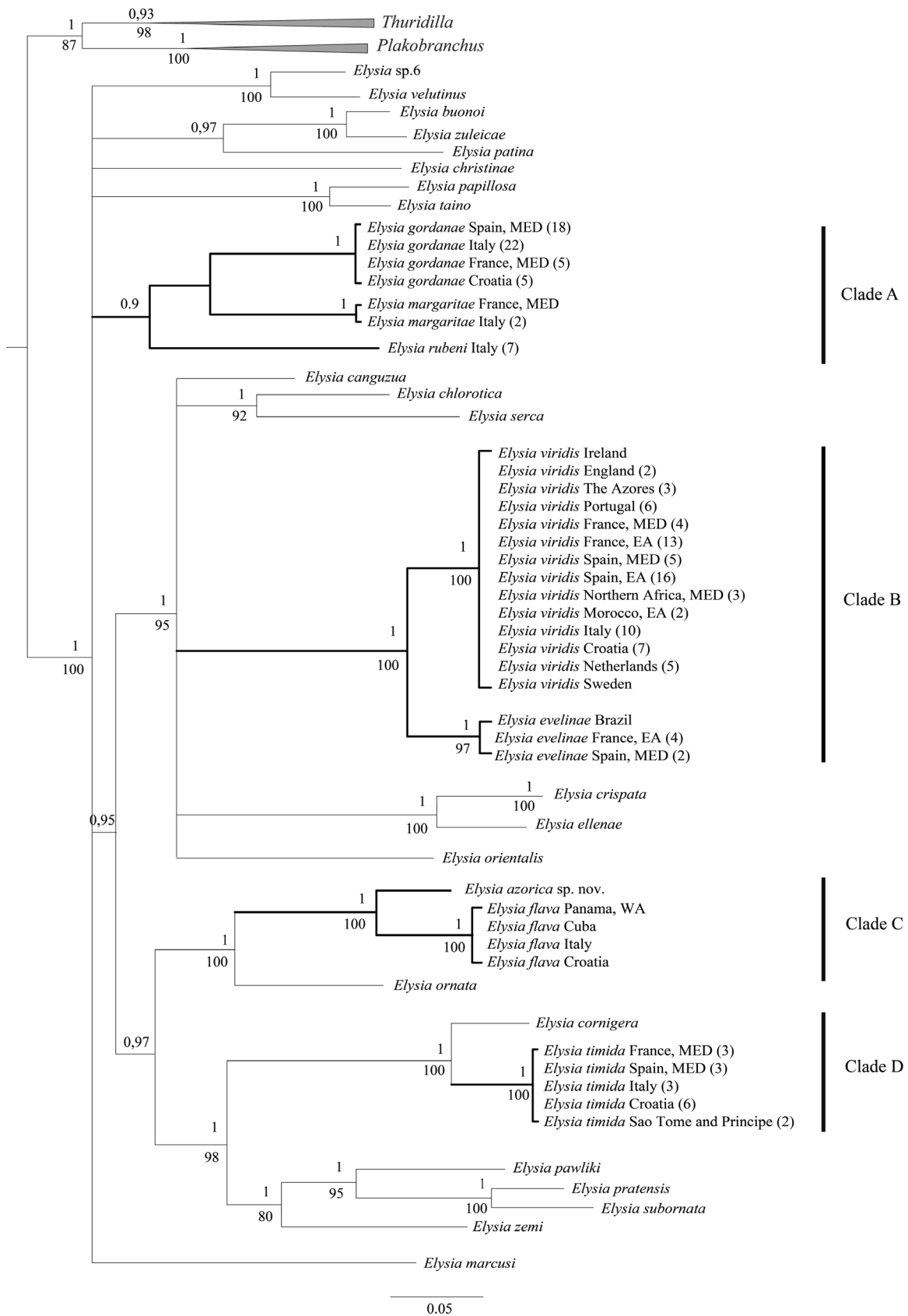


Figure 3. Molecular phylogeny of Atlantic and Mediterranean *Elysia* species rooted on genus *Bosellia* (not shown), based on the combined dataset (*H3* + *COI* + 16S) inferred by Bayesian inference analysis. Numerals in parentheses indicate the number of specimens from the same locality. Bold branches represent the *Elysia* species present in European waters arranged in four subclades labelled from A to D. Significant support values are given as ML bootstrap percentages (below branch) and BI posterior probabilities (above branch). Not supported branches are not labelled. Abbreviations: EA, eastern Atlantic Ocean; MED, Mediterranean Sea; WA, western Atlantic Ocean.

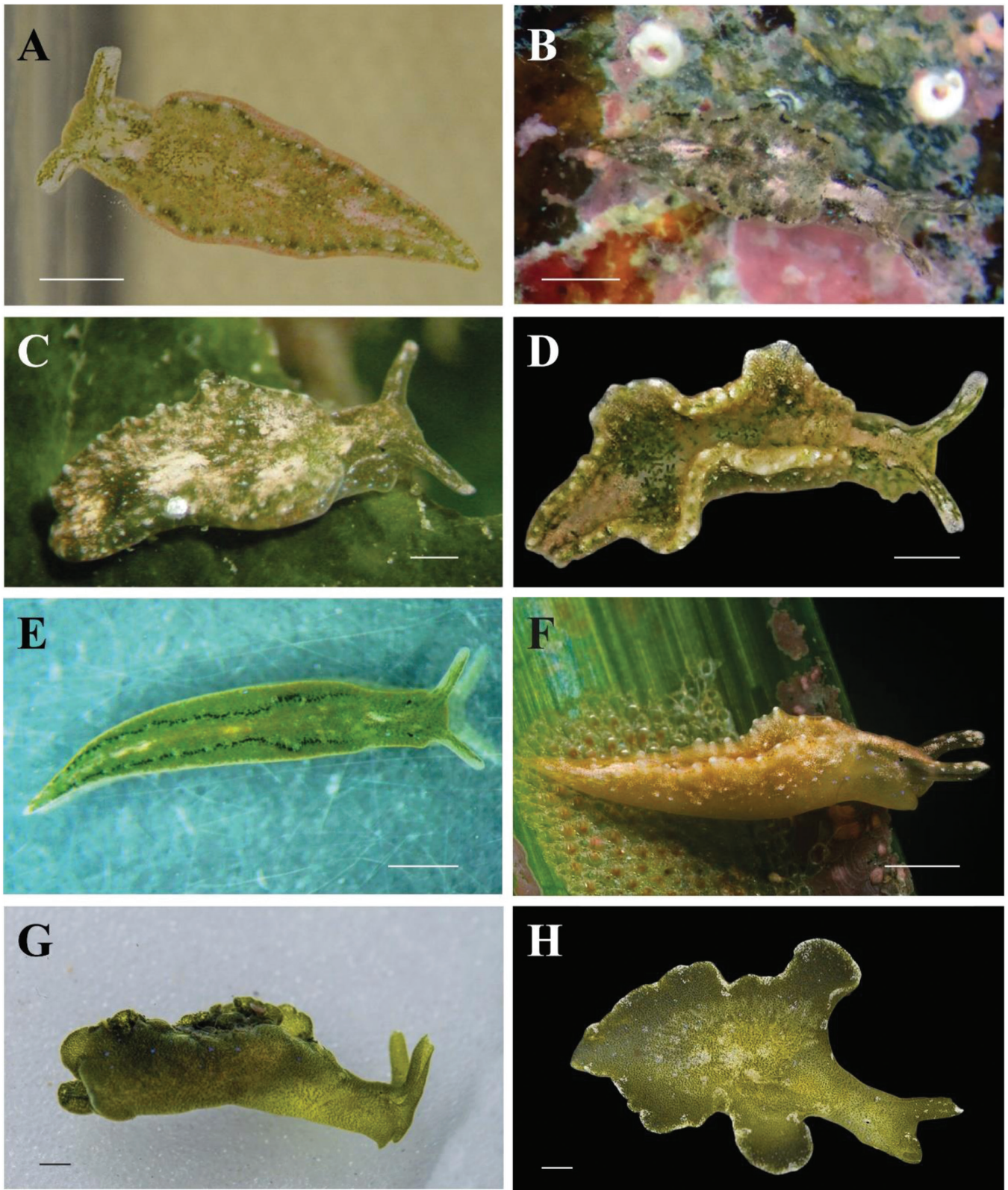


Figure 4. Living individuals of the species *Elysia gordanae* (A–F) and *Elysia margaritae* (G–H) used in this study. A, MNCN15.05/90861; B, MNCN15.05/90854; C, MNCN15.05/90855; D, MNCN15.05/90829; E, MNCN15.05/90841; F, MNCN15.05/90839; G, MNCN15.05/90816; H, MNCN15.05/90817. Photos taken by Fabio Vitale (A, G–H); Marina Poddubetskaia (B–C, E) and Alen Petani (D). Scale bars = 1 mm.

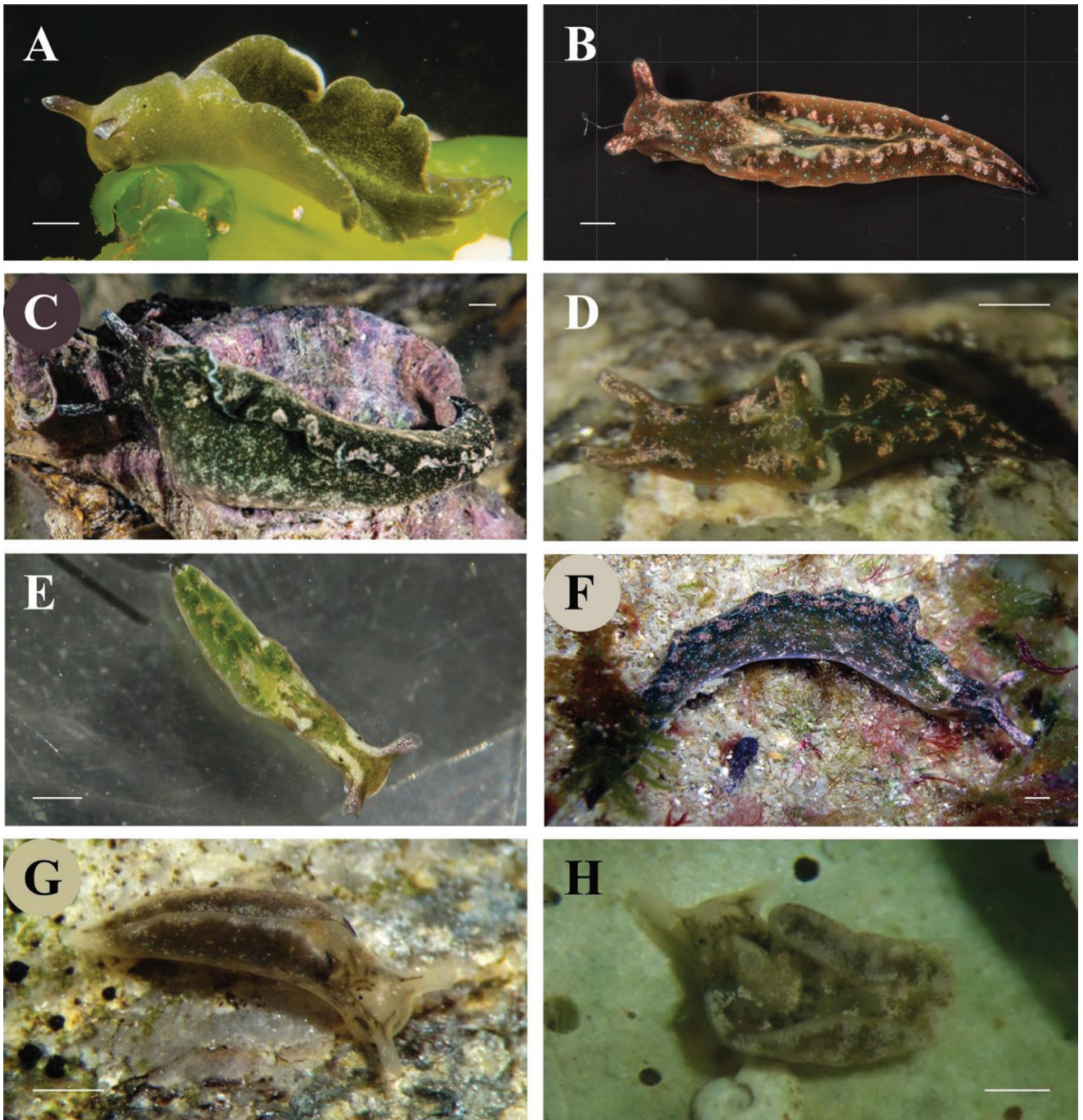


Figure 5. Living individuals of the species *Elysia viridis* (A–F) and *Elysia evelinae* (G–H) used in this study. A, MNCN15.05/90901; B, MNCN15.05/90894; C, MNCN15.05/90910; D, MNCN15.05/90898; E, MNCN15.05/90906; F, MNCN15.05/90889; G, MNCN15.05/90924; H, MNCN15.05/90923. Photos taken by Peter H. van Bragt (A); Leila Carmona (B); Gianni Colucci (C); Marina Poddubetskaia (D, G–H); Alen Petani (E) and D’Onofrio (F). Scale bars = 1 mm.

face of each parapodium. Large parapodia extending to posterior end of body, with undulated edges. Foot sole slightly narrowing to the rounded posterior end of body.

Internal anatomy:

Pharynx large and oesophagus with a smaller muscular pouch. Radula with 14–15 teeth (MNCN15.05/90817), four in ascending limb and seven in descending limb with three to four discarded teeth in the ascus (not shown). Leading tooth is about 125 μ m long

with blade-shaped cusp bearing at least 31 very small, rounded denticles (Fig. 7I, J). Shallow housing depression for interlocking teeth extending half total tooth length (Fig. 7K). Base of tooth approximately one-third of total tooth length. Penis elongate and cone-shaped, devoid of stylet with a narrow and thin vas deferens.

Geographical distribution:

Species known from the eastern Atlantic, specifically from the port of Valencia, Spain (Fez 1962); Banyuls-sur-Mer, France

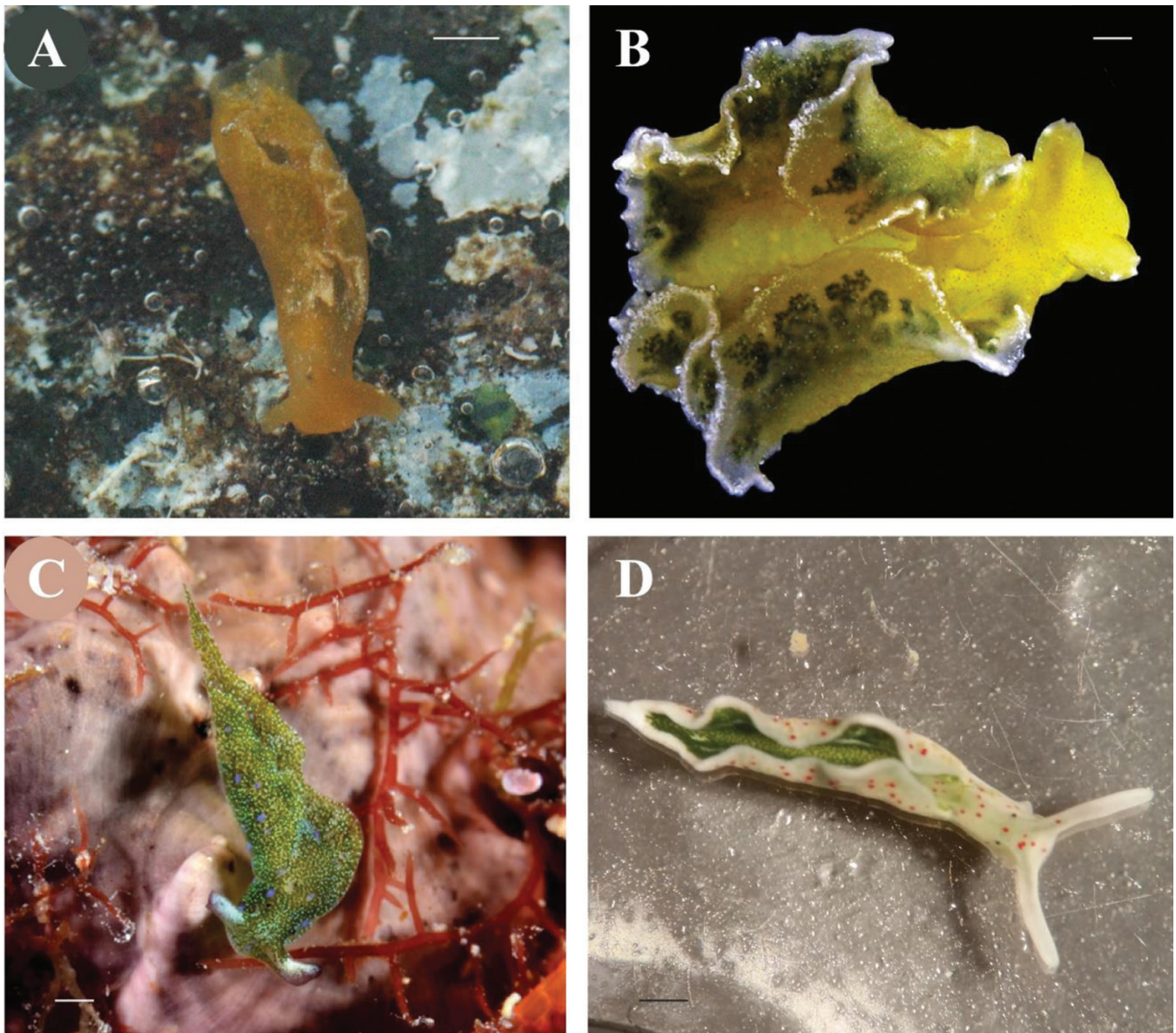


Figure 6. Living individuals of four *Elysia* species from European waters used in this study. A, *Elysia azorica* sp. nov. (MNCN15.05/47823); B, *E. flava* (MNCN15.05/90931); C, *E. rubeni* (MNCN15.05/200080); D, *E. timida* (MNCN15.05/90934). Photos taken by Manuel Malaquías (A); Jakov Prkic (B); Fabio Vitale (C) and Giulia Furfaro (D). Scale bars = 1 mm.

[specimen identified as *Elysia translucens* #845 in Christa *et al.* (2014b); present study]; the western and eastern coast of the Puglia region, Italy (Furfaro *et al.* 2020; present study).

Remarks:

The type locality of *E. margaritae* (port of Valencia, eastern coast of Spain) has been modified by anthropogenic impacts since De Fez (1962) found three large specimens of this species. The lack of specimens collected in this area has suggested some doubts about its true taxonomic identity. The three specimens of *E. margaritae* of this study were collected in Lecce, south-eastern Italy, and those sequenced clustered phylogenetically with one more specimen from GenBank previously identified as *E. translucens* collected in Banyuls-sur-Mer, France (Mediterranean Sea).

In agreement with the original description of *E. margaritae*, the author identified radular teeth with a smooth lower cutting

edge in the dissected specimen, but in contrast fine and small denticles were noted by SEM along the edge in the present work (Fig. 7I, J). They probably went unnoticed in observations using binocular magnifying glass.

Elysia azorica sp. nov.

(Figs 6A, 7A)

urn:lsid:zoobank.org:pub:C01F2876-A12F-4B37-8841-3D19D8FC617D.

References:

Elysia flava (non Verrill, 1901): Malaquias *et al.* (2009).

Material examined:

Holotype: MNCN15.05/47823 one specimen, dissected, 6 mm length alive, Praia de Porto Pim, (38°31'27.2"N,

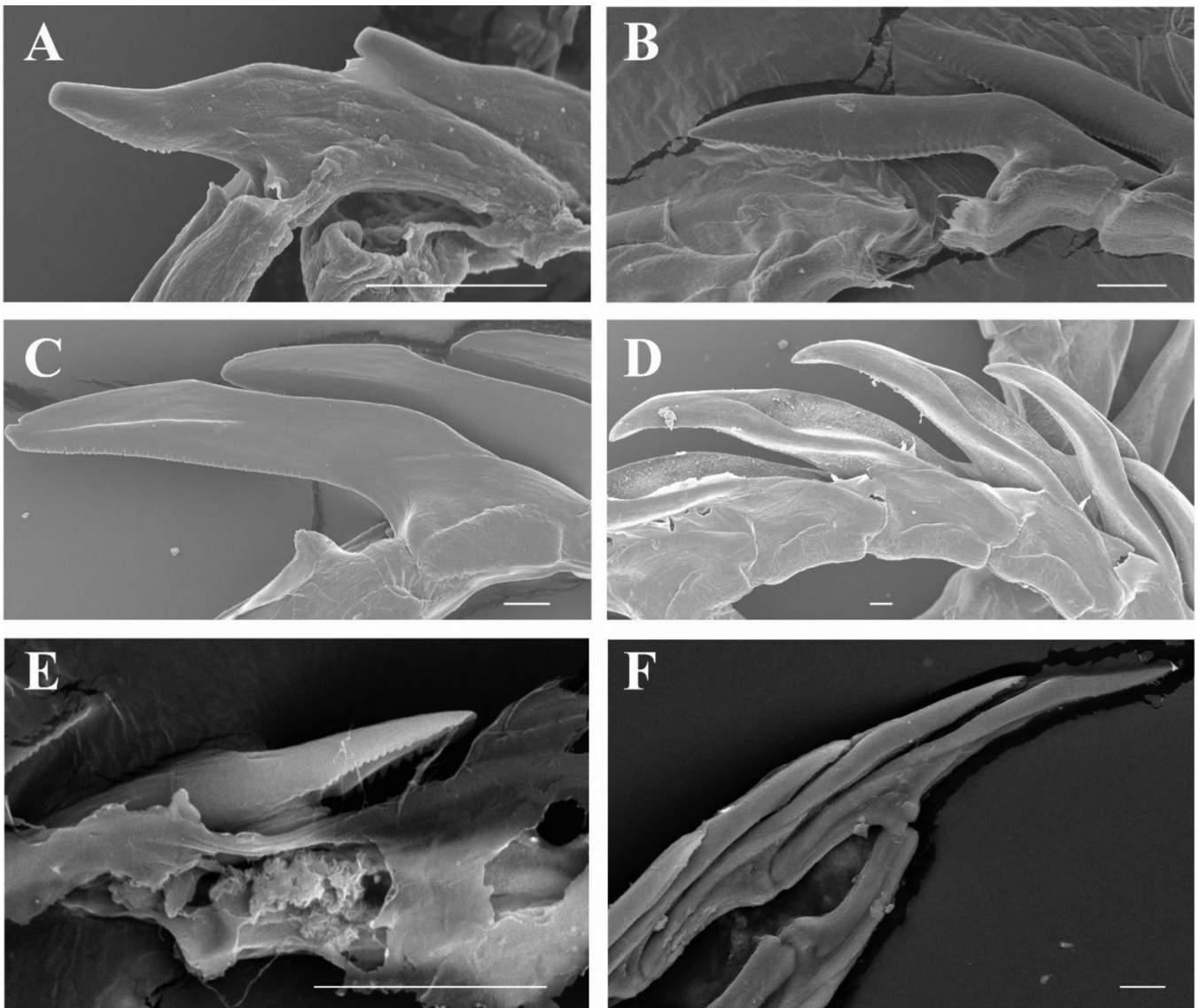


Figure 7. Scanning electron micrographs of *Elysia* spp. radular teeth. A, *E. azorica* sp. nov. (MNCN15.05/47823); B, *E. rubeni* (MNCN15.05/200080); C, D, *E. viridis*: C, MNCN15.05/90903; D, MNCN15.05/90912; E, *E. flava* (MNCN15.05/90929); F, *E. timida* (MNCN15.05/90933); G, *E. gordanae* (MNCN15.05/90843); H, *E. gordanae* (MNCN15.05/90836); I, J, K, *E. margaritae*: I, J, MNCN15.05/90817; K, MNCN15.05/94854; L, *E. evelinae* (MNCN15.05/90924). Scale bars = 10 μ m.

28°37'32.4''W), Ilha do Faial, the Azores, August 2007. Paratypes: MNCN15.05/47823A, one specimen, 3.5 mm preserved length, Praia de Porto Pim, (38°31'27.2''N, 28°37'32.4''W), Ilha do Faial, the Azores, August 2007; MNCN15.05/47823B, one specimen, 3 mm preserved length, Praia de Porto Pim, (38°31'27.2''N 28°37'32.4''W), Ilha do Faial, the Azores, August 2007. All specimens were collected by Manuel Malaquias in the infralittoral zone at 6 m depth.

External morphology:

Adults with total length about 6 mm (present study). Overall coloration orangish, with opaque white papillae on edges of parapodia (Fig. 6A). Some scattered white dots also present on head and sides of parapodia. Digestive gland visible through skin as a dark-green pigment. Body relatively short and wide. Small eyes located behind the rhinophores. Rhinophores relatively long, rolled and uniform with body colour.

Renopericardial prominence small with bright white spots. Branching of dorsal 'vessels' not evident on preserved specimens. Large parapodia extend to posterior end of body, with undulated edges. Foot sole not distinctly demarcated and the posterior end of body blunt-ended.

Internal anatomy:

Pharynx elongated and very small. Oesophagus with a small pouch. Radula with 17 teeth (MNCN15.05/47823), seven in ascending limb and 10 in descending limb. Ascus lost during preparation. Leading tooth wide, with a slightly curving cusp tip, bearing a fine, blunt denticulation on cusp and one smooth lateral edge (Fig. 7A). Housing depression for interlocking teeth extending half total tooth length. Base of tooth approximately half total tooth length.

Reproductive system triaulic (Fig. 8) with a robust and cone-shaped penis, devoid of stylet, and with a long and thin vas deferens. Female gland complex consisted of different arrangements

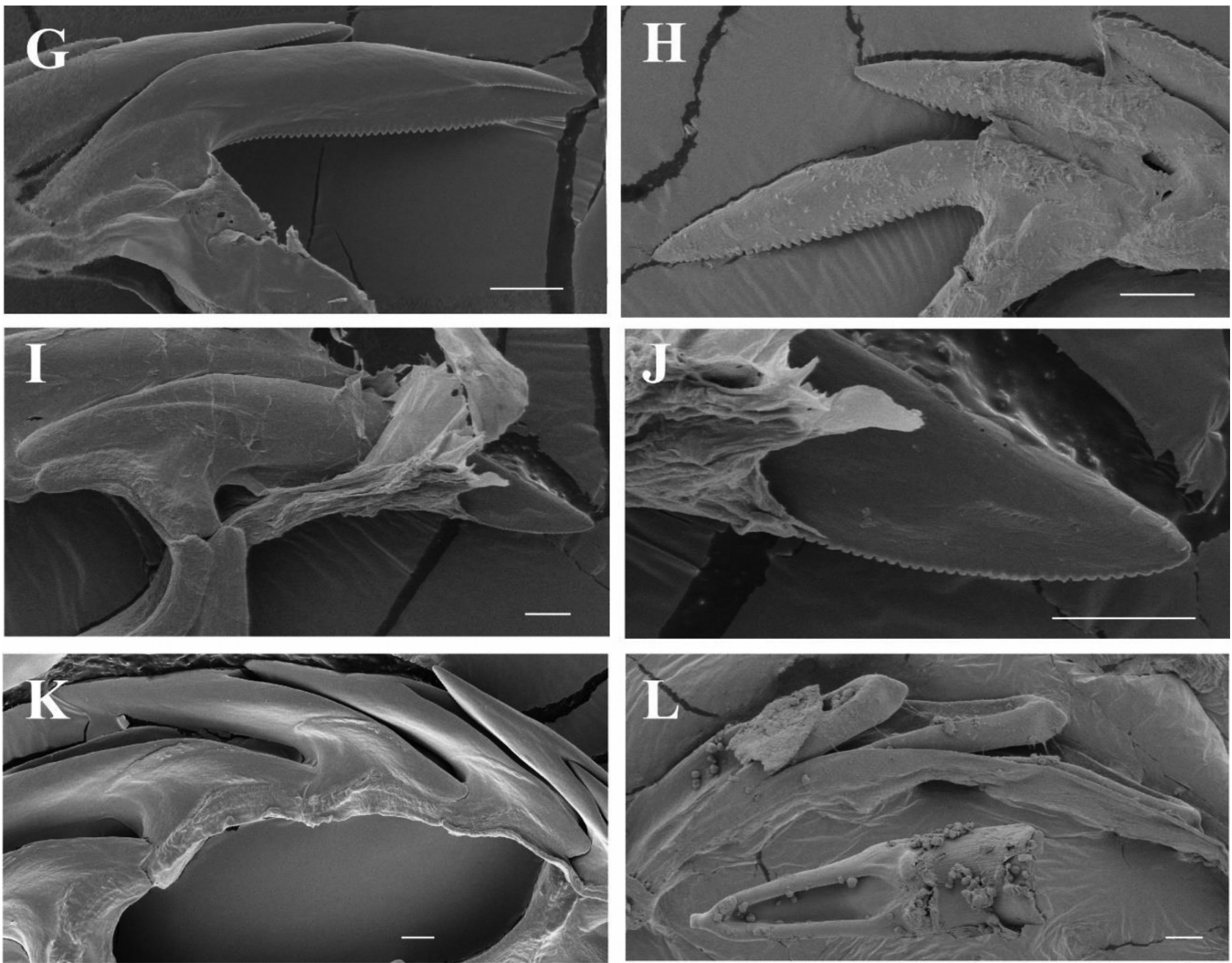


Figure 7. Continued

of glandular tissue, where the mucous gland constituted the largest portion. Hermaphroditic follicles densely distributed throughout parapodia. Rounded ampulla present and a small, spherical genital receptacle under female gland complex. Albumen gland formed by a branched system whose connections were diffuse in some sections.

Etymology:

The species name *azorica* refers to the collection site from which specimens were found (Ilha do Faial, the Azores) and is a Latin feminine adjective in the nominative case, in gender agreement with the genus.

Type locality: Praia de Porto Pim, Ilha do Faial, the Azores.

Geographical distribution:

Species only known from the eastern Atlantic, Praia de Porto Pim, Ilha do Faial, the Azores (present study).

Remarks:

Elysia azorica sp. nov. is easily recognizable alive by its bright and translucent orange colour, which allows the digestive gland to be observed through the skin, and the presence of opaque white

rounded papillae along the edge of the parapodia. Externally this species resembles the Atlantic *E. flava* and the Pacific species *E. obtusa* Baba, 1938, but they mainly differ in the external coloration. Aforementioned species have an overall translucent pale-yellow body colour with a greenish inner surface and white spots on the tips of the rhinophores, unlike the uniformly orange-yellow rhinophores of *E. azorica* sp. nov. In addition, *E. flava* and *E. obtusa* have a broader bright white line on the margins of the parapodia formed by white papillae, which may also be found in small clusters on the pericardial swelling. Also, white patches may appear scattered on the outer side of the parapodia of both species, not visible in *E. azorica* sp. nov.

The radula in *E. azorica* sp. nov. has shorter and wider blade-like teeth than those of *E. flava* and *E. obtusa*. Both *E. azorica* sp. nov. and *E. flava* show short denticulations on the cutting edge but differ in a lateral smooth edge present in *E. flava*. Likewise, *E. obtusa* possesses a different tooth shape with smooth edges without denticles or 'serrulations' (according to its original description).

Our molecular phylogenetic analyses confirm that *E. obtusa* from Indo-Pacific and Atlantic specimens of *E. flava* are genetically distinct in accordance with the analysis by [Krug *et al.*](#)

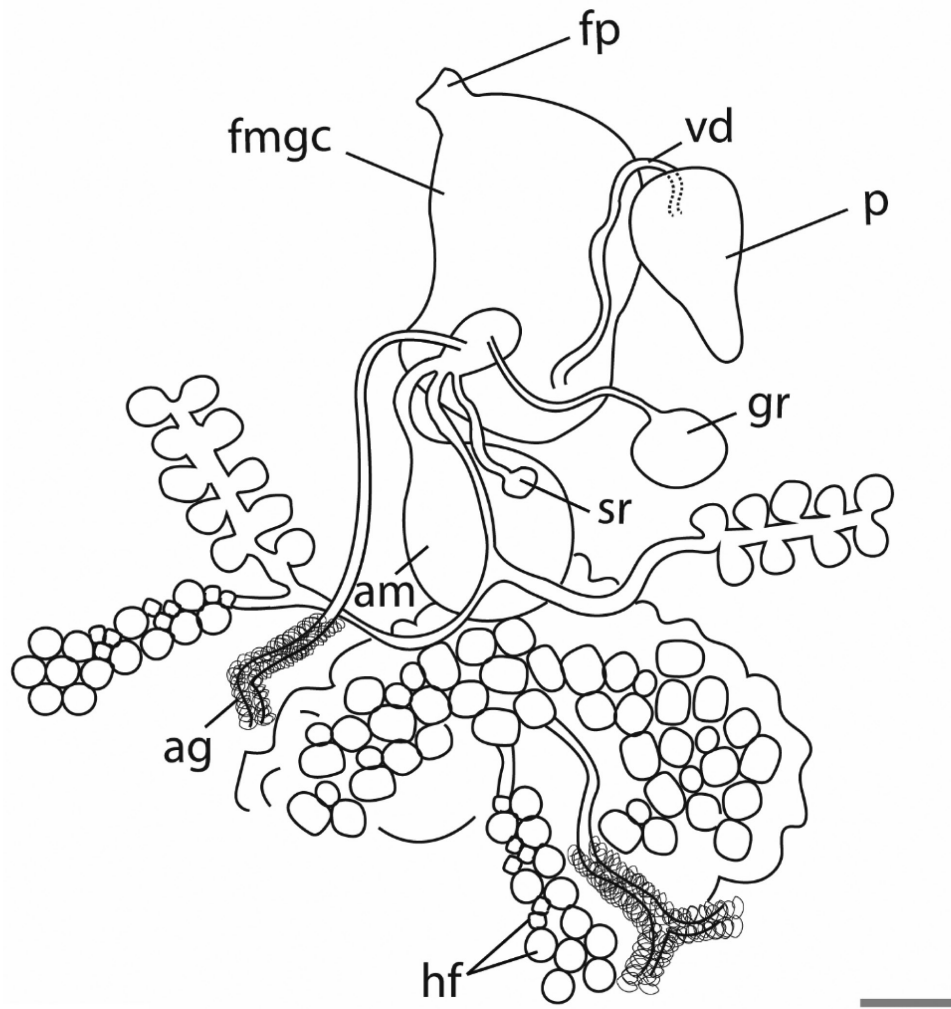


Figure 8. *Elysia azorica* sp. nov. reproductive system (MNCN 15.05/47823), scale bar = 100 μ m. Abbreviations: ag, albumen gland; am, ampulla; fmgc, female gland complex; fp, female pore; hf, hermaphroditic follicle; gr, genital receptacle; p, penis; sr, seminal receptacle; vd, vas deferens.

(2016), and both species are different from *E. azorica* sp. nov. (Fig. 1). Indeed, the minimum uncorrected *p*-distances for *COI* between *E. azorica* sp. nov. and the sister-species *E. flava* were 10.6% (see Table 1) and 10.2% between *E. azorica* sp. nov. and *E. obtusa* (not shown).

DISCUSSION

Phylogeny of *Elysia* and geographical patterns

Here, we present the most comprehensive phylogenetic hypothesis proposed for *Elysia* to date, although our taxon sampling still represents only about 60% of recognized species. While including specimens and species sampled widely across their geographical range, we focused on the European diversity and taxonomy of *Elysia* spp.. The reconstructed phylogenetic relationships resulted in numerous clades within the genus recovered with strong support; however, the EES were not recovered as a monophyletic group.

With the exception of *E. rubeni*, whose phylogenetic position is unresolved, and *E. timida*, whose sister-species is found in the western Atlantic, the remaining EES each have a sister-taxon

from the eastern Atlantic and/or the Mediterranean. It should be noted that the sibling pairs *E. flava*–*E. azorica* sp. nov. and *E. evelinae*–*E. viridis* present a particular distribution with an amphiatlantic–eastern Atlantic species, respectively. Furthermore, the pairs of sister-lineages *E. flava*–*E. azorica* sp. nov. and *E. timida*–*E. cornigera* seem to have closer affinities to Indo-West Pacific species, whereas *E. evelinae*–*E. viridis* are closely related to western Atlantic species, suggesting a possible shared evolutionary history between some members of the eastern and western Atlantic faunas, as well as the isolation of an ancestral lineage in the eastern Pacific after the closure of the Isthmus of Panama.

Little is known about data on larval development mode or host alga in the Atlantic subclade comprising *E. flava* and *E. azorica* sp. nov., but *E. flava* may feed on *Cladophora* sp. (Marín and Ros 1988). The Indo-Pacific species *E. obtusa* is the sister-taxon of the Atlantic subclade and feeds on *Bryopsis* sp. (Krug et al. 2016).

The sister-taxa *E. cornigera* and *E. timida* are allopatric within the Atlantic: *E. timida* is restricted to the eastern Atlantic and Mediterranean, while *E. cornigera* is distributed throughout the Caribbean. Both species feed on the alga *Acetabularia*, but *E. timida* shows highly efficient photosynthesis with long-term

retention of plastids, unlike the short-term retention observed in *E. cornigera* (Händeler et al. 2009, Laetz and Wägele 2018, Hirokane et al. 2022). Larval development is lecithotrophic in both species but *E. timida* also can have intracapsular development with metamorphosis of larvae before hatching (Rahat 1976, Marin and Ros 1993, Jensen 2001). Although the subclade formed by *E. cornigera*–*E. timida* is fully supported, the relationship between these two species and their possible sister clades in the Pacific Ocean have not been resolved in our analyses.

Overlapping ranges also occur in sister-taxa *E. viridis*–*E. evelinae* in the eastern Atlantic and Mediterranean Sea. *Elysia evelinae* was originally described from Brazil (Er. Marcus 1957, García et al. 2008) but also found in Costa Rica (Espinosa and Ortea 2001) and Florida (Ev. Marcus and Er. Marcus 1960, 1967, Jensen and Clark 1983, Clark 1994). In the present study, *E. evelinae* was collected from the eastern Atlantic (Cap Ferret, France) and the Mediterranean Sea (Rosas, Spain) and so inhabits both sides of the Atlantic, whereas its sister-taxon *E. viridis* is restricted to the eastern Atlantic. Both species share a common ancestor with congeners from the western Atlantic. In terms of ecology, *E. viridis* has a variety of different algal food sources, including siphonaceous species such as *Codium tomentosum* Stackhouse, 1797 and *Bryopsis hypnoides* Lamouroux, 1809 and septate species such as *Cladophora dalmatica* Kützinger, 1843 and *Chaetomorpha* spp. (Jensen 1989, 1993, Händeler et al. 2009, De Vries et al. 2014, Cartaxana et al. 2017, Rauch et al. 2018). The co-occurring *E. evelinae* primarily feeds on benthic diatoms of the genus *Biddulphia* (Jensen 1981), but also on diatoms that may be epiphytic on *Bryopsis* sp. and *Caulerpa* sp.. It is noteworthy that *E. evelinae*, which has lecithotrophic or intracapsular development, has an ampho-Atlantic distribution, whereas its sister-species, *E. viridis*, with planktotrophic development and thus presumably higher dispersal potential, has a wide distribution in the eastern Atlantic Ocean and the Mediterranean Sea, but has not been found west of the Azores (Clark and Jensen 1981, Trowbridge 2000, Trowbridge and Todd 2001, Sotka 2005). Thus, anthropogenic activities or transoceanic rafting on floating debris because of climatic phenomena (i.e. tsunamis, cyclones, storms, etc.) could facilitate long-distance distribution patterns of non-planktotrophic species and explain the invasion of *E. evelinae* in the eastern Atlantic (Carlton et al. 2017, Rech et al. 2018, Waters and Craw 2018, Lindo 2020, Póvoa et al. 2021).

The sister-pair *E. gordanae*–*E. margaritae* display overlapping ranges along European coasts of the Mediterranean Sea. A process of trophic segregation and a possible ecological speciation may occur whereby species coexist through specializing on different host algae (Simmonds et al. 2020, Rodriguez and Krug 2022). *Elysia gordanae* has been found on *Bryopsis* sp., *Derbesia* sp., and *Flabellia petiolata* (Turra) Nizamuddin, 1987 and feeding on *Acetabularia acetabulum* (Linnaeus) P.C.Silva 1952 (Poursanidis and Koutsoubas 2015) and other filamentous algae like *Cladophora* sp. (Marin and Ros 1988). Little is known about the host alga of *E. margaritae* but in the present study the species was found on *Dictyota dichotoma* (Hudson) J.V.Lamouroux, 1809, *Sphaerococcus coronopifolius* Stackhouse, 1797, and probably on *Chaetomorpha* sp.. The former two species have complex thallus microstructure, and it is highly unlikely that a sacoglossan would feed on these. Nevertheless, it is possible that the species feeds on some filamentous epiphytes. Finally, *E. rubeni* was found

on *F. petiolata* or crawling on *Peyssonnelia* sp., but its feeding has not been observed.

Assessment of diversity of European *Elysia* species

From the 13 currently accepted species within *Elysia* in the eastern Atlantic and the Mediterranean Sea, the species delimitation analyses consistently recovered six known species: *E. viridis*, *E. timida*, *E. flava*, *E. margaritae*, *E. gordanae*, and *E. rubeni*. A new species of *Elysia* was also identified in Azores, is here described as *Elysia azorica* sp. nov.. The uncorrected *p*-distances for *COI* among known members of *Elysia* from European coasts and *Elysia azorica* sp. nov. ranged from 10.6% (vs. *E. flava*) to 19% (vs. *E. rubeni*). *Elysia azorica* sp. nov. has an orangish body colour with white papillae on the parapodial edge, also visible in *E. flava*, although its general colour is yellowish-green.

The present study includes the first record of the *E. evelinae* species on European coasts of the Atlantic and Mediterranean. Only *H3* and *28S* genes of *E. evelinae* from the Caribbean were available from GenBank and agreed with those obtained in our analyses, although future work should compare *COI* sequences from both sides of the Atlantic. The molecular information is a valuable tool to minimize possible misidentifications and to facilitate the detection of trans-Atlantic species. For this reason, unless the morphological differences were very noticeable, the report of an unnoticed species originally from distant areas is inconclusive if its validity is not molecularly confirmed. For example, *Elysia amuravela* Ortea 2017, was described from the coast of Ghana. Morphologically it showed strong resemblance to *E. evelinae*, but molecular data are unavailable for this species, so it cannot be compared to our specimens of *E. evelinae* from the Atlantic coast of France and the Mediterranean.

As stated previously, *E. gordanae* has been considered a junior synonym of *E. margaritae* (see: Ortea et al. 1997, 2017, MolluscaBase 2023c), but this is not supported by either our molecular or morphological data. Despite the external resemblance of *E. margaritae* and juvenile specimens of *E. gordanae*, which have fewer nodules on the edge of the parapodia (F. Vitale, pers. obs.), these species have several distinguishing morphological features in the adult stage that differentiate them. Most noticeable are conspicuous white pustules on the parapodial edge and on the sides in *E. gordanae*, absent on the smooth edge of *E. margaritae*, and also, the large size difference of these species: *E. margaritae* was described from a few specimens 33–40 mm long, while *E. gordanae* is usually <10 mm. The overall body colour of *E. gordanae* is translucent greenish with superficial iridescent red, white, and blue specks, which sometimes gives them an orange appearance, whereas *E. margaritae* presents a bright green ground colour with small light-blue spots irregularly arranged on the outer side of the parapodia. Several internal differences have also been documented in the radulae. *Elysia margaritae* has wider teeth with smaller denticles than those of *E. gordanae*, which are noticeably thicker. Additionally, our phylogenetic results showed that *E. margaritae* and *E. gordanae* are sister-species, 14.2% divergent at *COI*, which is significantly higher than the interspecific divergence previously reported for elysiids from the Caribbean (Krug et al. 2016); thus, both species must be considered valid and distinct.

Regarding the validity of *E. translucens*, we have reviewed the literature on this species. Its original description was based on a specimen from Banyuls-sur-Mer, France (Mediterranean Sea) preserved in formalin that showed a totally translucent appearance, which Pruvot-Fol (1957) interpreted as a possible new species, without complete certainty. However, the appearance of this translucent individual, compared to other *E. viridis* specimens that the author had also fixed in formalin, finally resulted in the description of a separate species. Previous studies have revealed that specimens of *E. viridis* can be dark green, light green, brown, yellow, or almost white, depending on what and when they last fed (Rauch *et al.* 2018); the type specimen could have spent several days or weeks of starvation before being collected and its appearance could thus have resulted from that plus the fixative effect.

The most important difference between *E. translucens* and *E. viridis* was that the radular teeth of *E. translucens* were much smaller and had coarser denticles on the cutting edge (Pruvot-Fol 1957). The tooth figured in Bouchet (1984) looks considerably more like that of *E. gordanae*, which was not described until four years later (Thompson and Jaklin 1988). Thompson and Jaklin's drawing of the tooth of *E. gordanae* showed a distinctly convex outline of the cusp, a prominent articulation knob, a lateral ridge, and denticles on the cutting edge, which do not appear to be coarser than those of *E. viridis*. All of these characters are also seen in the figure of *E. translucens* in Bouchet (1984). Thompson and Jaklin (1988) also figured a tooth of a specimen identified as *E. translucens*, which was somewhat larger than that of *E. gordanae*, but had coarser denticles, less convex outline, and no visible lateral ridge. In the present study, three species have radular teeth with prominent articulation knobs: *E. margaritae*, *E. gordanae*, and *E. rubeni* (Fig. 7B, G–K; Clade A in Fig. 3). The teeth of *E. margaritae* are larger than those of the other two species, and the denticles are much finer. The teeth of *E. gordanae* show some variability concerning presence or absence of the lateral ridge and coarseness of denticles, but the size is about the same. These differences could be caused by differences in diet, as previously described for *E. viridis* (Jensen 1993, Rauch *et al.* 2018).

Externally the specimen from Bouchet (1984, fig. 2–*E. translucens*) looks somewhat similar to *E. rubeni* (Fig. 6C) and *E. margaritae* (Fig. 4G, H), and it is photographed on *Flabellum petiolata*, on which *E. rubeni* has also been found (Martín-Hervás *et al.* 2020). The drawing of *E. translucens* in Thompson and Jaklin (1988, fig. 4B, C) is not helpful in identifying the species, indeed it looks identical to the drawing of *E. viridis* in Thompson (1981: fig. 5c, d). The description of specimens identified as *E. translucens* from the Adriatic and Aegean Seas could be almost any species of *Elysia*, and the drawing of a juvenile specimen (Thompson and Jaklin 1988, fig. 5C) looks more like *E. rubeni*. Unfortunately, there is no description of external or radular morphology of the specimen identified as *E. translucens* in the molecular analysis of Christa *et al.* (2014b); this specimen came from Banyuls-sur-Mer, France, and we have included three specimens from this locality in the present study. All specimens, including the sequence from GenBank, grouped with specimens of *E. gordanae* from all other localities in the Mediterranean. Thus, with the information about *E. translucens* presently available, it is not possible to determine whether it is a separate species, and therefore it should be considered a *taxon inquirendum*.

The status of the Mediterranean *Elysia hetta* as a valid species is questioned. This taxon is characterized by underdeveloped parapodia and large white papillae on the edge of parapodia and on the sides, cephalic region and pericardium with whitish coloration and numerous red granules distributed on both sides, internal and external to the parapodia (Perrone 1990). Although all these characteristics are consistent with those visible in *E. gordanae*, Perrone (1990) described *E. hetta* as a different species because of its rounded and thickened foot that in *E. gordanae* seems thin and elongated, but this is not a determining character since the animal can contract and show such an aspect. Also, Perrone (1990) argued the presence of pinkish granules in *E. gordanae* gave it a fleshy appearance, not visible in *E. hetta*. Upon examining photographs of several specimens of *E. gordanae* included in our phylogenetic study, a high degree of intraspecific chromatic variability was evident. Thus, most likely, the description of *E. hetta* was based on a specimen of *E. gordanae* and thus *E. hetta* should be considered a junior synonym of that species. Based on these observations, the images of nominal *E. hetta* reproduced by Micaroni (2015) and Trainito and Doneddu (2016) in their contributions presumably correspond to *E. gordanae*. The egg mass illustrated in Trainito and Doneddu (2016) looks identical to one in the Sea Slug Forum (Koehler 2001; <http://www.seaslugforum.net/find/4160>).

Several old names for *Elysia* species in the Mediterranean and north-east Atlantic exist. Some of these species were originally described under different genus names as well. These include *Aplysiopterus neapolitanus* Delle Chiaje 1830, *Elisia* [sic] *marmorata* Cantraine 1835, *Actæon minuta* M.Sars, 1835, *Elysia fusca* Philippi 1844, *Acteon elegans* Quatrefages 1844, *Elysia albomarginata* Trinchese 1869, and *Elysia viridissima* Trinchese 1869. The latter two species were described in a summary of a presentation on the use of a binocular microscope for zoological studies (Trinchese 1869), and hence have been overlooked by most subsequent malacologists. Mazzarelli (1903) listed *E. viridis* and *E. viridissima* as separate species. The identity of these two species remains uncertain. *Elysia albomarginata* has white papillae on parapodial margins and could be *E. gordanae* or *E. flava*. *Elysia viridissima* could be *E. viridis* or *E. margaritae* due to its large size. At the present time both should be considered *taxa inquirenda*. The name *Rhyzobranchus temminckii* Cantraine, 1827 apparently was not properly published; there are some references in the literature to either genus *Rhyzobranchus* without a specific epithet (Cantraine 1835) or *Rhyzobranchus* [sic] *Temminckii* Cantr. Correspondance en 1827 (Philippi 1844: 101). Cantraine had realized already in 1835 (p. 384) that Risso's genus name *Elysia* predated his unpublished *Rhyzobranchus*, and in 1840 (p. 66) he added the species name *temminckii* to the list of synonyms for *Elysia viridis*. Pruvot-Fol (1946) listed most of the above species as (questionable) synonyms of *E. viridis*, but she left *E. minuta* as a separate species based on the figure of radular teeth given in Bergh (1872: pl. 22, fig. 25). The figure, however, is very similar to teeth of *E. viridis* feeding on *Chaetomorpha* (see: Jensen 1993: fig. 2C, D), and hence *E. minuta* is a synonym of *E. viridis*, as listed in MolluscaBase (2023d). Bouchet (1984) also listed most of the old names as synonyms of *E. viridis*, and these are also listed as such in MolluscaBase (2023e). Based on the original illustrations (Delle Chiaje 1830: pl. 51, fig. 5), *Aplysiopterus neapolitanus* is almost certainly a synonym of *E.*

viridis. The un-illustrated *E. marmorata* (see: Cantraine 1835: 385) may also be a synonym of this species, but it could also be *E. gordanae* or *E. margaritae*, since both have blue dots and pale parapodial margins. The tiny outline sketch accompanying the description of *E. fusca* (see: Philippi 1844: 100–101, pl. 22, fig. 4) may be a synonym of *E. viridis*, but the spotted appearance and mention of red spots could also be *E. timida*. *Acteon elegans* (= *Elysia elegans*), based on the original illustration (Quatrefages 1844: pl. 3, fig. 5), appears to be a synonym of *E. timida* rather than *E. viridis*, as already pointed out by Fischer (1867).

In order to perform a complete revision of European *Elysia* species, it would be necessary to compare all original descriptions with newly collected material, photographed and properly preserved for molecular analysis. Thus, it would be possible to determine if the taxonomical status of other *Elysia* species cited or described for European coasts (i.e. *E. ornata*, *E. subornata*, *E. tomentosa*, *E. grandifolia*, *E. cf. nealae*, *E. fezi*, and *E. manriquei*), as well as species from other regions in the Eastern Atlantic as Cape Verde and the Gulf of Guinea (*E. amuravela* and *E. sanfermin* Ortea 2017), are valid taxa, and/or actually occur in European waters.

Although material of the aforementioned species could not be collected from the Eastern Atlantic for the present study, sequences from Caribbean representatives of *E. ornata* and *E. subornata*, and from the Indian and Pacific Oceans specimens of *E. tomentosa* and *E. grandifolia*, were included from GenBank. Most of the above-mentioned species are taxonomically problematic or parts of cryptic species complexes (Krug et al. 2013, 2016), and records of these from European waters may be based on misidentifications or recent introductions from other regions by anthropogenic activities.

Colour variations and morphological similarities impede simple and quick identification of many *Elysia* species, but colour details allow some species to be differentiated; others require integrative approaches combining molecular and morphological data for species identification. High intraspecific polymorphism in morphology was observed in European species such as *E. gordanae* and *E. viridis*. The present work includes a range of morphotypes that reveal the chromatic polymorphisms of both species, facilitating their identification. By providing comprehensive descriptions of these species, we establish a solid taxonomic framework that will facilitate accurate identifications, clarify distribution patterns, and enable future work to better understand the ecological roles of this group in marine ecosystems.

CONCLUSION

This study provides the most complete phylogenetic hypothesis proposed for *Elysia* until now. Molecular and morphological analyses of the genus have revealed a new undescribed pseudocryptic species in the course of reviewing the taxonomic status of nine to 15 species identified on the European Atlantic and Mediterranean coasts. We have updated the phylogenetic systematics of this highly diverse genus in a global phylogenetic framework, including about the 60% of the valid species of *Elysia*. However, further work is needed to fully elucidate the diversity of this genus, since about 40 species are undescribed and others are only distinguished by their molecular divergence.

SUPPLEMENTARY DATA

Supplementary data is available at *Zoological Journal of the Linnean Society* Journal online.

Table S1. Dataset of sacoglossan specimens used for this study including locality, voucher number and GenBank accession numbers. Novel sequences are marked with an asterisk (*). Specimens whose sequences were retrieved from GenBank appear here with the specific names assigned by their authors.

Figure S1. Dataset of recovered groups from the output of the Automatic Barcode Gap Discovery (ABGD) web-interface (<http://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>) for all available NCBI *Elysia* COI sequences (n = 524, bolded NCBI accession labels) and those of the specimens collected in this study (n = 114) showing results of the Kimura (K80) model.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

DATA AVAILABILITY

The data underlying this article are available in the article and in its online Supporting Information.

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