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Molecular Updates on the 'Warty Dorid' *Doris verrucosa* Linnaeus, 1758 (Mollusca, Nudibranchia) from the Mediterranean Sea

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Abstract

Basic and applied research reveals the importance of sea slugs as a source of new bioactive molecules or of still little-known intra/intercellular processes, mainly linked to the highly specialised defensive strategies typical of this group of shell-less molluscs. In this context, the nudibranch *Doris verrucosa* (Gastropoda, Mollusca), commonly known as 'warty dorid', is particularly interesting due to its ability to produce de novo biochemical compounds with pharmacological properties and being the type species of the genus *Doris*, one of the oldest and richest in species, currently characterised by a troubled systematics. Despite its wide distribution across the Eastern Atlantic Ocean and the Mediterranean Sea, this species has not yet been characterised from a genetic point of view. Considering the importance of assessing species identity to correctly investigate the systematics and to properly unravel potentially useful applications, results from a molecular assessment of such interesting species are provided. Genetic analysis involved species delimitation, phylogeny and haplotype network methods carried out on specimens of *D. verrucosa* collected from highly anthropised areas of Southern Italy (central Mediterranean Sea). Furthermore, in situ observations allowed us to fill some gaps in knowledge on the ecology and the morphological variability of this species that could be useful for future comparisons.

Keywords: Heterobranchia; systematics; haplotype analysis; marine drugs; genetic diversity

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1. Introduction

The study of Mediterranean marine Heterobranchia is an intriguing field of research that has gained renewed attention in recent decades due to the increased attention to conservation of neglected biodiversity and marine environments. Marine Heterobranchia molluscs are known for their unique adaptations and highly specialised defensive strategies, being model species in different fields of applied research such as neuroscience, biomedicine, and pharmaceutic [1–5]. In fact, some of the marine natural products currently tested as drugs were originally identified and isolated from sea slug molluscs [3]

that can obtain, accumulate, and sometimes modify chemical compounds from their prey or directly biosynthesise them de novo [6]. Potential anticancer activity is currently known for compounds such as 'kahalalide F' obtained from sacoglossans, and 'aplyronines' and 'dolastatins' from anaspideans and from several different compounds in the Nudibranchia order and especially in Doridoidea [2,7–11]. However, the presence of biochemically active compounds is only one of the specialised traits evolved in response to the reduction and/or loss (in the adult stage) of the defensive shell [12]. Symbiotic relationships with different taxa [13–15], extraordinary regeneration ability and autotomy [16–18], and kleptopredation behaviour [19–21] are just a few examples of the great potential of these extremely specialised gastropods.

In this context, a nudibranch belonging to the Doridiidae family, *Doris verrucosa* Linnaeus, 1758, has gained great scientific relevance, as it produces metabolites in the mantle, namely verrucosins (Verrucosins 1–9, Verrucosin A, and other diterpene glycerides) with ichthyotoxic and pharmacological properties [11,22–24]. This species biosynthesises these molecules de novo following a pathway not yet totally depicted [25] and can be considered an emergent model species. *Doris verrucosa* is a benthic nudibranch inhabiting rocky substrates around 0–12 m depth but also found in tide pool habitats where it may feed upon sponges of the genera *Halicondria* Fleming, 1828 and *Hymeniacidon* Bowerbank, 1858 [26–28]. Even if this species is one of the oldest known nudibranchs, originally described by Linnaeus in 1758 in the *Systema Naturae*, it was the object of a very complicated taxonomy that was solved only after the designation of a neotype from one specimen collected in Castropol, Asturias (Atlantic coast of Spain) that is currently deposited in the Museum National d'Histoire Naturelle in Paris (MNHN) [29]. To date, *D. verrucosa* is the type species of the genus *Doris* Linnaeus, 1758, one of the oldest and richest nudibranch genera with about 56 currently accepted species [30]. *Doris verrucosa* is characterised by some morphological diagnostic features, like the dorsum covered with simple, rounded tubercles, two tubercles around the rhinophoral cavity, and the presence of eight tubercles around the gills [31]. This species has also lateral, triangle-shaped extensions with a ventral furrow around the mouth, approximately 13 rhinophoral lamellae, tubercles covering the dorsum more elongated than rounded and distant from each other, a foot width that does not reach the edge of the notum, and with the same width/length of the anterior–posterior proportion [31,32]. Several authors have considered *D. verrucosa* a widespread species, with a geographic range including the western and eastern Atlantic Ocean; however, recent comparative anatomical studies have demonstrated that populations from the western Atlantic belong to a different species, namely *Doris januarii* (Bergh, 1878), while *D. verrucosa* is restricted to the Atlantic coast of Europe and the Mediterranean Sea [29,31,32]. The eastern Atlantic area of occurrence is wide since it ranges from the North Sea [33] to the Canary Islands [34–36] and probably to Ghana [37] and South Africa [38]. Within the Mediterranean Sea, *D. verrucosa* has been detected from the Spanish coast, with two specimens in the Barcelona Forum bathing area [27] and in the Catalan coast [35]; along the French coast, in Thau lagoon [39]; from Malta [40]; and in the Adriatic basin in Croatia [41] and in the Sicily Channel from the Tunis harbour, Tunisia [42]. In Italy it was collected in the central Tyrrhenian basin, from the Gulf of Naples, where it has been frequently sampled [23–25] and in the southern Tyrrhenian area from Ustica Island (reported as *Doris* cf. *verrucosa*) [43] and from the Faro Lake in north-eastern Sicily Island [44]. It was registered in the Ionian Sea along the Salento Peninsula, from the Mar Piccolo of Taranto, Santa Maria di Leuca [39,45], and close to Gallipoli (Lecce), where its spawning was observed in August [26].

Despite being a common species reported throughout the Mediterranean basin, noteworthy in applied research, and the type species of a representative nudibranch genus, it has been explored very little from a molecular point of view, with only two individ-

uals, for the whole range of distribution, partially analysed so far [24,46]. Considering the importance of a correct identification of this species to properly interpret and discuss its potential application in other fields of research, a molecular study was conducted on specimens observed and collected from highly anthropised areas of Southern Italy. The main goals of the present study are to (i) characterise *D. verrucosa* from a molecular point of view, filling the gap of knowledge still existing on the genetics of this important species, (ii) provide molecular data on the nuclear *H3* (histone *H3*) and the two mitochondrial *COI* (Cytochrome Oxidase subunit I) and 16S markers (the three most commonly used molecular markers in nudibranchs) that could be useful for future systematic studies, and (iii) add some data on the diagnostic characters, like the external colour variability and the shape of the egg masses that could be useful for a more effective species detection and identification in the future.

2. Materials and Methods

2.1. Field and Laboratory Sampling

A preliminary bibliographic study was conducted to define the geographic range of the known distribution of this species. Monitoring took place in two different sectors of the Italian seas (according to [47]), namely the north-eastern part of Sicily Island (Strait of Messina) and the Salento peninsula in southern Apulia (Ionian Sea). The studied areas consisted of semi-closed basins characterised by a high level of anthropogenic impact and pollution and by fish and mussel farms [44,48,49].

The coastal Faro Lake (North-Eastern Sicily) was studied using a visual census followed by the collection, cataloguing, and storing of some individuals for future molecular analyses. The Sicilian specimens were observed through snorkelling and SCUBA diving activities, and the pictures were taken in situ or in the laboratory.

In the framework of a project of the marine zoology group of the University of Salento, the Heterobranchia fauna from the Mar Piccolo of Taranto (Ionian Sea) was investigated between 2020 and 2024. A monthly SCUBA diving activity allowed us to observe and photograph the specimens in situ and in the laboratory and to collect and store them in EtOH 96% for further morphological and molecular analyses. All the collected specimens were catalogued (vouchers 'RM3') and stored in the Heterobranchia collection deposited at the Department of Biological and Environmental Sciences and Technologies (DiSTeBA) of the Salento University.

The photographic in situ documentation was obtained using a Nikon D500 camera (Tokyo, Japan) with a Tokina 10/17 lens (Tokyo, Japan), in an Isotta underwater housing with an 8" dome by Isotecnic (Castelnuovo del Garda, Italy) and two Sea&Sea YS-D3 underwater flashes (Tokyo, Japan), and post-produced with Camera Raw V7.0 and Photoshop CS6. Observation in the laboratory at a high magnification level was carried out under the stereomicroscope Nikon SMZ800N equipped with the Nikon Digital Sight 1000 camera.

2.2. Molecular Analysis

Total genomic DNA was extracted from a small piece of tissue of five collected specimens by using the 'salting out' procedure [50]. The primer pairs LCO1490 and HCO2198 [51], 16Sar-L and 16Sbr-H [52], and H3AD-F and H3BD-R [53] were used for the amplification of the two mitochondrial markers, cytochrome oxidase subunit I (*COI*) and 16S and the nuclear histone *H3*, respectively. The PCR reaction mix and the temperature profile used to amplify the three molecular markers were the same already reported in [54]. The amplified products were sequenced at the European Division of Macrogen Inc. (Milan, Italy). The newly obtained sequences were edited with Staden Package 2.0.0b9 [55] and checked using the BLASTN V2.17.0 [56] to exclude contaminations and to confirm the iden-

tity of the sequenced fragments. Newly obtained sequences were deposited in GenBank (available at https://www.ncbi.nlm.nih.gov/). Consensus sequences were aligned together with sequences already available in GenBank using the Muscle algorithm implemented in MEGA version 11 [57]. Four different alignments were generated: three single-gene datasets (*COI*, 16S, and *H3*) and one with the three genes concatenated and partitioned (ConcDNA). The program Gblocks 0.91b with less stringent options [58,59] was used to eliminate poorly aligned positions or hyper-divergent regions of the multiple sequence alignment of the 16S rDNA. The best-fitting evolutionary model for each of the four datasets (three single genes and one concatenated and partitioned) was determined using JModelTest version 2.1.10 under the BIC model [60]. To generate the concatenated and partitioned dataset, the program DnaSP 6.12.03 [61] was used. Analysis of the different haplotypes was carried out to reconstruct the *COI* network by using the program PopArt (Population Analysis with Reticulate Trees) (available at https://popart.maths.otago.ac.nz/, accessed on 4 May 2024) with the TCS inference method [62]. Prior to conducting phylogenetic analyses using *COI* sequence data, we evaluated substitution saturation in the *COI* alignment using the entropy-based index of substitution saturation developed by Xia et al. [63], as implemented in DAMBE v7.3.32 [64]. The proportion of invariant sites (Pinv) was set to 0.1468 based on the estimate obtained in DAMBE. Bayesian inference and maximum likelihood phylogenetic analyses were carried out to investigate the evolutionary relationships. The Bayesian inference analysis (BI) was performed using the program MrBayes (v. 3.2.6) [65], applying a Bayesian posterior likelihood methodology. Each of the four runs was conducted with four MCMC (Markov chain Monte Carlo) for five million generations, a sample frequency of one tree per 1000 generations and a burn-in of 25%. Maximum likelihood analysis was performed using raxmlGUI 1.5b2 [66], a graphical front-end for RAxML 8.2.1 [67], with 100 independent ML searches and 1000 bootstrap replicates. The species *Goniobranchus vibratus* (Pease, 1860) was selected as the outgroup species for both analyses.

3. Results

The results from the bibliographic study on the geographic range of distribution of this species and the on-field investigations allowed us to reveal the presence of many individuals of *Doris verrucosa* from the two marine sectors investigated here (Figure 1).

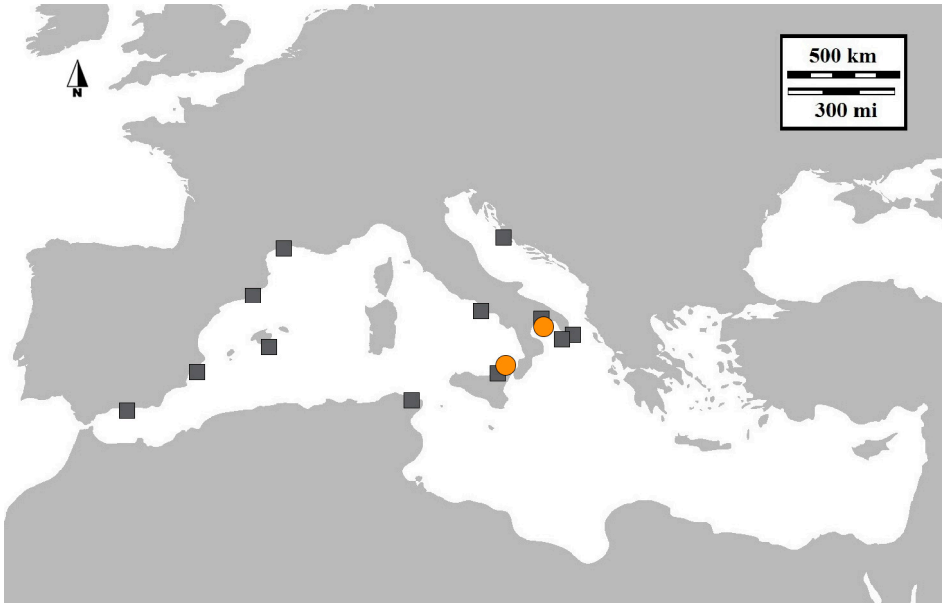

Figure 1. Maps of known (dark grey squares) and new (orange circles) Mediterranean records of *D. verrucosa* reported in the present study.

Four specimens were photographed from the Faro Lake, three of them collected, and many were observed from the Mar Piccolo of Taranto: six were photographed in situ and collected (Table 1).

Table 1. Date, locality of collection, voucher (except for the individuals that were non-collected 'n.c.'), and notes on the *Doris verrucosa* specimens of the present study. The asterisk (*) highlights specimens that were molecularly analysed.

Species	Locality	Date	Voucher	Note
Doris verrucosa	Sicily, Messina, Faro Lake	7 July 2018	RM3_1419 *	Depth: 1 m; Temperature: 26 °C
		7 July 2018	RM3_1432	Depth: 1 m; Temperature: 26 °C
		7 July 2018	RM3_1438 *	Depth: 1 m; Temperature: 26 °C
		26 June 2023	n.c.	Depth: ca. 0.5 m; Temperature: 27 °C
	Apulia, Taranto, Mar Piccolo	13 October 2022	RM3_2592 *	Depth: 4 m; Temperature: 22 °C
		15 September 2021	RM3_2282 *	Depth: 4 m; Temperature: 25 °C
		27 June 2024	RM3_3525	Depth: 3 m; Temperature: 27 °C
		27 June 2024	RM3_3526	Depth: 3 m; Temperature: 27 °C
		27 June 2024	RM3_3527	Depth: 3 m; Temperature: 27 °C
		27 June 2024	RM3_3528 *	Depth: 3 m; Temperature: 27 °C

In situ observations allowed us to obtain and document (see Table 1) useful information on the bathymetric range, preferred season, and water temperature of *D. verrucosa*, along with images of mating and egg-laying behaviours (Figure 2).

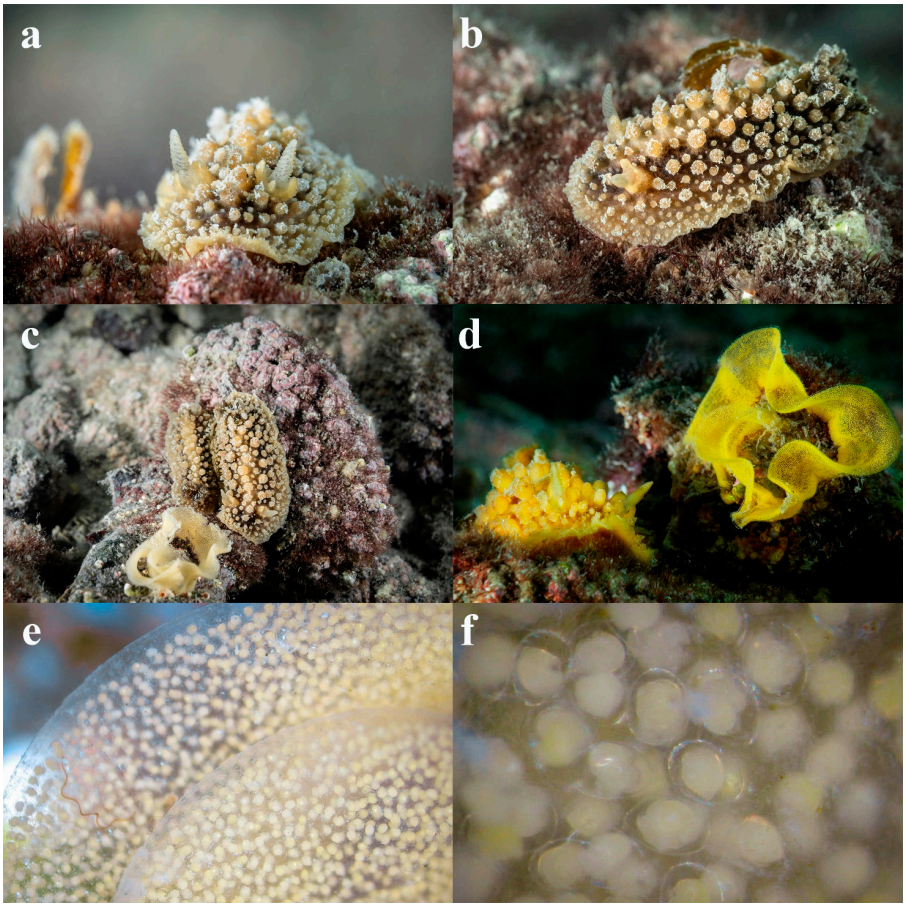

Figure 2. Images in situ (**a**–**d**) and in laboratory (**e**,**f**) of *Doris verrucosa* specimens and its egg masses observed during this study; (**a**,**b**) frontal and upper view of *D. verrucosa* (voucher RM3_ 3528) from Mar Piccolo of Taranto; (**c**) two specimens mating and with their newly laid egg mass, visible at the bottom left; (**d**) the yellow morphotype (voucher RM3_2282) from Mar Piccolo of Taranto and its yellow egg mass; (**e**) detail of the egg mass at a higher magnification; (**f**) images of the veliger larvae inside the egg capsule.

The body colour pattern is variable, going from a brown morphotype, through an orange one, and to the more common morphotype with bright yellow and pale creamy colours (Figure 3). The general shape of the body and the diagnostic features perfectly match those reported in the original and subsequent descriptions of this species (see the Introduction Section); however, it is noteworthy that a lighter area extends centrally along the entire dorsum, flanked by darker lateral areas. This pattern is present regardless of the colour of the different morphotypes (Figure 3).

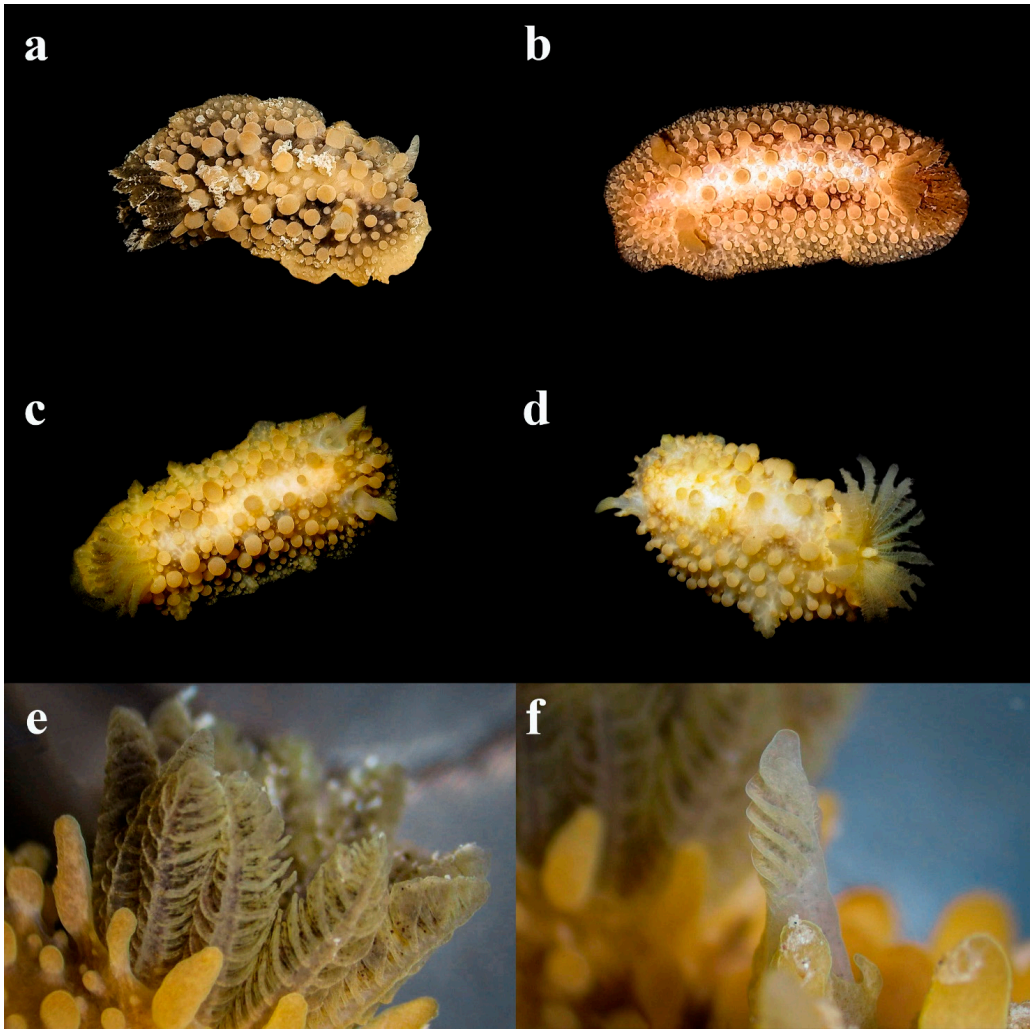

Figure 3. Images of *Doris verrucosa* specimens observed and photographed in laboratory; (**a**) the brown morphotype (voucher RM3_3526) observed from the Mar Piccolo of Taranto; (**b**) the light brown morph showed by the specimen voucher RM3_2592; (**c**,**d**) two of the three creamy coloured specimens collected from the Faro Lake (vouchers RM3_1419 and RM3_1438 in (**c**,**d**), respectively); (**e**,**f**) details of the gills (**e**) and the rhinophore (**f**) photographed from the specimen with voucher RM3_3528.

All the *D. verrucosa* individuals were found in transitional water systems used as aquaculture sites, which are very complex ecotones characterised by high spatial and temporal variability of physico-chemical characteristics and productivity patterns [68]. The Sicilian specimens were found in an environment characterised by a high turnover in benthic communities and with sediments at the bottom that are in part anthropogenized, being reworked by the mollusc farmers, and very rich in remnants of shells of cultivated bivalves [68,69]. The Apulian specimens were found in a rhodolite bed habitat, extending

over a sandy bottom without any significant cover of algae or phanerogams, down to a depth of approximately 3 m [70].

Molecular Analysis

A total of 15 sequences, one for each of the three molecular markers, were obtained from five specimens of *D. verrucosa* collected in the Tyrrhenian (Sicily) and Ionian Seas (Apulia) (Table 2). These newly obtained sequences were compared with those already present in GenBank and belonging to the same species or to congeneric and/or closely related taxa for a final dataset consisting of 67 total sequences (Table 2).

Table 2. Species name; voucher; locality; and GenBank *COI*, 16S, and *H3* accession numbers of the specimens included in the molecular analyses. Highlighted with an asterisk '*' are the individuals included in the concatenated and partitioned dataset (ConcDNA). In bold are the specimens collected and analysed in the present study.

Species	Voucher	Locality	COI	16S	H3
Doris berghi	G04 *	Playa Viveiro, Galicia, Spain	OR286435	OR286514	OR340969
	GC40 *	Piscinas de Agaete, Gran Canaria, Spain	OR286436	OR286515	OR340970
	ZSM20210024 *	Coves Cala Maset, Sant Feliu de Guíxols, Girona, Spain	OR286438	OR286516	OR340971
	MCZ395161	Aigua Freda, Begur, Girona, Spain	OR286437	–	–
Doris bertheloti	ZSM20240264/B7	La Herradura, Granada, Spain	OR286428	–	–
	ZSM20240263/B2 *	Blanes, Girona, Spain	OR286430	OR286510	–
As *Doris verrucosa*		–	ON716048	–	–
Doris marmorata	**RM3_3419 ***	**'La Depuradora', Punta del Romaní, l'Escala, Girona, Spain**	**PX123159**	**PX128874**	**PX148116**
	ZSMMol20210023 *	Coves Cala Maset, Sant Feliu de Guíxols, Girona, Spain	OR286431	OR286511	OR340966
	ZSMMol20210045 *	Caleta Caballo, Lanzarote, Spain	OR286429	OR286509	OR340965
Doris montereyensis	BICSIOM12334 *	USA: California, La Jolla, La Jolla Canyon	KC153022	KC153024	–
	CASIZ174493 *	USA: Battery Point, Crescent City, Del Norte Co., California	MF958425	MF958294	–
Doris nobilis	Gastr 8481V	–	MG935354	–	–
Doris ocelligera	X396 *	Coves Cala Maset, Sant Feliu de Guíxols, Girona, Spain	OR286433	OR286512	OR340967
	X447 *	Coves Cala Maset, Sant Feliu de Guíxols, Girona, Spain	OR286434	OR286513	OR340968
	MCZ395160	Punta del Romaní, l'Escala, Girona, Spain	OR286432	–	–
Doris odhneri	CASIZ188014	USA: Duxbury Reef, Marin Co., California	–	MF958295	–
	–	Munamjin-ri, Gangwon-do, South Korea	OL800585	OL800585	–
Doris verrucosa	**RM3_1438 ***	**Faro Lake, Messina, Sicily**	**PX123160**	**PX128875**	**PX148117**
	RM3_1419 *	**Faro Lake, Messina, Sicily**	**PX123161**	**PX128876**	**PX148118**
	RM3_2282 *	**Mar Piccolo of Taranto, Apulia**	**PX123162**	**PX128877**	**PX148119**
	RM3_2592 *	**Mar Piccolo of Taranto, Apulia**	**PX123163**	**PX128878**	**PX148120**
	RM3_3528 *	**Mar Piccolo of Taranto, Apulia**	**PX123164**	**PX128879**	**PX148121**
	ZSM20210044 *	Étang de Thau, Sète, France	OR286439	OR286517	OR340972
	DVCM1	Capo Miseno, Naples, Italy	–	HE861892	–
Homoiodoris japonica	Isolate 11	China	OQ573572		

Table 2. *Cont.*

Species	Voucher	Locality	COI	16S	H3
Avaldesia albomacula	CAS:IZ:181136 *	Bigej-Meck, Kwajalein Atoll, Marshall Islands, Pacific Ocean	MN720286	MN722434	MN720314
Chromodoris sp.	OS-Ss2	Iran	MN548837	_	_
Halgerda meringuecitrea	CASIZ 231100 *	South Africa: KwaZulu-Natal	MW223058	MW220923	MW414987
Goniobranchus vibratus	CASIZ 175564 *	USA: Hawaii, Maui	JQ727859	JQ727741	_

The *COI* single dataset included 29 sequences, and the alignment was 647 bp long. The proportion of invariant sites (Pinv) was set to 0.1468 based on the estimate obtained in DAMBE. The calculated Iss value was 0.3301, which is significantly lower (p = 0.0000) than the critical Iss.c value of 0.7289 (assuming a symmetrical tree topology), indicating limited substitution saturation. These results suggest that the *COI* sequences are informative and appropriate for phylogenetic inference. The 16S single dataset contained 23 sequences with a final alignment of 359 bp after GBlock processing. The histone *H3* single gene dataset had 16 sequences and was 328 bp long. The concatenated (*COI* + 16S + *H3*) and partitioned dataset (ConcDNA) consisted of 20 taxa, and the alignment was 1334 bp. The best evolutionary models selected for the *COI*, 16S, and *H3* were, respectively, TIM3 + I + G, TPM3uf + G, and K80 + G for the single gene datasets and TIM3 + I + G, TPM3uf + G, and TPM2 + G for each partition of the ConcDNA dataset. All the resulting trees were congruent with each other but showed a different ability to resolve phylogenetic relationships at different taxonomic levels. In fact, the *COI* single gene analysis proved powerful to investigate at the species level (Figure 4), while the concatenated analysis was the best to investigate at deeper phylogenetic relationships (Figure 5). The single gene analyses carried out using the 16S and the *H3* molecular markers were congruent with *COI* and the concatenated and partitioned analysis, but, as expected [71–73], with low statistical support; for this reason, these analyses are not shown here. Values of posterior probability (from the Bayesian analysis) higher than 0.90 and of bootstrap (from the maximum likelihood) higher than 70% were considered, while values lower than 0.50 and 50, respectively, were not reported in the final topologies. The *COI* single-gene dataset was used to investigate at the species taxonomic level, with all the species showing high support values except for the *D. berghi* (Vayssière, 1901) clade, which was supported only by Bayesian analysis and not the maximum likelihood inference. The monophyletic clade consisted, with high support (BPP = 1, BP = 99), of all the *D. verrucosa* individuals (Figure 4). Furthermore, an additional specimen belonging to the *Doris marmorata* Risso, 1818 species helped us to clarify a case of misidentifications that had occurred in a previous study focused on this species and the closely related *Doris bertheloti* A. d'Orbigny, 1839 [46]. In fact, the addition of the newly collected specimen (voucher RM3_3419) in the present molecular study revealed that the individual with voucher ZSMMol20210045 (*COI* accession = OR286429) previously identified as '*Doris bertheloti*' indeed belongs to *D. marmorata* (Figure 4) as well as the specimen with voucher ZSM20240263 (*COI* accession = OR286430) wrongly assessed to *D. marmorata* is indeed *D. bertheloti* (Figure 4). The TCS haplotype network analysis was performed on the *COI* single-gene alignment and revealed each five investigated *D. verrucosa* being characterised by their own unique haplotypes, suggesting a high genetic variability within this species and a reduced genetic flow between different populations (Figure 4).

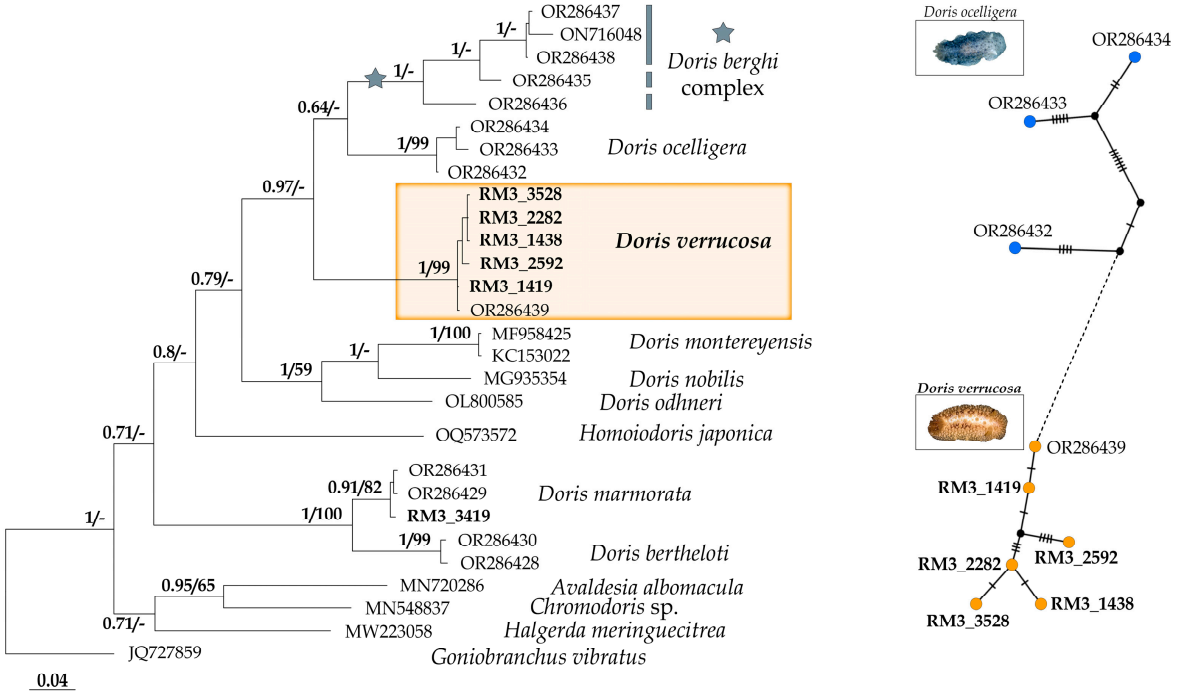

Figure 4. Bayesian inference tree based on the *COI* dataset. Numbers at nodes indicate Bayesian Posterior Probability (BPP; **left**) and bootstrap support from maximum likelihood analysis (BP; **right**). The '-' symbol indicates BPP < 0.50 and BP < 50%. On the right side is reported the *COI* haplotypes analysis (based on the TCS network) of the *Doris verrucosa* (orange) and its closely related *D. ocelligera* (blue).

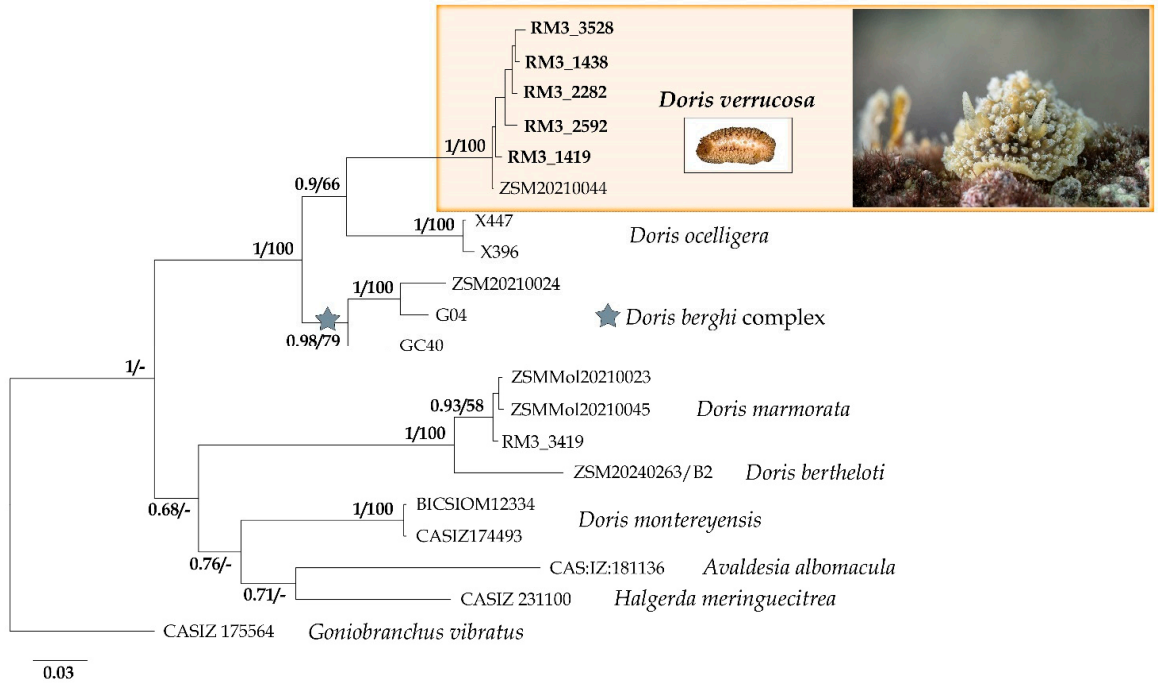

Figure 5. Bayesian phylogenetic tree based on the ConcDNA dataset (*COI*, 16S, *H3*). Bayesian posterior probability (BPP; **left**) and bootstrap support from maximum likelihood analysis (BP; **right**) are indicated at each node. The '-' symbol indicates unsupported values.

The Bayesian and maximum likelihood analyses carried out on the concatenated and partitioned dataset (ConcDNA) gave congruent topologies and were useful to unravel phylogenetic relationships at higher taxonomic levels (Figure 5). In fact, the results suggest that *D. ocelligera* (Bergh, 1881) is sister to *D. verrucosa* (BPP = 0.9, BP = 66), evidence that was

not revealed in the recent systematic study carried out on this group [46], and with *D. berghi* complex as sister to them (BPP = 1, BP = 100). *Doris marmorata* and *D. bertheloti* confirmed as sister taxa (BPP = 1, BP = 100) and grouped (with no statistical support) (BPP = 0.68, BP = <50) with a clade non supported (BPP = 0.76, BP = <50) that includes the conspecific *D. montereyensis* J.G. Cooper, 1863 (BPP = 1, BP = 100) and the outgroups *Avaldesia albomacula* (J. M. Chan & Gosliner, 2007) and *Halgerda meringuecitrea* Tibiriçá, Pola & Cervera, 2018 (BPP = 0.71, BP = <50) (Figure 5).

4. Discussion

Ten specimens of *Doris verrucosa* were studied from two localities of Southern Italy, consisting of areas highly impacted by human activities, like coastal lakes and semi-closed basins.

In this context, *D. verrucosa* shows a wide distribution across the whole Mediterranean basin; however, evidence from the present study suggests that the area of coverage is the western and central Mediterranean Sea, but with an important contribution coming from localities used as aquaculture sites, with high human impact. In fact, *D. verrucosa* is an euryhaline species as it lives from brackish [44] to marine waters and prefers shallower basins characterised by low hydrodynamics and eutrophic water. The ability shown by this species to live in a polluted environment with highly variable biotic and abiotic factors is noteworthy and becomes even more appealing if considering the biochemical compounds that are synthesised by *D. verrucosa* and that are characterised by antimicrobial and antiviral activities [74], abilities that could be particularly useful for the well-being of this species in such difficult environments. The variability observed in the body colour pattern of *D. verrucosa* (Figure 3) is in line with what is reported for other nudibranchs that can have different background body colours according to the prey they feed on [75,76]. In these regards, detailed images of the wide body colour variability (Figure 3) and of the shape of the egg masses with details of the veliger larvae (Figure 2c–f) are documented here and shown for the first time. These characters are important to define the ranges of intraspecific variability and could also be significant at higher taxonomic levels. In fact, the shape of the egg mass was revealed as a powerful diagnostic character also at the genus and family taxonomic levels [77]. For this reason, images of the egg masses of *D. verrucosa*, the type species of the genus *Doris*, are even more important.

Molecular investigations carried out in this study using the molecular markers mostly used in nudibranchs (i.e., *COI*, 16S, and *H3*) revealed a complicated phylogenetic history that is far from being fully understood yet (Figures 4 and 5). In fact, future analyses including a more representative dataset are required to better understand the systematics of the *Doris* genus, especially considering that it is one of the first genera to be described and in which many species with unknown taxonomy were included, causing a consequent general confusion. However, the present study allowed us to add some important insights, like the possible sister relationship between *D. verrucosa* and *D. ocelligera* (Figure 5) that was not revealed from the recent phylogenetic study carried out on the family [46]. Finally, it is noteworthy to mention that *D. berghi* is a possible case of species complex since the molecular results show a high genetic intraspecific diversity at the *COI* barcoding marker (going from 0.5% to 13.1% of minimum and maximum uncorrected *p*-distances, respectively) with values that fall within the range of interspecific variability commonly accepted for nudibranchs [71,78,79]. Moreover, the *D. marmorata* specimen (voucher RM3_3419) observed and collected in Girona (Spain) and included in the present study (Figure 6) helped us to clarify the incorrect identifications made in the past between *D. marmorata* and *D. bertheloti* that have generated confusion in the phylogenetic analyses (and in the relative table) reported in Renau and collaborators [46].

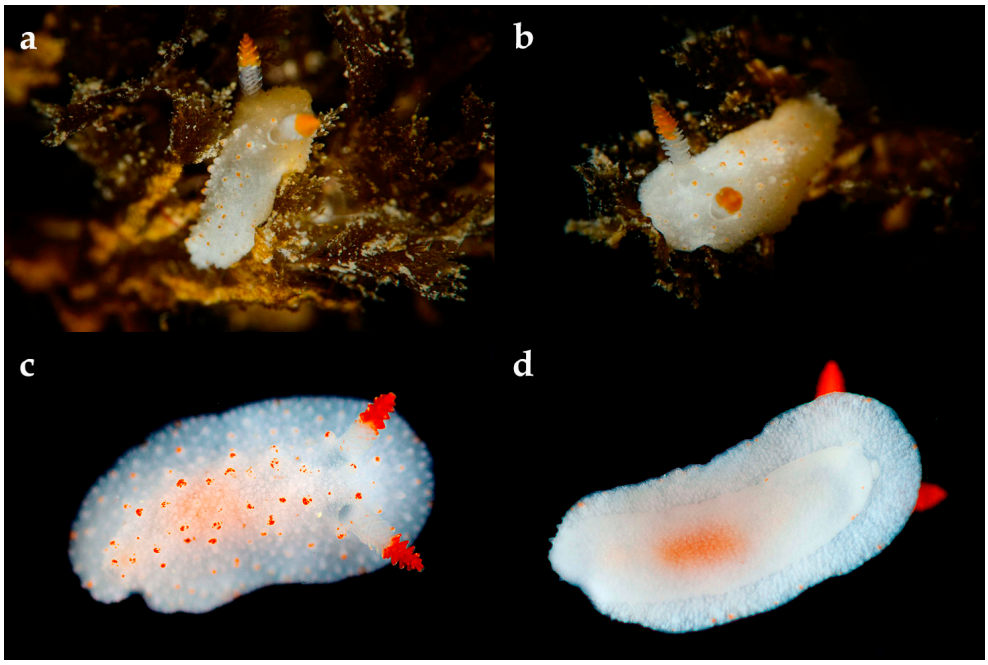

Figure 6. Images of *Doris marmorata* specimens with voucher RM3_3419 collected in Girona, Spain (Mediterranean Sea), and analysed in the present study. In situ dorsal (**a**) and frontal (**b**) view of the collected specimen. In (**c**,**d**) are the dorsal and ventral pictures taken in the laboratory.

Some speculations can be proposed based on the results of the present study and particularly regarding the importance of monitoring highly variable marine environments such as transitional waters and coastal areas with high human impact. In fact, these overlooked habitats, like ports, coastal lakes and brackish environments, have recently been revealed to be important to unravel rare or unknown diversity [80], to detect non-indigenous species early [81], and to study Heterobranchia species that are adapted to live under polluted and stressed conditions.

Finally, monitoring the future changes in the distribution range, as well as in other ecological traits of *D. verrucosa*, can highlight possible shifts in the conditions of natural environments and the presence of hidden environmental stressors. In this regard, it is interesting to note that we observed a potential anticipation in egg laying compared to what was previously observed for *D. verrucosa*. In fact, the specimens from Mar Piccolo of Taranto laid their eggs in June, while Perrone [26] observed individuals from nearby Gallipoli (about 80 Km south of the Mar Piccolo of Taranto) with eggs in August. This anticipation from late summer to early summer could be a mirror of the increasing sea water temperature due to global warming, confirming the Heterobranchia as useful bioindicators of the environmental status [82].

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Data Availability Statement: Newly produced DNA sequences will be submitted to GenBank (at https://www.ncbi.nlm.nih.gov/) after paper acceptance.

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Abbreviations

The following abbreviations are used in this manuscript:

NIS	Non-Indigenous Species
COI	Cytochrome Oxidase Subunit I
H3	Histone *H3*

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