Abstract

The evolutionary relationships among genera within the nudibranch family Discodoridídae remain poorly understood, with comprehensive molecular studies still largely absent. *Discodoris*, the most species-rich genus within this family, has historically represented a wastebasket taxon where species with 'discodoridid' morphology were placed. In this study, we present molecular data to evaluate the taxonomic classification of the family and to investigate potential (pseudo)cryptic diversity. Our molecular analyses comprised a multilocus phylogenetic analysis of 199 specimens, including 20 type species. The dataset included 142 specimens belonging to Discodoridídae, 24 from Doridídae, and 33 outgroup taxa. A total of 52 specimens from 30 different species of Discodoridídae, including six type taxa, were sequenced from the Mediterranean Sea, Eastern Atlantic Ocean, and Central and South America. Phylogenetic analyses recovered the monophyly of the family Discodoridídae but including the doridid *Aphelodoris*, revealing evidence of hidden diversity in several genera such as *Taringa*, *Tayuva*, *Paradoris*, and *Geitodoris*. Our study unveiled the paraphyly of the genera *Discodoris* and *Diaulula,* which warrant a critical appraisal of their morphology. Overall, we provide relevant molecular information to infer the most complete phylogeny of Discodoridídae to date and identify new questions for future studies.

Keywords: Cryptic diversity; Doridina; Doridoidea; Heterobranchia; Marine biodiversity; Phylogenetics; Systematics

1. Introduction

Heterobranch slugs and snails constitute a highly diverse group of gastropod molluscs, distributed ubiquitously in marine, limnic, and terrestrial environments all over the world (Jörger *et al*., 2010). Every year, new molecular systematic studies allow the scientific community to understand better the relationships among marine heterobranch species as well as their diversity, resulting in a continuous stream of new genera and species descriptions (e.g., Korshunova *et al*., 2020; Furfaro & Mariottini, 2020) and the synonymisation or resurrection of various taxa (e.g., Moles & Riesgo, 2019; Fernández-Vilert *et al*., 2020). One of the most important barriers to increasing knowledge is the existence of cryptic diversity, which is one of the reasons why existing biodiversity has been underestimated (e.g. Araujo *et al*., 2021; Korshunova *et al*., 2019; Moles *et al*., 2021).

One of the largest and most underestimated groups of sea slugs is the Doridina nudibranchs (Thompson & Brown, 1984; Rudman, 1998; Willan, 1998). Within the Doridina, Doridoidea is differentiated from its sister group, Phyllidiodea, by the presence of a radula (Valdés, 2002). Doridoidea comprises the families Discodorididae and Dorididae, while the former possesses a notched labium, small conical oral tentacles, a prostate with two differentiated portions, and a blood gland with two parts, the latter has small and blunt oral tentacles at both sides of the mouth area and a smooth labial cuticle (Valdés, 2002). The morphological synapomorphies of Discodorididae support the monophyly of its 29 genera (Valdés, 2002: Dayrat & Gosliner, 2005). Nevertheless, the family lacks comprehensive molecular studies, as most research has mostly focused on specific genera so far (Torres & Gosliner, 2018; Donohoo & Gosliner, 2020; Neuhaus, 2021; Donohoo et al., 2023; Innabi et al., 2023), including the recently erected *Avaldesia* (Donohoo & Gosliner, 2024). To date, conflicting phylogenies or inadequate taxon sampling have not confirmed the monophyly of

the family (Hallas *et al*., 2017). While some genera are supported by synapomorphies, such as the spatulate outermost teeth in *Geitodoris* Bergh, 1891 (Schrödl, 2000), a flattened body in *Platydoris* Bergh, 1877 (Dorgan *et al*., 2002), or jaw with three plates, and grooved oral tentacles in *Paradoris* Bergh, 1884 (Padula & Valdés, 2012), others are poorly diagnosed, such as *Discodoris* Bergh, 1877, *Rostanga* Bergh, 1879, or *Peltodoris* Bergh, 1880 (Dayrat, 2005; 2011).

The type taxon *Discodoris* is the most species-rich genus of Discodorididae, with 22 accepted species distributed in temperate and tropical waters (Dayrat, 2010; Ahyong et al., 2024). Early on, Pruvot-Fol (1934) noted that *Discodoris* was a wastebasket taxon. Species with typical 'discodoridid morphology' (i.e. body oval, depressed, granulate dorsum) that could not be placed in any other well-characterized genus were placed therein (Dayrat, 2005). In the last decades, some species have been transferred to genera such as *Geitodoris* and *Paradoris* (Schrödl, 2000; Dayrat, 2006). Moreover, many species of *Discodoris* are only known from their original description, sometimes without traceable type material. Dayrat (2005) recognised that a systematic revision of *Discodoris* was critically needed. After almost two decades since its last review, we aim to provide new molecular data for a larger number of discodorid genera and species covering the entire distribution range of this group. The newly collected data are included in a wider phylogenetic context of the family to provide some clarification at both genus and species levels. We assess the systematic status of Discodorididae by shedding light on the problematic genera, further unravelling potential pseudo-cryptic diversity in the Mediterranean Sea in particular.

2. Materials and Methods

2.1. Sampling

A total of 52 specimens belonging to 30 different species were collected while SCUBA diving and freediving at 0.5–10 m depth, and one specimen was collected at 105 m by trawling, mostly along the Catalan coast (Mediterranean Sea), the Canary Islands (Eastern Atlantic Ocean), and the Caribbean and Pacific coasts of Panama. Individuals were fixed in 95% EtOH for molecular purposes. Live specimens were photographed underwater with a Nikon D90 and Nikon D7200 coupled with a 60 mm and 105 mm macro lens. Specimens were deposited at the SNSB-Bavarian State Collection of Zoology in Munich (ZSM, Germany) and the Museum of Comparative Zoology (MCZ, Harvard University, USA). Additional specimens from Peru, Chile, and Polynesia were obtained from ZSM. The Catalonian Government issued permits for the collection of samples from the Mediterranean (SF/0589/2018 and SF/0495/2019) and the Environmental Ministry for Panama (ARGB-0033-2023), besides the ones obtained by the ZSM and MCZ.

A total of 199 specimens were included in the final phylogenetic analyses. The outgroups comprised species of each family within the infraorder Doridoidei, i.e. Actinocyclidae, Aegiridae, Cadlinellidae, Cadlinidae, Calycidorididae, Chromodorididae, Corambidae, Dendrodoridídae, Goniodoridídae, Gymnodoridídae, Hexabranchidae, Onchidoridídae, Phyllididae, Polyceridae, and Showajidaiidae, and two species from the infraorder Bathydoridoidei (*Bathydoris aioca* and *Prodoris clavigera*), following Korshunova *et al.,* (2020). All trees were rooted assuming Bathydoridoidei was the sister group to Doridoidei (Martynov & Korshunova, 2011).

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from a small piece of the foot using the DNeasy Blood and Tissue Kit (Qiagen, USA) and the E.Z.N.A.® Mollusc DNA Kit (Omega Bio-Tek, USA) following the manufacturer's protocols. Four markers were amplified including the

mitochondrial genes cytochrome *c* oxidase subunit I (COI), using the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994), and 16S rRNA, using the primers 16S ar-L and 16S br-H (Palumbi *et al.*, 1991); and the nuclear genes histone-3 (H3), using the primers H3AD5'3' and H3BD5'3' (Colgan *et al.*, 1998) and 28S rRNA, using the primers LSU5 (Littlewood *et al.*, 2000) and ECD2S (Williams & Ozawa, 2006). Polymerase chain reactions (PCR) were carried out in 20 µL volume with 5 µL 5x MyTaq™ Red DNA Polymerase, 0.2 µL of each primer (10 µM), and 0.2 µL MyTaq DNA Polymerase (Bioline, UK).

PCR conditions included an initial 5 min Hot Start step at 94 °C, 35 cycles of 30 s at 94 °C for denaturation, 30 s at 50 °C for annealing, and 1 min at 72 °C for extension, with a 5 min final extension at 72 °C. Some PCRs were carried out in 10 µL volume with 5 µL QIAGEN Multiplex PCR Master Mix, containing HotStarTaq® DNA Polymerase, Multiplex PCR Buffer, and dNTP Mix, 1 µL of each primer (2 µM), and 1 µL Q-solution. These PCRs had an initial 15 min Hot Start step at 95 °C, 40 cycles of 30 s at 94 °C, 90 s at 50 °C, and 90 s at 72 °C, with a 10 min final extension. Annealing temperatures for COI and 28S were 48–50°C and 50–52°C for 16S and H3. Successful amplifications were cleaned up using ExoSAP-IT (Affymetrix, CA, USA) and sequenced by Macrogen Inc. (Madrid, Spain) and by the Sequencing Service of the Department of Biology Genomic Service Unit (GSU) of the Ludwig Maximilians Universität (Munich, Germany).

2.3. Sequence assembly, alignment, and masking

Sequences were visualised, edited, and assembled in Geneious Prime v. 2022 (https://www.geneious.com). Contamination was checked against the GenBank nucleotide database using the BLAST algorithm (Altschul *et al.*, 1997). All new sequences are deposited in GenBank (see Table S1 for sampling information). Sequences of each gene were individually aligned with MAFFT v. 7 (Katoh & Standley, 2013) implemented in Geneious, using the G-

INS-I (COI and H3) and L-INS-I (16S and 28S) algorithms. Each alignment was trimmed of primer regions, and missing data were coded as 'N'. The impact of poorly aligned positions and divergent regions in the 16S and 28S genes on the results was investigated by constructing trimmed matrices under two alternative methods: (1) Gblocks v. 0.9b (Talavera & Castresana, 2007) under relaxed parameters (retaining 74% of the original alignment in both genes) and (2) ZORRO, a probabilistic masking program that accounts for alignment uncertainty by assigning confidence scores to each alignment position (Wu *et al.*, 2012).

2.4.Phylogenetic analyses

Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference approaches (BI). The analyses were carried out for both single-gene and concatenated alignments. ML analyses were performed using IQ-TREE v. 2.1.2 (Nguyen *et al.*, 2015). The best partition scheme and corresponding evolutionary model were selected automatically with ModelFinder (Kalyaanamoorthy *et al.*, 2017) by merging partitions to reduce overparameterisation and increase model fit (TESTMERGE), also accounting for codon position for the protein-coding genes. Bootstrap support values (BS) were estimated via UFBoot v. 2 (Hoang *et al.*, 2017) with 1,500 replicates. Bayesian analysis was performed using MrBayes 3.2.7a (Ronquist *et al.*, 2012), and the nucleotide substitution model selected for the Bayesian analysis of each gene in the concatenated dataset was GTR+I+G (Tavaré, 1986; Yang, 1996). Four parallel runs of four coupled Markov chain Monte Carlo (MCMC) chains were run for 20 million generations, sampling every 1000 generations, discarding the first 25% trees as burn-in. Topological robustness was assessed using posterior probabilities (PP). All analyses were remotely run on the CIPRES Science Gateway v. 3.3 (Miller et al., 2015). Trees were visualised in FigTree v. 1.4.4 (Rambaut, 2017) and edited in Illustrator CC 2019. Only support

values of BS ≥ 80 for ML and PP ≥ 0.80 for BI are depicted. Otherwise, these are nodes considered unsupported.

2.5. Species delimitation tests

Species delimitation tests were conducted on the aligned COI dataset on each genus separately. Analyses were conducted using different methods, including distance and character-explicit approaches (DeSalle & Goldstein, 2019). The Assemble Species by Automatic Partitioning analysis (ASAP; Puillandre *et al.*, 2021) was run using the web interface at https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html with default parameters and Jukes-Cantor (JK69) as a substitution model to compute the distances. This is a method to build species partitions from single-locus sequence alignments that overcomes the limitations of the Automatic Barcode Gap Discovery (ABGD; Puillandre *et al.*, 2012). Species delimitation analyses were also carried out using the Poisson Tree Processes (PTP) and the Bayesian implementation of the PTP model (bPTP; Zhang *et al.*, 2013) on the webserver (https://species.h-its.org/ptp/) using 100,000 MCMC generations on a rooted tree with a 0.25 of burn-in. We also used the Generalized Mixed Yule Coalescent approach (GMYC; Fujisawa and Barraclough, 2013) on ultrametric trees generated in BEAST v. 1.10.4 (Drummond & Rambaut, 2007) selecting a coalescent tree prior (constant population size), which has been suggested to provide a more rigorous test of delimitation since the model assumes a single species as the null option (Monaghan *et al*., 2009). The best partition schemes and evolutionary models were selected by PartitionFinder and assigned a single lognormal uncorrelated relaxed clock model of Drummond *et al*. (2006), fixing the clock rate to 1.0. Three chains were run for 10 million generations. TreeAnnotator was used to discard the initial 25% of trees as burn-in. The GMYC clusters were identified with the help of the R package SPLITS (Ezard *et al*., 2017).

3. Results

The final sequence dataset contained 199 specimens, including 142 Discodorididae, 24 Dorididae, and 33 outgroup taxa. From these, we newly sequenced 52, and sequences of 140 specimens were obtained from GenBank. Out of the new sequences provided, we added six type species never sequenced before to 20 type species. The final concatenated alignment included 2,383 bp, including 967 parsimony-informative and 229 singleton sites (ca. 647 bp for COI, ca. 498 characters for 16S, ca. 328 bp for H3, and ca. 910 characters for 28S). The best substitution models and partition schemes, according to ModelFinder, were GTR+F+I+G4 for COI, 16S, and H3, and TIM3+F+I+G4 for 28S. ML and BI analyses of the concatenated alignment yielded similar results. After comparing Gblocks and ZORRO masking methods, we decided to avoid filtering the 16S and 28S alignment since this has been shown to impact tree accuracy (Tan *et al*., 2015; Moles *et al*., 2023). The monophyly of Dorididae and Discodorididae was compromised by the position of the doridid genus *Aphelodoris* Bergh, 1879 as sister to the discodoridid genus *Atagema* (BS = 81, PP = 0.83). The monophyly of the two remaining clades received high support in the ML analysis (remaining Discodorididae: BS = 99, PP = 0.34; remaining Dorididae: BS = 92, PP = 0.52) (Fig. 1).

Within Dorididae, the genus *Doris* Linnaeus, 1758 split into three clades, one including two groups, on one side the type species of the genus, *D. verrucosa* Linnaeus, 1758, *D. ocelligera* (Bergh, 1881) and *Doris berghi* (BS = 99, PP = 1) (Fig. 2, C–E). On the other side, a group including the genera *Doriopsis* Pease, 1860 (BS = 100, PP = 1) and *Homoiodoris* as sister taxa to *Rostanga poddubetskaiae* Innabi, Stout & Valdés, 2023 and an unrecognised species of *Doris* (BS = 99, PP = 0.77). A second clade with maximum support included *Doris* species closely related to the former *A. pseudoargus* Rapp, 1827, the type species of the currently unavailable genus *Archidoris* Bergh, 1878 from the Artic, included in this study for the first time (Fig. 2, F). The third clade (Fig. 2, A–B) and included specimens of *D. marmorata* Risso, 1818 and *D. bertheloti* (d'Orbigny, 1839) from NE Spain and the Canary Islands, which

were recovered as sister to the genus *Austrodoris* Odhner, 1926 from Antarctica (BS = 73, PP = 68).

Regarding Discodorididae, we found *Discodoris* to be polyphyletic. On the one hand, the type species *D. boholiensis* Bergh, 1877 from the Philippines clustered with *D. cebuensis* Bergh, 1877 from New Guinea (BS = 100, PP = 1), and both were recovered sister to a clade of *Carminodoris* Bergh, 1889 species (BS = 98, PP = 0.98). On the other, two *D. branneri* MacFarland, 1909 from the Caribbean side of Panama (Fig. 3, A) and the Dominican Republic were recovered along with *D. coerulescens* Bergh, 1888 from the Philippines as sisters to a clade formed by and representatives of the genus *Tayuva* Er. Marcus & Ev. Marcus, 1967, albeit low support (BS = 77, PP = 0.73). Moreover, *Discodoris rosi* Ortea, 1979 from NW and NE Spain (BS = 100, PP = 1) clustered with *C. boucheti* Ortea, 1979 from NE Spain. Both are sister taxa with a clade composed of *Rostanga elandsia* and *Gargamella immaculata* (Fig. 4, A–D) (BS = 94, PP = 0.99) and are transferred to *Gargamella* (see discussion). The latter clade clustered with the clade composed of other *Rostanga* species according to the BI (PP = 0.92), including the type species *R. rubra*, sister to *R. muscula* and *R. calumus*. As for the species *R. byga* and *R. pulchra* relationships were inconclusive.

Carminodoris species were found monophyletic (BS = 98, PP = 1), comprising *C. flammea* (Fahey & Gosliner, 2003) from the Philippines, *C. armata* Baba, 1993 from South Korea, *C. nodulosa* (Angas, 1864) from New Zealand, and *C. grandiflora* (Pease, 1860) from Mauritius.

The genus *Geitodoris* (Fig. 7, A–H) was also found monophyletic (BS = 100, PP = 1), having *Carminodoris* and *Discodoris* as sister taxa (BS = 99, PP = 1). Two specimens of *G. heathi* (MacFarland, 1905) from Panama (Pacific) and California were not clustered together, thus evidencing a possible case of cryptic diversity. These specimens clustered with the clade

composed of *G. reticulata* Eliot, 1906 from Senegal and *G. portmanni* (Schmekel, 1972) from NE Spain (BS = 97, PP = 0.95), forming the sister group to the type species *G. planata* (Alder & Hancock, 1846) from both sides of the Iberian Peninsula and sequenced here for the first time. Our two possible morphotypes of *G. bacalladoi* Ortea, 1990 clustered within *G. planata* specimens, a result supported by all species delimitation tests (see Fig. 1), yet separated into two clusters according to the GMYC.

Thordisa was found monophyletic (BS = 70, PP = 0.74) with *T. azmanii* as sister taxa to a well-supported clade comprising *T. nielseni, T. bimaculata, T. sanguinea*, and *T. oliva* (BS = 98, PP = 1). *Halgerda* species clustered as sister taxa of *Thordisa*, although not supported (BS = 61, PP = 0.73).

Jorunna Bergh, 1876 was recovered monophyletic with maximum support, except for *J. funebris* (Kelaart, 1859). The rest of the species included *J. osae* Camacho-García & Gosliner, 2008 from the Pacific side of Panama, *J. artsdatabankia*, *J. onubensis*, and the type species *J. tomentosa* (Cuvier, 1804) also including the two lineages A and B from Neuhaus et al. (2021) (BS = 100, PP = 1). The samples sequenced in this study from the Mediterranean and Eastern Atlantic clustered with linage B (BS = 95, PP = 0.99) (Fig. 6, A–D).

Paradoris species (Fig. 3, C–F) were recovered monophyletic (BS = 95, PP = 0.99) and the type species *P. indecora* (Bergh, 1881) from NE Spain, sequenced here for the first time, clustered with two possible unrecognised species, namely *Paradoris* sp. 1 (BS = 61, PP = 0.99) from Croatia as sister species to both *Paradoris* sp. 2 (BS = 100, PP = 1). The species delimitation tests separate these morphotypes from the Mediterranean as three distinct species. Sister to all these species, there is a clade composed of the Indo-Pacific species *P. liturata* (Bergh, 1905) from the Philippines and an undetermined species from the Oman Sea (BS = 95, PP = 0.99).

Regarding the genus *Tayuva*, analyses recovered with high support as sister taxa to *Peltodoris atromaculata*, the type species of the genus (BS = 98, PP = 1). Within *Tayuva*, two specimens of the type taxon *T. lilacina* (A. Gould, 1852), one from the Indo-Pacific and one from the Oman Sea, were clustered together (BS = 100, PP = 1), and the species delimitation tests considered them different. Moreover, the latter *Tayuva* species clustered with an undetermined species from China and *Discodoris* sp from the North Atlantic (BS = 78, PP = 1), all together as sister taxa to a clade comprising specimens also identified as *T. lilacina* from the Atlantic and Mediterranean (Fig. 3, B) (BS = 100, PP = 1). However, since the species delimitation tests differentiated *T. lilacina* specimens into four different species, the latter clade from the Mediterranean Sea has been proposed to be transferred to ***T. maculosa* stat. rest. comb. nov.** and the Atlantic clade to ***T. confusa* stat. rest. comb. nov.**

The genus *Platydoris* was found to be monophyletic (BS = 100, PP = 1). The type species *P. argo* (Linnaeus, 1767) from the Mediterranean was sequenced here for the first time and clustered with *P. sanguinea* Bergh, 1905 from the Philippines. These were sister taxa to the genus *Sclerodoris* (BS = 98, PP = 1) comprising the type species *S. tuberculata* Eliot, 1904 from Papua New Guinea and *S. faninozi* from New Caledonia. Another two undetermined species of *Sclerodoris* Eliot, 1904 clustered very distantly to the type species along with *S. dutertrei* (BS = 100, PP = 0.98) as sister taxa to *Taringa sublutea* (BS = 100, PP = 0.79) that clustered separately from the other *Taringa* species.

Taringa Er. Marcus, 1955 species (Fig. 5, A–H) were recovered as three distinct groups. One with two different undetermined *Taringa* species from Panama (BS = 100, PP = 1) and the Galapagos Islands (BS = 100, PP = 0.98), the second one comprising the type taxon *T. telopia* Er. Marcus, 1955 from Panama, and *T. armata* Swennen, 1961 from the Mediterranean (BS = 100, PP = 1). Regarding *T. armata*, several colour morphotypes commonly identified as

Aporodoris millegrana (Alder & Hancock, 1854) clustered together (BS = 100, PP = 1) and were identified as the same species. The species delimitation tests identified *T. armata* and '*A. millegrana*' specimens from the NE Iberian Peninsula and the Canary Islands as the same species. The third group included the single species *T. aivica* as the sister taxon to the rest (BS = 100, PP = 0.78).

Avaldesia species were found as sister taxon to *Taringa* with high support only in the ML (BS = 100). The genus was found to be monophyletic and comprising *A. albomacula*, *A. tamatoa*, and *A. thala* (BS = 100, PP = 1).

Hoplodoris Bergh, 1880 (BS = 100, PP = 0.9) and *Asteronotus* Ehrenberg, 1831 (BS = 99, PP = 0.76) were both recovered monophyletic. They were supported as sister groups on the ML (BS = 100), but no support was found on the BI (PP = 0.64).

The genus *Diaulula* Bergh, 1878 was recovered in two additional, non-related clades. One clade comprised *D. nayarita* (Ortea & Llera, 1981) from the Pacific side of Panama and two different *D. greeleyi* (MacFarland, 1909), one from the Pacific side of Panama and the other one from Florida (BS = 100, PP = 1). The second clade comprised *D. punctuolata* (d'Orbigny, 1837) from Chile and the type species of the genus *D. sandiegensis* (J. G. Cooper, 1863) from the Eastern Pacific (BS = 100, PP = 1). Interestingly, *Atagema* Gray, 1850 (Fig. 6, F–G) and the genus *Aphelodoris* Bergh, 1879, belonging to Doridiidae, were recovered as sister taxa, albeit with low support (BS = 81, PP = 0.83). Additionally, ML and BI analyses agreed in supporting the inclusion of *Diaulula hispida* (d'Orbigny, 1834) (Fig. 6, E) within the genus *Atagema* (BS = 99, PP = 0.89), along with *A. alba* (O'Donoghue, 1927) and *A. notacristata* Camacho-García & Gosliner, 2008 from Central and South America. All these species clustered with a clade with two groups with high support (BS = 100, PP = 1) comprising *A.* cf. *osseosa* (Kelaart, 1859) from Hawaii, with an undetermined species of *Atagema* from Polynesia (BS =

100, PP = 1) on one side, and *A. sobanovae*, *A. kimberlyae*, and *A. spongiosa* on the other side (BS = 93, PP = 0.73).

4. Discussion

The systematics of Discodorididae has been a matter of debate since Bergh (1891) erected the family. The reasons are that (1) almost all the existing phylogenies are solely based on morphology, and (2) molecular data have only been generated for a few genera (Torres & Gosliner, 2018; Donohoo & Gosliner, 2020; Neuhaus, 2020; Donohoo et al., 2023). The present paper is the first thorough molecular phylogenetic analysis of the Discodorididae, including many genera, type species, and species covering a wide distributional range.

In the following two sections, we discuss potential taxonomic changes derived from the phylogeny produced herein. Some of the conclusions of this study are tentative and will require further confirmation with morphological diagnostic characters and larger sample sizes.

4.1. Systematics of Doridiidae

The families Doridiidae and Discodorididae *sensu* Valdés (2002) are paraphyletic because *Aphelodoris* and *Atagema*, which were traditionally considered to belong to Doridiidae and Discodorididae, respectively, cluster together in a separate clade outside both families. Similar results were found in other molecular analyses highlighting *Aphelodoris* as a problematic group (Hallas *et al*., 2017) against morphological odds (Valdés, 2002). Another conflicting result with morphological data is the fact that the caryophyllidia-bearing dorids are not monophyletic (Valdés, 2002; Fahey & Healy, 2003), which contradicts the conclusions by Valdés and Gosliner (2001). These results suggest that these complex dermal organs have evolved multiple times independently or have evolved early in Discodorididae (based on the early split of *Atagema*) and have been lost multiple times. The family Doridiidae contains a

substantial amount of diversity, suggesting that the proposal by Valdés (2002) to synonymise several taxa with the genus *Doris* was overly simplistic. More work is needed in this group to provide a more up-to-date taxonomy consistent with both molecular and morphological data. Since *Doriopsis*, *Conualevia*, and *Homoiodoris* are accepted genera within Dorididae, the Northern Hemisphere genus *Archidoris* and the Southern Hemisphere *Austrodoris*, synonyms of *Doris*, should be re-erected. Within Dorididae, our results support recent findings by Renau et al. (2024), except for the inclusion of *Rostanga poddubetskaiae*. The morpho-anatomical characters that define the species were recognised as unusual, but molecular data placed the species in the genus *Rostanga* (Innabi *et al*., 2023). Here, we suggest transferring the species to Dorididae under the name **"*Doris*" *poddubetskaiae* comb. nov.** pending a thorough taxonomic assessment of Dorididae.

4.2. Systematics of Discodoridídae

Within the Discodoridídae, some genera were recovered monophyletic in our analyses, i.e., *Asteronotus*, *Avaldesia*, *Geitodoris*, *Halgerda* Bergh, 1880, *Hoplodoris*, *Paradoris*, and *Platydoris*, yet some others were not. For example, the genus *Atagema* was recovered monophyletic with the caveat that it also includes the South American species *Diaulula hispida*. However, as Valdés and Muniain (2002) indicated, *D. hispida* is characterised by having a dorsal longitudinal ridge, which is unique to members of the genus *Atagema*. This, together with our molecular evidence, leads us to propose ***Atagema hispida* comb. rest.** Additional species traditionally assigned to *Diaulula* appear in separate clades, suggesting this group needs reassessment. The type species *D. sandiegensis* was included in a well-supported clade containing *D. punctuolata*. This clade needs further data and sequences from additional species of *Diaulula*, but if the name *Diaulula* is restricted to the clade containing the type species, other

329 species previously assigned to *Diaulula,* such as *D. greeleyi* and *D. nayarita* need a new generic
330 placement.

331 *Discodoris* is paraphyletic, a clade including the type species *D. boholiensis* as well as
332 *D. cebuensis* is well supported. Other species traditionally assigned to *Discodoris*, such as *D.*
333 *lilacina* (A. Gould, 1852), as well as *D. coerulescens* and *D. branneri*, form a distinct clade
334 currently accepted as *Tayuva* (Dayrat, 2011). Based on molecular evidence and the overall
335 morphological similarities in external morphology, colouration, mantle autotomy (as in *T.*
336 *lilacina*), and egg mass shape (Valdés et al., 2006; Dayrat, 2010), we transfer the latter two
337 species to *Tayuva* as ***T. coerulescens*** **comb. nov.** and ***T. branneri*** **comb. nov.** Moreover, the
338 present study suggests the existence of a species complex of five different species under the
339 name *T. lilacina*, which also requires further research. Since we do not have material from the
340 type locality (Hawaii), we cannot determine which specimen represents the true *T. lilacina* until
341 further studies. The specimens assigned to *T. lilacina* from the Mediterranean Sea should be
342 transferred to *D. maculosa* Bergh, 1884 (Dayrat, 2011), which is the oldest name available for
343 this region (type locality Naples, Italy), under the new name ***T. maculosa*** **stat. rest. comb. nov.**
344 A single specimen from Jamaica was here assigned in the latter clade, originally identified as
345 ***Discodoris hummelincki*** **syn. nov.** (Ev. Marcus & Er. Marcus, 1963) (Lindsay et al., 2016),
346 and represents here a synonym of *T. maculosa*. An additional *Tayuva* species from the Nort
347 Eastern Atlantic is here recognised as a separate species, which most likely corresponds to the
348 name *Discodoris confusa* Ballesteros, Llera & Ortea, 1985 here resurrected as ***T. confusa*** **stat.**
349 **rest. comb. nov.** The genus *Peltodoris* is also enigmatic since the type species *P. atromaculata*
350 forms a clade with near maximum support with *Tayuva*. In fact, within the genus *Tayuva*, some
351 species were previously described as *Peltodoris* and later synonymised, such as *P. crucis*
352 (Mörch, 1863) *sensu* Bergh, 1880 (Dayrat, 2010) and *P. hummelincki* (this study). More

genomic data are needed to assess this group and to assess the possible synonymy of *Tayuva* to *Peltodoris*.

Another clade with high support, including taxa previously regarded as members of different groups, is the one comprising *Discodoris rosi* and *Carminodoris boucheti*, which were recovered sister to *Gargamella immaculata* Bergh, 1894 and *Rostanga elandsia*. Previous morphological studies have already proposed the inclusion of *D. rosi* in *Rostanga* because of the possession of elongate, slender lateral teeth, a character traditionally used to recognise *Rostanga* species (Rudman & Avern, 1989; Dayrat & Gosliner, 2005; Dayrat, 2011). Also, like the species of *Rostanga*, *D. rosi*, and *C. boucheti* possess caryophyllidia, which is otherwise missing in *Discodoris* (Ortea *et al*., 2014). However, the labial cuticle is smooth in *D. rosi* but armed with jaw elements in *Rostanga* and *Discodoris* (Ortea *et al*., 2014), except for *R. elandsia*, which has a smooth jaw lacking elements (Garovoy et al., 2001). Thus, some authors maintained *D. rosi* in *Discodoris*, waiting for further clarification (Sánchez-Tocino, 2003; Cervera *et al*., 2004). Since both *D. rosi* and *C. boucheti* also cluster together with the type species *G. immaculata* and because both *C. boucheti* and *Gargamella* present a smooth labial cuticle (Perrone & Doneddu, 1996; Ortea *et al*., 2006), as mentioned above for *D. rosi* and *R. elandsia*, we transferred this species to *Gargamella* as ***G. boucheti*** **comb. nov.**, ***G. elandsia*** **comb. nov.**, and ***G. rosi*** **comb. nov.**

On the other hand, *Carminodoris* and *Hoplodoris* have been considered synonymous by Fahey and Gosliner (2003), but Valdés (2002) suggested that *Carminodoris* was distinct and possibly a synonym of *Discodoris*. However, Dayrat and Gosliner (2005) retained both *Hoplodoris* and *Carminodoris* as valid genera because of differences in the penial spine, referring to unpublished research. In the present study, all *Hoplodoris,* including the type species *H. desmoparypha* Bergh, 1880, form a well-supported clade, completely unrelated and

distant to the monophyletic *Carminodoris* clade (the clade *C. armata*, *C. nodulosa*, *C. flammea*, and *C. grandiflora*), albeit lacking the type species *C. mauritiana* Bergh, 1889 to confirm the status of the genus.

Molecular data confirm *Jorunna* as monophyletic, as previously supported by morphological synapomorphies (Valdés & Gosliner, 2001; Camacho-García & Gosliner, 2008). The only exception is a specimen identified as *J. funebris*, which clustered with *Discodoris*, *Carminodoris*, and *Geitodoris*, albeit lacking support. Therefore, it is unclear if this specimen was correctly identified, although it has a very unique pattern (Kelaart, 1858). Additional work on tropical dorid systematics is needed before these species can be confidently identified based on morphological traits.

Geitodoris presently has seven valid species in the Mediterranean Sea: *G. joubini* (Vayssière, 1919), *G. portmanni*, *G. bonosi* Ortea and Ballesteros, 1981, *G. planata*, *G. pusae* (Marcus, 1955), *G. reticulata*, and *G. bacalladoi* (Sánchez-Tocino *et al*., 2006; Ahyong et al., 2024). We defined genetically three of the species, namely *G. portmanni*, *G. reticulata*, and *G. planata*, while the morphotype assigned to *G. bacalladoi,* originally described in the Atlantic by Ortea (1990), turned out to be *G. planata*. Regarding the species from the Indo-Pacific and Eastern Pacific, we only had two specimens of the species *G. heathi* that did not cluster in the same clade, indicating the possibility of a species complex. More sampling will be required to improve support values and better understand the diversity and evolution within that region.

Paradoris indecora was the only valid species in the Amphi-Atlantic and Mediterranean regions within this genus and, after the present study, constitutes a species complex. We found three different morphotypes assigned to this species due to colour variability. One has a translucent white body and several small black spots on the dorsum (*P. indecora*; Fig. 3, C–D), another with a translucent white body without the presence of black spots (*Paradoris* sp. 1; Fig.

3, E), and the last one with an opaque grey body (*Paradoris* sp. 2; Fig. 3, F). The two latter are pending morphological study to determine whether they are undescribed species or belong to already described species. *Paradoris aviles* Ortea & Moro, 2022 and *P. rafaherreroi* Ortea & Moro, 2021, both described from Cape Verde, are externally similar to *Paradoris* sp. 1 and sp. 2, respectively. Alternatively, these could represent the currently synonymised species *P. porri* (Vérany, 1846), *P. granulata* Bergh, 1884 or *P. cavernae* (Starmülhner, 1955) studied in Trainito and Doneddu (2014), which should therefore be resurrected.

Sclerodoris Eliot, 1904 appears to be polyphyletic. The type taxon *Sclerodoris tuberculata* Eliot, 1904 was recovered as the sister group to *Platydoris*, but seemed to be unrelated to two unidentified species of *Sclerodoris* and *S. dutertrei.* This could be due to misidentifications, and further taxon sampling is needed to establish the species' identity. Along the same lines, *Taringa sublutea*, probably another misidentification, is sister to a clade including *Hoplodoris* and *Asteronotus*. Other morphologically similar genera, such as *Otinodoris* White, 1948 are potentially members of this clade, but additional samples are needed to resolve these relationships.

The genus *Taringa* is identified as monophyletic if considering *T. sublutea* as a misidentification or a species that does not belong to this genus and, thus, should be redefined. Valdés and Gosliner (2001) invoked ICZN (1999) Art. 23.9 and considered *Aporodoris* a *nomen oblitum* and *Taringa* a *nomen protectum*. However, Dayrat (2010) argued that this reversal of precedence is nullified by the usage of the name *Aporodoris* by various authors and considered *Aporodoris* valid. This has led to the current situation in which the two names are used for each particular species, *Taringa* being the most used (e.g., Alvim & Pimenta, 2013). The present study confirms that *Taringa* and *Aporodoris* together are monophyletic. Moreover, the data presented here supports that the Mediterranean dark brown morphotype associated with *A.*

millegrana is *T. armata* instead (note names have been modified in Fig. 1, but see Table S1). Valdés and Gosliner's (2001) morphological study reinforces this hypothesis since *A. millegrana* (based on specimens from the Canary Islands) was also regarded as a sister to *T. telopia*, thus placing *T. armata* and *A. millegrana* in the same clade. This confirms that *T. armata* displays substantial chromatic variation within specimens from the Canary Islands to the western Mediterranean, with white, orange, or dark brown body colour (see Fig. 5, C–F). Regarding the unnamed species *Taringa* sp. 1 and *Taringa* sp. 2 from the Galapagos Islands and Panama, respectively, a morphological revision is needed to determine whether these are new species.

4.3. Biogeographic distribution

Most genera examined possess two well-differentiated groups corresponding to different biogeographic regions; genera such as *Tayuva*, *Paradoris*, and *Atagema* are divided into Mediterranean–Atlantic and Indo-Pacific clades. In contrast, genera such as *Taringa*, *Geitodoris*, and *Jorunna* are differentiated into Mediterranean–Atlantic and Eastern-Pacific clades. This has already been discussed in previous studies for other heterobranch groups (Gosliner & Johnson, 1999; Garovoy *et al.*, 2001; Dorgan *et al*., 2002; Alejandrino & Valdés, 2006; Padula & Valdés, 2012) and was attributed to one or two consecutive vicariant events: (1) the closure of the Tethys Sea approximately 24–21 million years ago (Mya) (Torfstein & Steinberg, 2020), which communicated the Indian with the Atlantic Ocean, and (2) the rise of the Isthmus of Panama approximately 3.1 Mya (see McCarthy *et al*., 2000; Valdés, 2004). These vicariant events would have led to a significant reorganisation of oceanic circulation and climate patterns on a global scale and driven effective isolation, eventually promoting allopatric speciation (Padula and Valdés, 2012; Torfstein & Steinberg, 2020).

4.4. Forthcoming research

Our multilocus, Sanger-based phylogenies provide good support at the species level as well as for certain genera such as *Asteronotus*, *Atagema*, *Avaldesia*, *Carminodoris*, *Gargamella*, *Geitodoris*, *Halgerda*, *Hoplodoris*, *Jorunna*, *Paradoris*, *Platydoris*, *Taringa*, *Tayuva*, and *Thordisa*. The genera *Diaulula*, *Discodoris*, *Peltodoris*, *Rostanga*, and *Sclerodoris* appear paraphyletic. However, the level of resolution, mainly at deeper nodes, was lower than expected despite the use of 4 different molecular markers. Despite the inclusion of a substantial number of specimens, the intricate taxonomy of Discodorididae needs a broader taxon sampling to delineate the boundaries and diversity within each genus more accurately. We propose that the incorporation of genomic data, employing methodologies such as target enrichment, has the potential to yield a more resolved and robust phylogeny, facilitating a deeper understanding of the evolutionary history of Doridina (Moles & Giribet, 2021; see Paz-Sedano *et al*. 2024). Such an approach holds promise not only for unravelling the phylogenetic complexities inherent in large clades like Discodorididae but also for refining the systematic classifications within genera. Through this investigation, we have contributed a wealth of novel genetic data that will serve as a cornerstone for future research endeavours encompassing both molecular and morphological dimensions. In essence, the advancement of Discodorididae systematics hinges upon the integration of broader taxon and molecular data to elucidate taxonomic uncertainties highlighted by morphological analyses and this study.

Credit Authorship Contribution Statement

R.F-V. and J.M. work in the conceptualisation. J.M., X.S. and M.S. collected specimens. R.F-V. performed lab work. R.F-V. and J.M. performed analyses. M.S., M.A.A., and J.M. contributed to resources. R.F-V., J.M. and A.V. wrote the original draft. All authors reviewed and edited the final version of the manuscript.

Declaration of Interest

None.

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723 **Figure 1.** Maximum likelihood phylogenetic tree of Doridoidea based on the concatenated COI,
724 16S, H3, and 28S genes (2,383 characters). Bootstrap support values (left) and posterior
725 probabilities (right), >80 BS and >0.8 PP, are shown on branches. Green dots indicate nodes
726 with maximum branch support in both analyses. Stars indicate type species. The outgroups
727 comprised species of each family within the infraorder Doridoidei and two species from the
728 infraorder Bathydoridoidei; the latter was used to root the tree. Specimens in bold were
729 sequenced in this study. The results of the species delimitation tests (ASAP, PTP, bPTP, and
730 GMYC) based on COI are represented by the vertical bars to the right side of the tree. The scale
731 bar indicates substitutions per site.

732 **Figure 2. A:** *Doris bertheloti* (ZSM Mol 20210045) from the Canary Islands; **B:** *Doris*
733 *marmorata* (ZSM Mol 20210023) from NE Spain; **C:** *Doris berghi* (ZSM Mol 20210024) from
734 NE Spain; **D:** *Doris ocelligera* (MCZ Mala 395160) from NE Spain; **E:** *Doris verrucosa* (ZSM
735 Mol 20210044) from SE France; **F:** *Doris pseudoargus* (ZSM Mol 20210038) from NW Spain.

736 **Figure 3. A:** *Discodoris branneri* (ZSM Mol 20210047) from Panama (Caribbean); **B:** ***Tayuva***
737 ***maculosa* stat. rest. comb. nov.** (ZSM Mol 20210012) from NE Spain; **C:** *Paradoris indecora*
738 (ZSM Mol 20210034) from NE Spain; **D:** *Paradoris indecora* (ZSM Mol 20210025) from NE
739 Spain; **E:** *Paradoris* sp. 1 (ZSM Mol 20210026) from NE Spain; **F:** *Paradoris* sp. 2 (ZSM Mol
740 20100138) from Croatia.

741 **Figure 4. A:** ***Gargamella boucheti* comb. nov.** (ZSM Mol 20210019) from NE Spain; **B:**
742 ***Gargamella rosi* comb. nov.** (ZSM Mol 20210037) from NW Spain; **C:** ***Gargamella rosi***
743 **comb. nov.** (ZSM Mol 20210018) from NE Spain; **D:** *Rostanga rubra* (ZSM Mol 20210043)
744 from NW Spain.

745 **Figure 5. A:** *Taringa* sp. 2 (ZSM Mol 20210050) from Panama (Pacific); **B:** *Taringa* sp. 2
746 (ZSM Mol 20210051) from Panama (Pacific); **C:** *Taringa armata* (ZSM Mol 20210011) from

747 NE Spain; **D:** *Taringa armata* (ZSM Mol 20210013) from NE Spain; **E:** *Taringa armata* (ZSM
748 Mol 20210030) from NE Spain; **F:** *Taringa armata* (ZSM Mol 20210016) from NE Spain; **G:**
749 *Taringa armata* (ZSM Mol 20210046) from the Canary Islands; **H:** *Taringa armata* (ZSM Mol
750 20210032) from NE Spain.

751 **Figure 6. A:** *Jorunna tomentosa* (ZSM Mol 20210042) from NW Spain; **B:** *Jorunna tomentosa*
752 (ZSM Mol 20210031) from NE Spain; **C:** *Jorunna tomentosa* (ZSM Mol 20210027) from NE
753 Spain; **D:** *Jorunna tomentosa* (ZSM Mol 20210020) from NE Spain; **E:** ***Atagema hispida***
754 **comb. rest.** (ZSM Mol 20090428) from Chile; **F:** *Atagema* sp. (ZSM Mol 20060303) from
755 Polynesia; **G:** *Atagema alba* (ZSM Mol 20090766) from Perú.

756 **Figure 7. A:** *Geitodoris heathi* (ZSM Mol 20210052) from Panama (Pacific); **B:** *Geitodoris*
757 *portmanni* (ZSM Mol 20210036) from NE Spain; **C:** *Geitodoris reticulata* (ZSM Mol
758 20190751) from Senegal; **D:** *Geitodoris planata* (MCZ Mala 395157) from NE Spain; **E:**
759 *Geitodoris planata* (ZSM Mol 20210039) from NW Spain; **F:** *Geitodoris planata* (ZSM Mol
760 20210040) from NW Spain; **G:** *Geitodoris planata* (MCZ Mala 395156) from NE Spain; **H:**
761 *Geitodoris planata* (ZSM Mol 20210041) from NW Spain.