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Integrative Taxonomy of the Bubble Snails (Cephalaspidea, Heterobranchia) Inhabiting a Promising Study Area: The Coastal Sicilian Faro Lake (Southern Italy)

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Abstract: The worldwide diffused bubble snails, Haminoeidae, although characterized by an extreme morphological homogeneity, display the most diverse radiation inside the order Cephalaspidea. This hidden diversity within the family Haminoeidae was recently unraveled by molecular studies, which helped to understand the evolutionary history of this group by clarifying some aspects of its systematics. In fact, the type genus *Haminoea* W. Turton and Kingston (1830) was proved to be polyphyletic and, consequently, the genus *Haminoea sensu stricto* was restricted to the Mediterranean, Atlantic and East Pacific species, with the Mediterranean *Haminoea hydatis* Linnaeus (1758) as the type taxon. However, at the specie rank, many aspects need to be clarified, especially concerning the Mediterranean fauna. Due to low reliability of macro-morphological characters, the minimal quantity of molecular data currently available on Mediterranean specimen adds to the lack of molecular comparison in most reports. Based on such considerations, *Haminoea* species from an interesting Mediterranean study area, Faro Lake, a Sicilian coastal lake that is considered a hot spot for both alien and endemic marine Heterobranchia, have been studied using an integrative taxonomic approach. Eleven Mediterranean specimens belonging to four *Haminoea* bubble snails have been collected, identified and compared with samples from other localities, integrating ecological, morphological, anatomical (reproductive apparatus) and molecular data. Based on molecular investigations carried out on three different molecular markers (H3, 16S and COI), the morphological identifications of the species collected in the Faro Lake have been confirmed, and 37 new sequences are provided for future comparisons. Furthermore, results from this integrative systematic study shed light on the phylogenetic relationships occurring in this group of bubble snails that could be useful in identifying valid diagnostic morphological characters. *Haminoea hydatis* and *H. navicula* were confirmed to be close to each other, with *H. orteai* as sister to them and with *H. orbignyana* as the basal taxon. Given external morphological features are unreliable with species identification in *Haminoea* genus open questions on the geographical distribution of the species and on their ranges of intraspecific variability have yet to be addressed and further in-depth studies are needed. Finally, the presence of three sympatric *Haminoea* species, two of which are considered native or long-time naturalized, along with other occasional congeneric species, and the absence of the introduced invasive *Haloa japonica*, reflects both the resilience and stochastic space-temporal dynamics of Faro Lake. This confirms it as an inexhaustible source of case-studies.

Keywords: phylogeny; Mediterranean Sea; mollusca; systematics; evolution; diagnostic characters

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1. Introduction

Despite their apparent morphological homogeneity, the bubble snails of the family Haminoeidae represent the most diverse radiation inside the order Cephalaspidea, both at the genera [1] and species level [2,3]. They inhabit shallow waters in almost all temperate and tropical regions worldwide. Molecular investigations have recently contributed to clarify some aspects of this important radiation, demonstrating that the type genus, *Haminoea* W. Turton and Kingston (1830), is a polyphyletic taxon [4]. Consequently, reinstating the genus *Haloa* Pilsbry (1921) for Indo-west Pacific species has been proposed, while maintaining the genus *Haminoea sensu stricto* only for the Mediterranean, Atlantic and East Pacific species. The Mediterranean Sea's *Haminoea hydatis* Linnaeus (1758) serve as the type taxon for the genus. At species level, color patterns are generally considered a useful characteristic for a quick identification. However, this is only valid for some tropical species, as different colorations generally represent phenotypic differences rather than traits at the species level [5]. This is particularly true when considering European temperate haminoeid species [6], which are typically characterized by similar body color patterns and by the lack of recognizable and characterizing external structures. For this reason, it is becoming evident that there is an increasing need to reevaluate, utilizing new innovative techniques, the diagnostic power of the morphological features that have been commonly used to investigate the Mediterranean *Haminoea* diversity. Although shell morphology has proven effective in delineating taxonomic ranks higher than the species level (e.g., genera and families), it could also serve as a useful diagnostic character and a valid support for delimiting species when an integrative taxonomic approach is employed. Integrating results from additional characters obtained through different methodologies revealed to be the best way to efficiently estimate diversity in marine Heterobranchia. This helped to unravel the evolutionary history of a group of species and to redefine the diagnostic morphological and ecological traits [5,7,8]. To date, the mostly used diagnostic characters in Cephalaspidea are the shell, the buccal apparatus (i.e., radula and gizzard plates), the Hancock's organ and the reproductive system with particular attention to the male portion [6,9,10]. The prostatic gland can be different shapes (oval, spherical, triangular) and is often divided in two lobes, proximal and distal. The cylindrical shaped prostatic duct can be short, thick and straight, rather than longer, thin and convoluted. The penis, thin or bulky, opens into the genital pore (atrium), which contains the papilla penis. The shape can differ among species and may have ornamental features such as crests or spins in an upper portion. It is important to note that the distal part of the male genitalia can be referred to as the "penial sheath", while the structure present inside is known as the "penis". However, under an anatomical point of view, it is more accurate to define the entire terminal part of the male genitalia closest to the genital atrium as "penis" and refer to the structure inside, in its apical portion, as the "papilla penis" (glans). For this reason, the latter terminology is adopted here. Both the prostatic gland and the papilla penis are considered diagnostic. Unfortunately, detailed examination of such characters is rarely pursued, and the variability of the other diagnostic characters often leads to incorrect identifications, as observed in the case of invasive Cephalaspidea, and misassignment of DNA sequences [11–13]. Incorrect determinations represent a problem that should not be overlooked, as underlined by [13] in relation to the emergence of cercarial dermatitis caused by schistosome parasites, which are notably associated with the invasive species *Haloa japonica* (Pilsbry, 1895). Moreover, Heterobranchia faunas are known for both stochastic and environmentally fluctuations [14], extensive colonization of new habitats [15] and the efficient spread of invasive species [16,17], often associated with Mytilus, clam farming and export [13,18,19].

Faro Lake (northeastern Sicily), in this respect, is an appropriate study area. Although belonging to a natural reserve, this coastal lake is subject to high pressure from clam and mussel farms, which contribute to the introduction of alien species [18]. Furthermore, in proportion to its small extent, the lake can be considered a hotspot for heterobranchs, as testified by the 47 species reported by [20], which includes four morphologically identified

species of the genus *Haminoea*. Prior to their study, only two previous [21] and dated [22] checklists had reported *Haminoea* species from Faro Lake; however, none of them included a molecular identification of the species recorded.

Taking into consideration the difficulty of identifying *Haminoea* species when relying only on external morphological features, the limited availability of molecular data on Mediterranean *Haminoea* specimens, and the lack of genetic identification of the *Haminoea* species from the studied area, the aims of this study are to: (i) identify the *Haminoea* species living in Faro Lake with an integrative taxonomy approach, which involves morphological, anatomical and molecular methods; (ii) compare the population living in Faro Lake with populations from other Mediterranean and Extra Mediterranean localities; (iii) investigate the phylogenetic relationships occurring between Sicilian species and other congenerics; (iv) provide insights on the morphological characters that could be useful for species identification purposes; (v) clarify some controversies about the identification and distribution of some disputed Mediterranean Haminoeidae species.

2. Materials and Methods

2.1. Study Area

Faro Lake, together with the connected Ganzirri Lake, belongs to 'The Capo Peloro Lagoon', a brackish system located in the northeastern point of Sicily (38°15′57″ N, 15°37′50″ E) (Figure 1) in an area which is highly anthropized except for a narrow riparian belt. The lagoon receives significant marine inflows through two canals that are characterized by strong tidal currents, contrasting with the low hydrodynamics of waters inside both lakes [23]. Faro Lake, which covers an area of just 263,600 m^2, is the deepest coastal basin in Italy, reaching 29 m depth in its eastern half, and not exceeding 3.5 m in the western area. Known for a peculiar meromictic regime, with anoxic waters below 10 m depth, surface waters are generally not disturbed by dystrophic phenomena and related anoxic crises [24]. Due to the brackish condition, hydrological parameters are subject to marked seasonal and interannual variability. Temperatures from 10 °C in February to 29.43 °C in August have been reported, together with salinities from 33.0 to 38.6 and oxygen concentration from 73.82 ± 3.40% to 92.55 ± 7.53% [25–27]. Such data agree with information provided by the Official Manager of the Reserve, which also recorded pH values between 7.94 and 8.40 [28]. High population density in the surrounding areas and human activities such as mollusk farming are the main causes of high anthropogenic pressure, the latter also being responsible for the introduction of remarkable non-indigenous species [18,29].

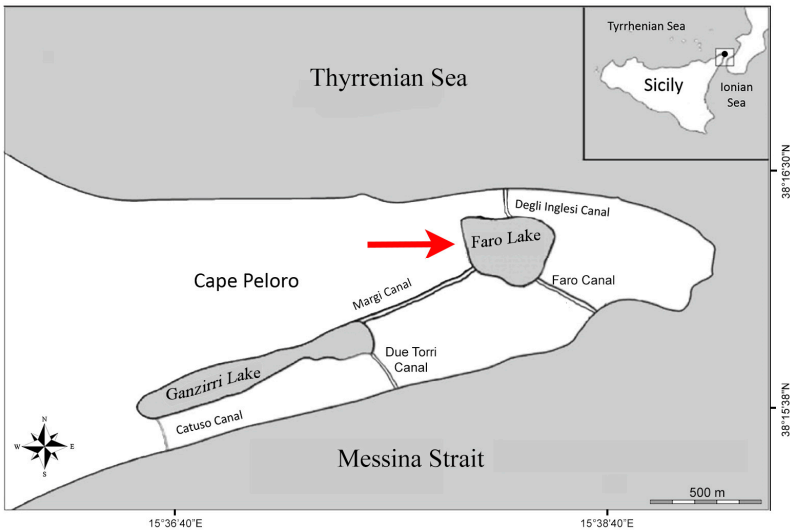

Figure 1. Map of the study area, Faro Lake, in the context of the 'Capo Peloro Lagoon' (Messina, Sicily, Italy). The red arrow highlights Faro Lake from where bubble snails subject to the present integrative taxonomic study were collected.

2.2. Shell Morphology and Anatomical Investigations

Living specimens have been collected by hand from 0.5 to 3 m depth throughout the whole shallow water area, photographed, preserved in 95% ethanol (EtOH) for further molecular analyses, and deposited in the Department of Science of the Roma Tre University collection (Vouchers RM3_ID number). The nomenclature used in the present study is based on the most up-to-date species names presented in the World Register of Marine Species (WoRMS) [30]. Once fixed, tissues were isolated by removing the shells and dissected under a light stereo microscope using very fine, pointed watchmaker's forceps. The genitalia were drawn using a light camera. Dry shells were also photographed using a digital camera. When possible, more than one specimen was analyzed to investigate the intraspecific variability of the anatomical characters selected.

2.3. Molecular Analyses

DNA was extracted and investigated from 13 newly collected Mediterranean specimens, and a total of 37 (13 COI, 12 16S and 12 H3) new sequences were analyzed and deposited in GenBank (Table 1). The final dataset included the newly obtained Mediterranean specimens together with specimens from other Mediterranean and extra Mediterranean localities, with a total of 104 individuals belonging to *Haminoea* and *Lamprohaminoea* genera. Molecular analyses involved a total of 326 sequences. In particular, the COI alignment was 627 bp and included 103 sequences, the 16S alignment was 415 bp from 69 individuals, the H3 alignment was 330 bp from 64 sequences while the concatenated and partitioned dataset included 68 individuals and was 1372 bp long. The best evolutionary models selected per each alignment were: TPM3uf+I+G, HKY+I+G and TrNef+G, respectively, for the mitochondrial COI and 16S and the nuclear H3 molecular markers.

Table 1. List of the species names, vouchers, sampling localities and COI, 16S and H3 GenBank accession numbers of the Cephalaspidea species included in the present study. Sequences included in the concatenated dataset are highlighted with an asterisk (*). In bold are the original data.

Species	Voucher	Locality	COI	16S	H3
Haminoea alfredensis	NHMUK 20070314	Kariega river estuary, Kenton, South Africa	KF615816*	MH933154*	MH933413*
	NHMUK 20070315	Kenton on Sea, Kariega river estuary, South Africa	KF615815*	MK474187*	MK492113*
	NHMUK 20070315	Kenton on Sea, Kariega river estuary, South Africa	KF615814*	MH933155*	MH933417*
	ZMBN 86406	Knysna lagoon, South Africa	MK473513*	MK474188*	MK473560*
	ZMBN 86406	Knysna lagoon, South Africa	MK473514*	MK474184*	MK473561*
Haminoea antillarum	NHMUK 20070094	Bocana, Sisal, Yucatan, Mexico	KF615811*	MK474186*	MK473562*
	NHMUK 20070093	Bocana, Sisal, Yucatan, Mexico	KF615820*	MH933150*	MH933410*
	NHMUK 20070316	Jupiter inlet, Palm Beach, Florida	KF615817*	MH933151*	MH933414*
	NHMUK 20070092	Mexico	KF615818	----------	----------
	NHMUK 20070091	Mexico	KF615819	----------	----------
	FTP_0224	Indian River Lagoon, Florida, USA	KP255198	----------	----------
Haminoea cf. *hydatis*	NHMUK 20060326	Port Barcarès, Salses-Leucaté Lake, Mediterranean France	KF615841*	KJ022796*	KJ022925*
	NHMUK 20060326	Mediterranean France	DQ974674*	MH933208*	MH933481*

Table 1. *Cont.*

Species	Voucher	Locality	COI	16S	H3
Haminoea hydatis	NHMUK 20070177	Italy	KF615840*	MH933152*	MH933411*
	RM3_2250	**Faro Lake, Messina, Sicily, Italy**	**OR197628***	**OR197605***	**OR208234***
	RM3_2252	**Faro Lake, Messina, Sicily, Italy**	**OR197629***	**OR197606***	**----------**
	RM3_2256	**Faro Lake, Messina, Sicily, Italy**	**OR197630***	**----------**	**OR208235***
	RM3_1675	**Faro Lake, Messina, Sicily, Italy**	**OR197631***	**OR197607***	**OR208236***
	RM3_1686	**Faro Lake, Messina, Sicily, Italy**	**OR197632***	**OR197608***	**OR208237***
Haminoea navicula	NHMUK 20070018	Canal de Mira, Aveiro, Portugal	KF615838*	MK474221*	MK473548*
	NHMUK 20070020	Portugal	KF615837	----------	----------
	NHMUK 20070020	Triângulo da Barra, Aveiro, Portugal	KF615839*	MH933144*	MH933405*
	NHMUK 20070021	Eight Acre Pond, Hampshire, UK	KF615836*	MH933145*	MH933406*
	BMNH 20070018	Aveiro, Portugal	EU314804	----------	----------
	BMNH 20060324	Fort Gilkicker Lagoon, England	DQ974676	----------	----------
	RM3_2240	**Faro Lake, Messina, Sicily, Italy**	**OR197633***	**OR197609***	**OR208238***
Haminoea orbignyana	NHMUK 20030296	Faro, Portugal	KF615813*	KJ022794*	MK473540*
	NHMUK 20030296	Faro, Portugal	KF615812*	MK474185*	MK473539*
	GeneBank 1	----------	EU314805	----------	----------
	ZMBN 81714	Naples, Italy	KC404964*	KC404959*	----------
	GeneBank 2	Lake Qarun, Fayoum, Egypt	KT339765	----------	----------
	GeneBank 3	Lake Qarun, Fayoum, Egypt	KT339766	----------	----------
	ZMBN 81791	Rabat, Morocco	MH933103*	MH933174*	MH933444*
	RM3_2254	**Faro Lake, Messina, Sicily, Italy**	**OR197634***	**OR197610***	**OR208239***
	RM3_2259	**Faro Lake, Messina, Sicily, Italy**	**OR197635***	**OR197611***	**OR208240***
	RM3_2247	**Faro Lake, Messina, Sicily, Italy**	**OR197636***	**OR197612***	**OR208241***
	RM3_2249	**Faro Lake, Messina, Sicily, Italy**	**OR197637***	**OR197613***	**OR208242***
Haminoea orteai	NHMUK 20030836	Barronco Hondo, Tenerife, Canary Islands	KF615846*	MK474239*	MK473555*
	NHMUK 20070458	Boca das Caldeirinhas, Faial, Azores	KF615844*	MH933160*	MH933422*
	NHMUK 20070458	Boca das Caldeirinhas, Faial, Azores	KF615845*	MK474238*	MK492112*
	ZMBN 81701	Lago Lucrino, Naples, Italy	KX383914*	MH933172*	MH933442*
	NHMUK 20070459	Faial, Azores	KC404963*	KC404960*	----------
	ZMBN 81701.1	Lago Lucrino, Naples, Italy	KX383912	----------	----------
	ZMBN 81701.3	Lago Lucrino, Naples, Italy	KX383913	----------	----------
	NHMUK 20070023	Sal Island, Cape Verde	KX383915	----------	----------
	CPIC 01878	Maliakos Gulf, Greece	KX683877	----------	----------
	CPIC 01879	Maliakos Gulf, Greece	KX683878	----------	----------
	CPIC 01880	Maliakos Gulf, Greece	KX683879	----------	----------
	RM3_2222	**Villa San Giovanni, Reggio Calabria, Italy**	**OR197638***	**OR197614***	**OR208243***

Table 1. *Cont.*

Species	Voucher	Locality	COI	16S	H3
Haminoea vesicula	CASIZ 97502	Bodega Harbor, Sonoma Co., California	KF615843*	MH933161*	MH933423*
	GeneBank 4	Washington, USA	JQ693571	----------	----------
	BMBM0081	False Bay, San Juan Island, Washington, USA	MH242779	----------	----------
	BIOUG12670G07	Indian Arm, British Columbia, Canada	MG423188	----------	----------
Haminoea virescens	GeneBank 5	Venice, California	AF156142*	AF156126*	----------
	10BCMOL-00152	Queen Charlotte City, Haida Gwaii, British Columbia, Canada	KF643269	----------	----------
	10BCMOL-00154	Queen Charlotte City, Haida Gwaii, British Columbia, Canada	KF643444	----------	----------
	10BCMOL-00307	Queen Charlotte City, Haida Gwaii, British Columbia, Canada	KF643501	----------	----------
	10BCMOL-00348	Skidegate Beach, Haida Gwaii, British Columbia, Canada	KF643861	----------	----------
	10BCMOL-00345	Skidegate Beach, Haida Gwaii, British Columbia, Canada	KF643877	----------	----------
	10BCMOL-00346	Skidegate Beach, Haida Gwaii, British Columbia, Canada	KF643968	----------	----------
	10BCMOL-00347	Skidegate Beach, Haida Gwaii, British Columbia, Canada	KF644011	----------	----------
Haminoea sp. 3	NHMUK 20070180	Florida, USA	KF615829*	MK474212*	MK473551*
	NHMUK 2007060	Pine Channel, Florida	KF615832*	MK474214*	MK473553*
	NHMUK 20070448	Banana River, Florida	KF615828*	MK474213*	MK473552*
Haminoea sp. 4	NHMUK 20070090	Bocana, Sisal, Yucatan, Mexico	KF615833*	MK474211*	MK473554*
Lamprohaminoea cymbalum	NHMUK 20030302	Pasar Wajo, Buton Is., Southeast Sulawesi, Indonesia	KF615842*	MH933149*	MH933409*
	ZMBN 81711	Magilao, Guam, Mariana Islands	KF992182*	KJ022812*	KJ022908*
	BNHS opistho 638	Lakshadweep, Kavaratti, India	MH638589	----------	----------
	ZMBN 81711	Magilao, Guam, Mariana Islands	MK473501*	MK474200*	MK492114*
	ZMBN 81711	Magilao, Guam, Mariana Islands	MK473495*	MK474201*	MK473587*
	MNHN 42249	Panglao, the Philippines	DQ974675*	MK474202*	MK473581*
	MNHN IM-2013-52940	Palikulo Peninsula, Vanuatu	MK473502*	MK474203*	MK473582*
	ZMBN 125446	Lakshadweep, India	MK473504	----------	MK473583
	ZMBN 125457	Lakshadweep, India	MK473499*	MK474204*	MK473584*
	ZMBN 125452	Lakshadweep, India	MK473500*	MK474205*	MK473585*
	MNHN IM-2013-52939	Segond Channel, vicinity of Marime College, Vanuatu	MK473497*	MK474206*	MK473586*
	ZMBN 125454	Tidal reef, Paindane, Mozambique	MK473503*	MK474199*	MK473534*
	ZMBN 125451	Paindane, Mozambique	MK473496	----------	----------
	ZMBN 125448	Paindane, Mozambique	MK473505	----------	----------

Table 1. *Cont.*

Species	Voucher	Locality	COI	16S	H3
Lamprohaminoea evelinae	UF 374145	Guam, Mariana Is.	MK473481	----------	MK473565
	MNHN 42264	Panglao, the Philippines	MK473457*	MK474189*	MK473566*
	UF 374127	Guam, Mariana Is.	MK473482*	MK474190*	MK473567*
Lamprohaminoea mikkelsenae	MNHN IM-2009-16288	Plage de Lavanono, Madagascar	MK473506*	MK474207*	MK473577*
	MNHN 42252	Panglao the Philippines	DQ974677*	MH933204*	MH933476*
	MNHN IM-2013-52902	Ranavalona, Madagascar	MK473507*	MK474209*	MK473579*
	BNHS opistho 1340	Andaman islands	MH638599	----------	----------
	BNHS opistho 1340	Andaman islands	MH638600	----------	----------
Lamprohaminoea ovalis	ZMBN 81762	Lazzaro, Reggio Calabria, Italy	MH933111*	MH933173*	MH933443*
	ZMBN 81762	Lazzaro, Reggio Calabria, Italy	MK473485*	MK474192*	MK473571*
	ZMBN 81803	Ihl Rabat, Malta	MH933112*	MH933175*	MH933445*
	ZMBN 125456	Split, Croatia	MK473487	----------	----------
	NHMUK 20070031	Airport Beach, Maui, Hawaii	MK473489*	MK474173*	MK473570*
	ZMBN 81689	Maui, Hawaii	KF992184*	KJ022814*	KJ022906*
	ZMBN 88230	Manza Beach, Okinawa	MK473491	MK474193	----------
	ZMBN 88230	Manza Beach, Okinawa	MK473488*	MK474194*	MK473568*
	AMS c.46947	South Moorena, Tahiti, French Polynesia	MK473490*	MK474195*	MK473575*
	MNHN IM, S12_MP588	Panglao, the Philippines	MK473492*	MK474196*	MK473572*
	MNHN IM-2013-52931	Malo, Vanuatu	MK473493*	MK474197*	MK473573*
	MNHN IM-2009-16293	Plage de Lavanono, Madagascar	----------	MK474210	----------
	CASIZ 192351	Red Sea, Saudi Arabia	MK473486*	MK474198*	MK473569*
	CASIZ 174196	Line Islands, Palmyra Atoll, West Lagoon	MK473494	----------	----------
	RM3_1175	**Cala Grande, Argentario, Tuscany, Italy**	**OR197639***	**OR197615***	**OR208244***
	RM3_2224	**Cittadella del Capo, Cosenza, Calabria, Italy**	**OR197640***	**OR197616***	**OR208245***
Lamprohaminoea vamiziensis	ZMBN 105076	Rock Walk, Vamizi Island, Quirimbas, Mozambique	MK473483*	MK474191*	MK473574*

DNA was extracted from a small piece of the foot tissue using the 'salting out' method [31] as reported in [7]. In particular, the tissue fragment is heated for one hour at 40 °C to remove the Ethanol alcohol in which it was stored. In the following step, 430 µL of Cell Lysis Buffer and 20 µL of Proteinase K are added to the dried tissue. The samples were then left in a thermoblock overnight at 56 °C. Next, samples were vortexed and centrifuged at 13,200 rpm for 10 min. After this first centrifugation, the supernatant liquid was carefully pipetted into new tubes. Afterwards, 160 µL of NaCl 5M was added to the samples, these were gently vortexed and centrifuged for 10 min at 13,200 rpm. The supernatant was moved into the final tubes and 500 µL of cold isopropanol added. Last, samples were gently vortexed and centrifuged under the same conditions used in the previous steps and finally the supernatant was discarded leaving the DNA pellet adhere to the wall of the tubes. One ml of 80% EtOH was added, and the tubes were centrifuged for the last time for 10 min at 13,200 rpm. The supernatant was carefully discarded again, and the samples were left to dry for 1–2 h at room temperature. Finally, dried samples were diluted with the 60–100 µL

of purified H_2O. Two different mitochondrial gene regions, COI and 16S, and the nuclear H3 were amplified. The universal primers LCO1490 and HCO2198 [32] and 16Sar-L and 16Sbr-H [33] were used for the COI and 16S mitochondrial markers, respectively, while H3AD-F and H3BD-R universal primers [34] were used for nuclear H3. Temperature profile for the PCR reactions was the same for the three molecular markers, starting with an initial denaturation step at 94 °C, which lasted 5 min. This step was followed by 35 cycles consisting of 30 s at 94 °C for the denaturation step, 60 s at an annealing temperature of 46–50 °C, and 60 s at an elongation temperature of 72 °C. After this cycle, temperature was hold for another 7 min at 72 °C. Once all these steps were finished, the whole reaction was cooled down to a temperature of 10 °C. The PCR reaction mix has a final volume of 20 µL and consisted of 14.6 µL of dH_2O, 4.0 µL of 5x FIREPol Mastermix [5x Reaction buffer (0.4M Tris-HCl, 0.1M (NH4)2SO4, 0.1% *w/v* Tween-20], 12.5 mM MgCl2, 1 mM dNTP), 0.2 µL of each forward and reverse primers (20 µM) and 1.0 µL DNA. The quality of all obtained PCR products was controlled on 1.2% agarose gel. Samples were sequenced by Macrogen Europe. Before the sequences were used for the alignment, they were controlled with the Basic Local Alignment Search Tool (BLAST) to exclude possible contamination. Sequences were aligned together with GenBank sequences using the Muscle algorithm implemented in MEGA 6.0 [35]. Four different alignments were generated, three single genes dataset (COI, 16S and H3) and one with the three genes concatenated and partitioned (ConcDNA). Primer regions were always removed from the final alignments. 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 [36]. To generate the concatenated and partitioned dataset, the program DnaSP 6.12.03 [37] was used. We used the ASAP species delimitation analysis [38,39] to detect the barcode gap in the distribution of pairwise distances calculated on the COI sequence alignment. Kimura Two Parameter (K80 Kimura) genetic distance method and the default settings parameters were used to carry out the ASAP analysis.

Bayesian Inference and Maximum Likelihood phylogenetic analyses were carried out to investigate on the phylogenetic evolutionary relationships. Bayesian Inference analysis (BI) was performed using the program MrBayes (v. 3.2.6) [40] applying a Bayesian posterior likelihood methodology. Each of the four runs were 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%. The Maximum Likelihood analysis was performed using raxmlGUI 1.5b2 [41], a graphical front-end for RAxML 8.2.1 [42], with 100 independent ML searches and 1000 bootstrap replicates. *Lamprohaminoea cymbalum* (Quoy and Gaimard, 1833) was used as the outgroup species for both BI and ML analyses.

3. Results

3.1. Morpho-Anatomical Characterization

Results from the anatomical analyses and particularly from the investigations of the male portion of the reproductive systems enabled the identification of three different morphospecies within collected specimens, which were representative of different classes size and color patterns. These three morphospecies were ascribed to *H. hydatis* (N = 5 dissected specimens), *H. navicula* (N = 1 dissected specimen) and *H. orbignyana* (N = 6 dissected specimens) based on the observation of the male part of the reproductive system since the analyzed specimens did not correspond to any size class, typical shell morphology or characteristic color pattern. In fact, even if the photographs depicting the three species (Figures 2–4), which were also validated by molecular analyses, show well distinct and easily recognizable body color patterns and shape of the shells, it could be underlined that they represent the extreme forms of a wide morphological variability that makes these two characters useless tools for a quick species determination. Thus, according to characteristics of the male genitalia, the under mentioned species have been recognized in Faro Lake.

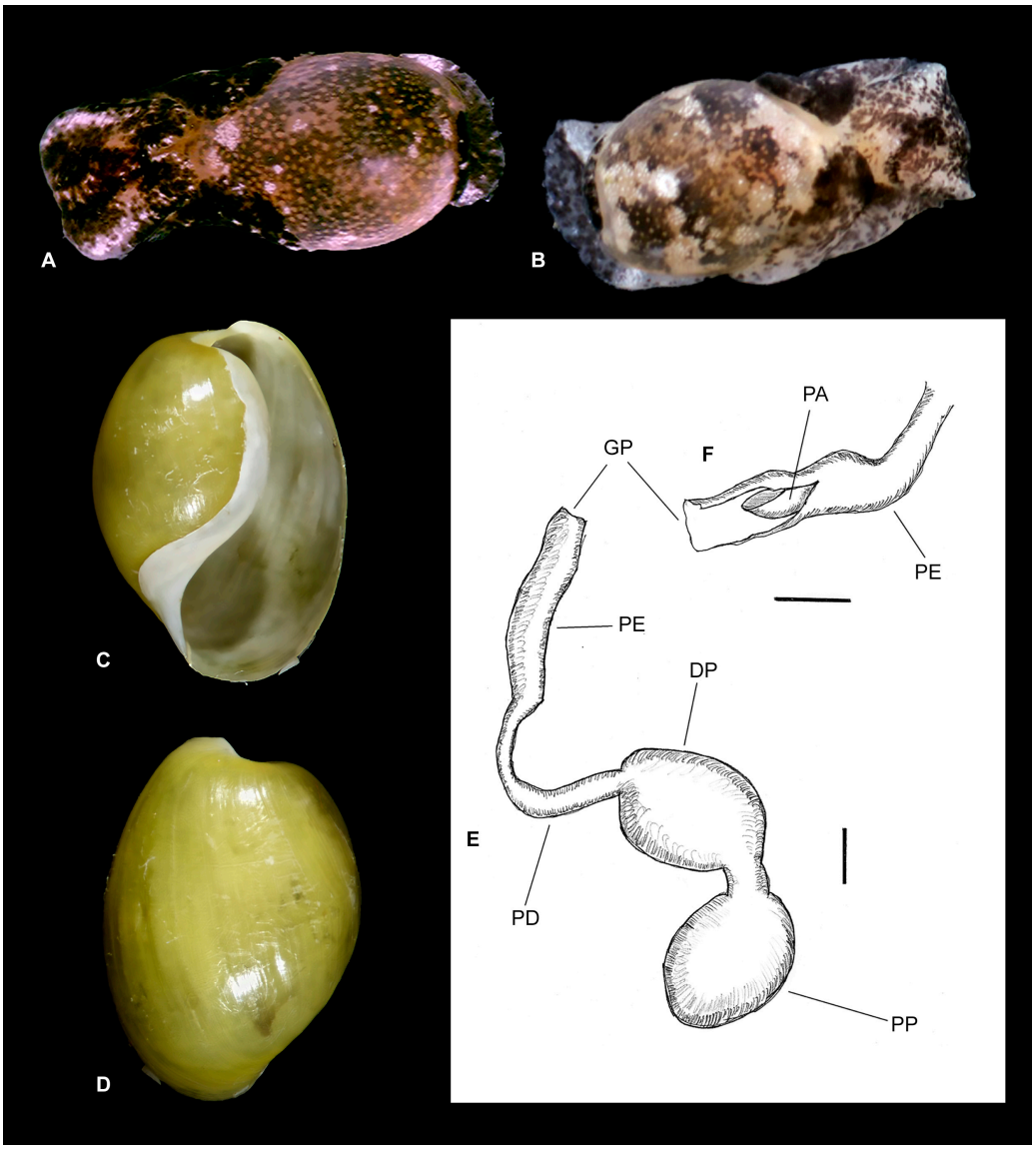

Figure 2. *Haminoea hydatis* (Linnaeus, 1758). (**A**) In vivo picture of the specimen voucher RM3_2250 with dark coloration of the body; (**B**) Individual of *H. hydatis* (voucher RM3_1686) showing a pale body color pattern; (**C,D**) Shell extracted from the specimen voucher RM3_2256 (L = 8.1 mm, Faro Lake, Messina, 17 June 2020); (**E,F**) Male genitalia of *H. hydatis*. Specimen voucher RM3_2256. (**E**) complete genitalia, (**F**) particular of distal penis with inner papilla penis. Acronyms: DP: distal prostatic gland; GP: genital pore (atrium); PA: penial papilla (papilla penis); PD: prostatic duct PE: Penis; PP: proximal prostatic gland. Scale bar 1 mm.

Haminoea hydatis (Figure 2E,F) shows a prostatic gland composed of two small parts (proximal and distal), spherical, similar in size, separated by a constriction (narrower zone); it follows the prostatic duct, short, cylindrical, not thickened. The penis is as long as the prostatic duct, cylindrical; the papilla penis is small, pointed at the top, truncated on one side (cf. [9] Figures 2 and 3). All the analyzed specimens showed a constant genital tract, perfectly matching the drawings published by [9] (Figures 2 and 3) for the Spanish fauna and the descriptions published by [43] (Table 1) and [6] (Table 1), for the Mediterranean and European faunas.

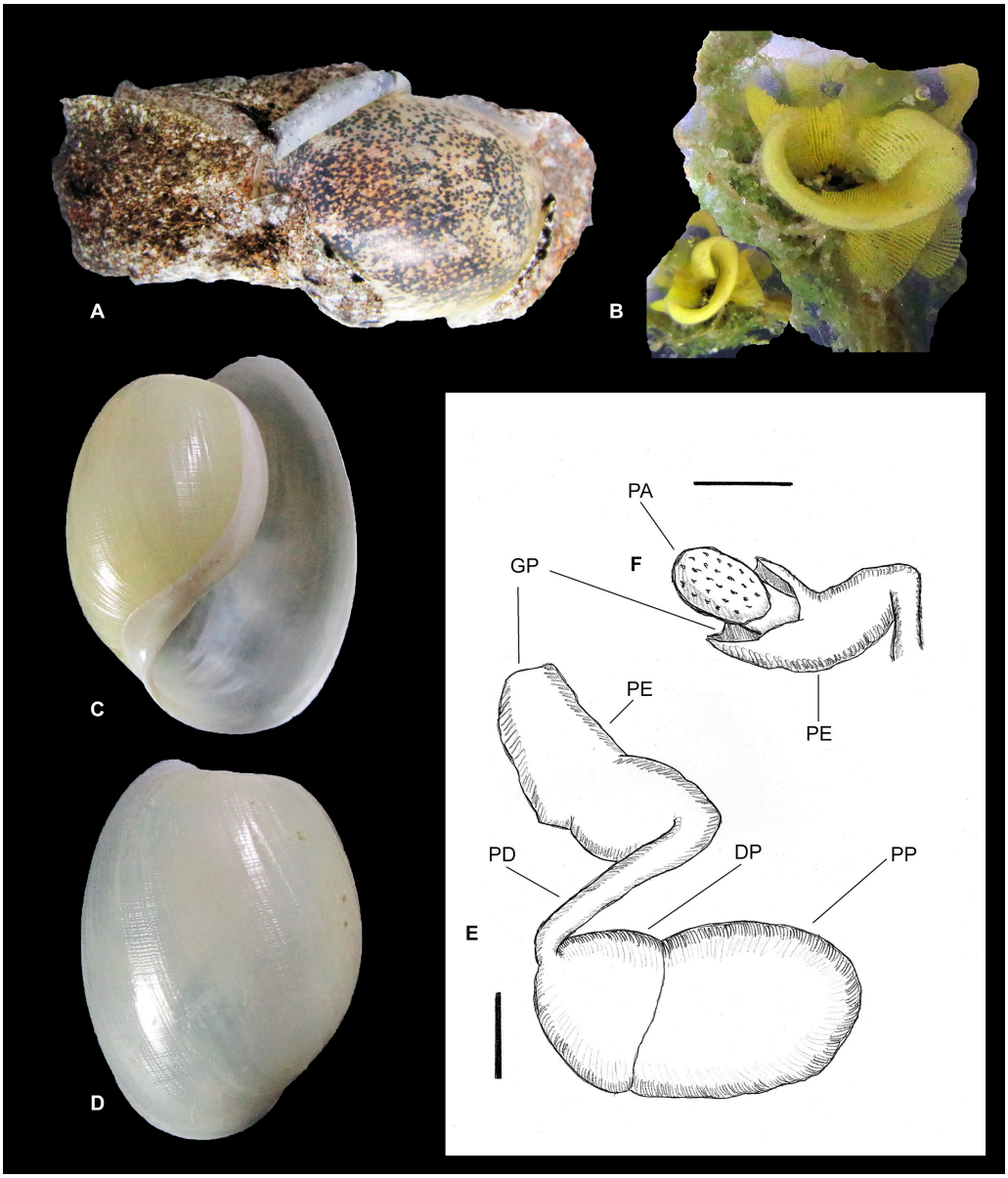

Figure 3. *Haminoea navicula* (Da Costa, 1778). (**A**) In vivo picture of the specimen voucher RM3_2240; **(B)** *In situ* picture of *H. navicula* egg mass; (**C,D**) Shell extracted from the specimen voucher RM3_2240 (L = 22.0 mm, Faro Lake, Messina) (18 April 2019); (**E,F**) Male genitalia of *H. navicula*. Specimen voucher RM3_2240. (**E**) complete genitalia, (**F**) particular of distal penis with inner papilla penis. Acronyms: DP: distal prostatic gland; GP: genital pore (atrium); PA: penial papilla (papilla penis); PD: prostatic duct; PE: penis; PP: proximal prostatic gland. Scale bar 1 mm.

Haminoea navicula (Figure 3E,F), shows a voluminous, oval prostatic gland; the proximal and the distal parts are not clearly separated, but the proximal one is about 2/3 of the total prostate length. The prostatic duct is short, cylindrical, not very thickened. The penis is large, as long as the prostatic duct, cylindrical; the papilla penis is small, oval, flattened on one side and covered by several very small, pointed spicules (cf. [9] Figures 6 and 7). Even if the analysis of the genital tract concerned only one specimen, the anatomical details observed leave no doubts as they perfectly matched those reported in the drawings published by [9] (Figures 6 and 7) for the Spanish fauna and the descriptions published by [43] (Table 1) and [6] (Table 1), for the Mediterranean and European faunas.

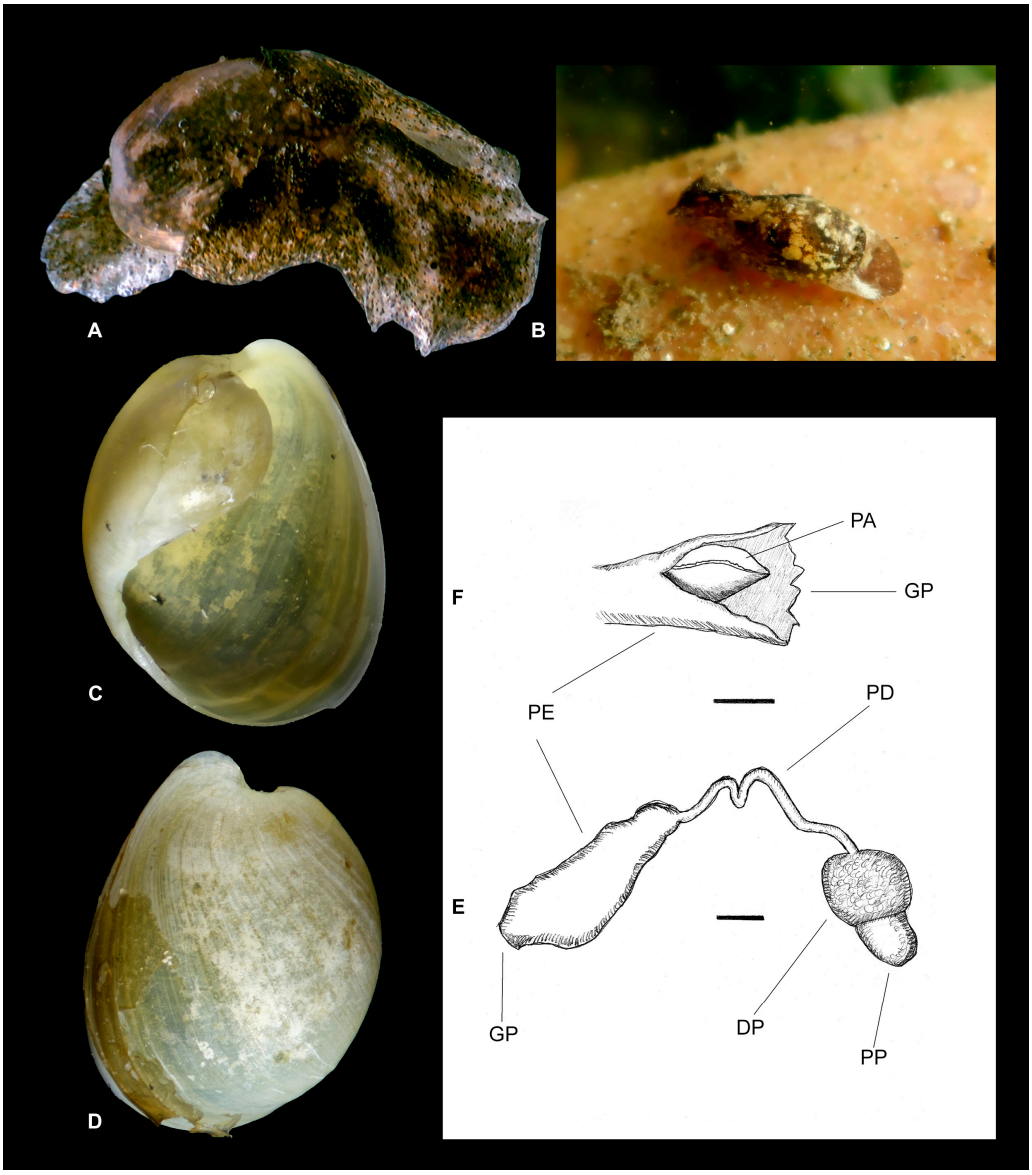

Figure 4. *Haminoea orbignyana* (Férussac, 1822). (**A**) In vivo picture of the specimen voucher RM3_2249; (**B**) *In situ* picture of *H. orbignyana* individual (voucher RM3_2259); (**C,D**) Shell extracted from the specimen voucher RM3_2249 (L = 8.3 mm, Faro Lake, Messina), 22 May 2019); (**E,F**) Male genitalia of *H. orbignyana*. Specimen voucher RM3_2249 (**E**) complete genitalia, (**F**) particular of distal penis with inner papilla penis. Acronyms: DP: distal prostatic gland; GP: genital pore (atrium); PA: penial papilla (papilla penis); PD: prostatic duct; PE: penis; PP: proximal prostatic gland. Scale bar 1 mm.

Haminoea orbignyana (Figure 4E,F), shows a rather oval prostatic gland, composed of a proximal part smaller than the distal one (wider); it follows a quite long prostatic duct, thin and twisted, longer than the penis length. The penis is cylindrical, a little larger in its distal portion (near the genital pore); the papilla penis is conical, pointed at the top, covered by one or two small longitudinal crests (cf. [9] Figures 12 and 13). All the analyzed specimens showed invariant features of the genital tract, confirming the absence of intraspecific variability in this character. Furthermore, this important diagnostic anatomical trait corresponds perfectly to the drawings published by [9] (Figures 12 and 13) for the Spanish fauna and to the descriptions published by [43] (Table 1) and [6] (Table 1), for the Mediterranean and European faunas, leaving no doubts on the species identity.

The comparison between the male genitalia of *Haminoea* collected in Faro Lake and those reported for other Mediterranean congeneric species clearly distinguished the three identified species, confirming this anatomical character as the only one morphological feature diagnostic at the species level. In fact, *H. fusari* L. A. Alvarez, F. J. García and Villani, 1993, considered endemic to Fusaro Lake (Naples, Italy), has the prostatic gland composed of two oval parts (proximal and distal), separated by a constriction (narrower zone) like *H. hydatis*, but in the former species, the proximal is a little bigger than the distal one and a long and thin prostatic duct is present in *H. fusari* that lacks in *H. hydatis* [44]. *Haminoea orteai* Talavera, Murillo and Templado, 1987, reported from several localities in the Mediterranean and northeastern Atlantic [45], presents a large and spherical prostatic gland, both oval penis and papilla penis with several perimeter crests that are not present in the specimens investigated here ([45] Figure 3B,C). *Haminoea exigua* Schaefer, 1992, *H. elegans* (Gray, 1825) and *H. templadoi* Garcia, Perez-Hurtado and Garcia-Gomez, 1991, have a typical shape of their papilla penis [43] (Table 1) ([6] Figure 6) that differ from those reported for the three species inhabiting Faro Lake.

3.2. Molecular Analyses

Species delimitation analyses, based both on genetic distances and monophyly, gave congruent results highlighting 17 preliminary species hypotheses within the final dataset (Figure 5). The *Haminoea* species from Faro Lake (Sicily, central Mediterranean Sea) were grouped with sequences already present in GenBank and ascribed to *H.* cf. *fusari*, *H. orbignyana* and *H. navicula*. These results were in line with identifications based both on phenotypes and on anatomical analyses, except for the specimens belonging to *H. hydatis*, which grouped with the GenBank sequence erroneously reported as *H.* cf. *fusari* (Voucher NHMUK 20070177).

Results from phylogenetic Bayesian Inference and Maximum Likelihood analyses were congruent in all the datasets, both the single genes (the COI Bayesian topology is shown in Figure 5) and the concatenated and partitioned one (Figure 6). However, the single-gene analyses carried out using the 16S and the H3 molecular marker were congruent with the COI and the concatenated and partitioned analysis but, as already reported on the other marine Heterobranchia [5,7,46], with low statistical support; for this reason, results of these analyses are not shown here. On the contrary, the single COI dataset showed higher ability to resolve at a lower taxonomical scale (as the species level) with respect to the concatenated and partitioned analysis, which had the better statistical support at the basal nodes and was the best one for describing the deep evolutionary history and the phylogenetic relationships occurring among *Haminoea* species.

BI and ML analyses on the concatenated and partitioned analyses revealed two main groups, the first one (BI = 1, ML = 100) including all the *Lamprohaminoea* taxa and the second one including all the *Haminoea* species (BI = 1, ML = 100) (Figure 6), with *Lamprohaminoea cymbalum*, the type species of the genus, as the out-group. *Lamprohaminoea mikkelsenae* (BI = 1, ML = 100) was the sister species of a big group which included the *Haminoea* clade and another monophyletic clade (BI = 1, ML = 100), grouping the rest of the *Lamprohaminoea* species. In particular, the latter clade had *L. evelinae* as the basal taxon (BI = 1, ML = 100) and *L. vamiziensis* and *L. ovalis* (BI = 1, ML = 70) as sisters to each other (BI = 1, ML = 84).

The *Haminoea* clade was divided in two other monophyletic clades, one showing high statistical support (BI = 1, ML = 100), which was further divided into two clades with *H. virescens* as basal to both and another with moderate support (BI = 0.89, ML = 61). The former *Haminoea* clade showed two groups respectively formed by two separated phylogenetic lineages identified as *H. antillarum* (BI = 0.59, ML = nd) and the sister species (BI = 0.9, ML = nd) *H. orbignyana* (BI = 0.97, ML = 54) and *H. alfredensis* (BI = 1, ML = 96). The second big *Haminoea* clade included two monophyletic highly supported groups. The first one (BI = 1, ML = 81) with *Haminoea* sp. 3 and *Haminoea* sp. 4 as sister species (BI = 1, ML = 100) and with *H. orteai* (BI = 1, ML = 99) and *H. vesicula* as sister to each other (BI = 1, ML = 100). The second monophyletic group showed *H. navicula* (BI = 1, ML = 98) as the

sister (BI = 1, ML = 100) to another clade grouping *H.* cf. *hydatis* (BI = 1, ML = 100) and *H. hydatis* (BI = 1, ML = 98) with strong statistical support (BI = 1, ML = 100).

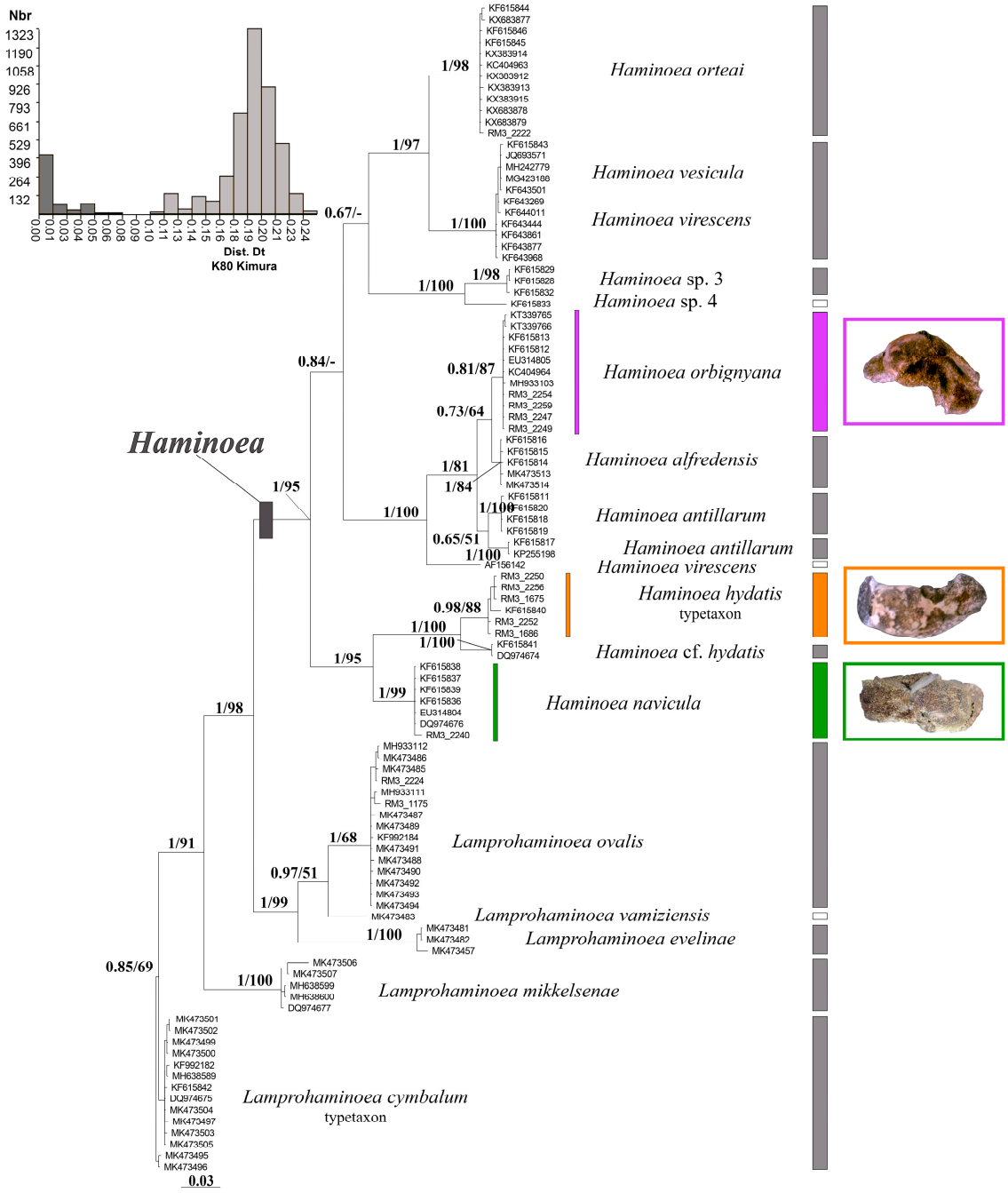

Figure 5. Bayesian Inference tree based on the COI sequence dataset of the Haminoeidae species analyzed in the present study. Numbers at nodes indicate Bayesian posterior probability (BI; left) and bootstrap support from Maximum Likelihood analysis (ML; right). BI < 0.50 and ML < 50% are not reported. The histogram shows the distribution of the pairwise genetic distances (K2P) in intraspecific (**left**, dark grey) and interspecific (**right**, light grey) comparisons. The "-" symbol indicates unsupported values. The vertical rectangles on the right side refer to the ASAP results.

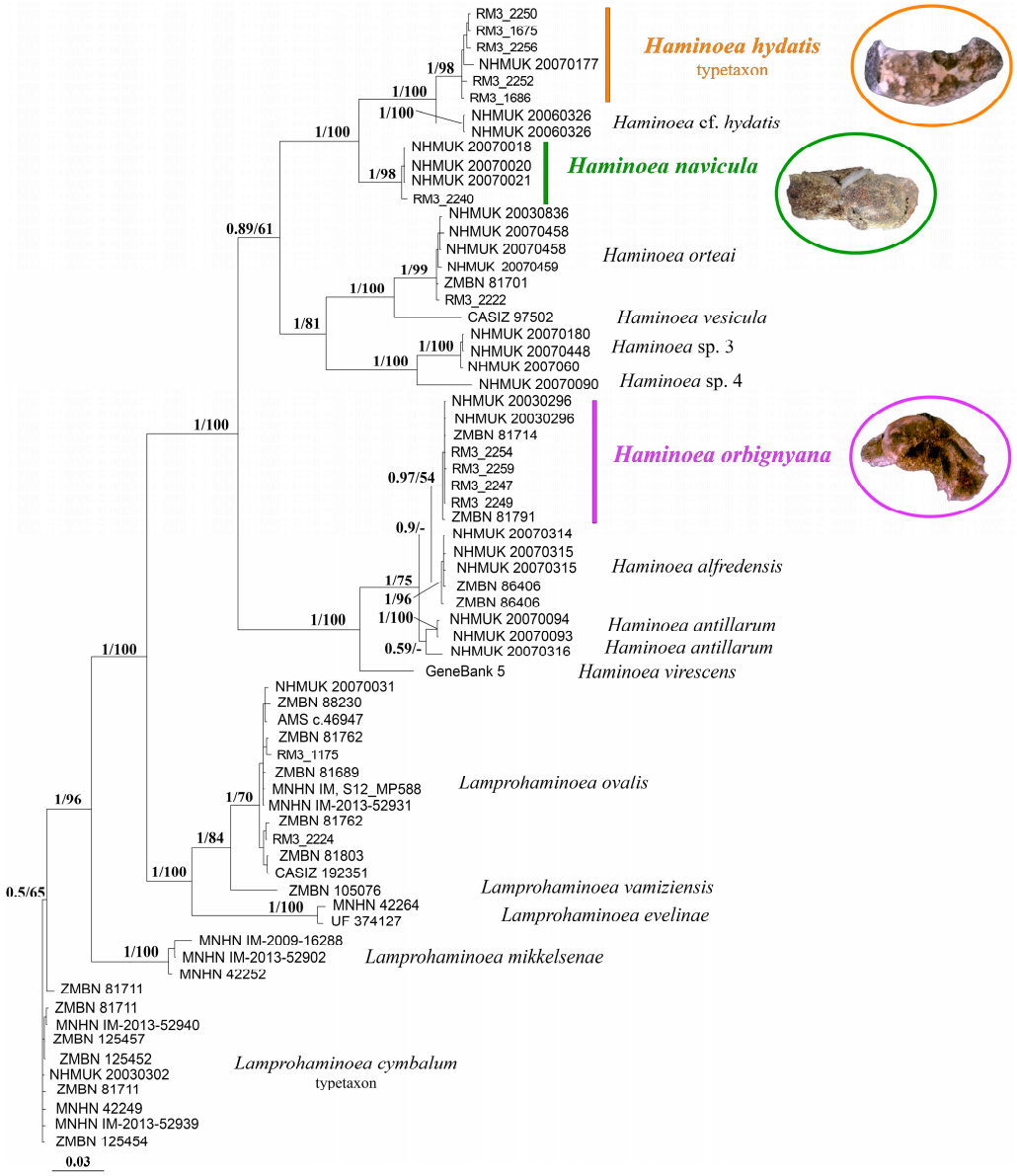

Figure 6. Bayesian phylogenetic tree based on the concatenated and partitioned dataset (COI, 16S, H3) of the Haminoeidae species analyzed in the present study. Bayesian posterior probability (BI; **left**) and bootstrap support from Maximum Likelihood analysis (ML; **right**) are indicated at each node. The "-" symbol indicates unsupported values.

3.3. Ecological Context

All the specimens sampled in the Faro Lake and examined to the aims of this investigation were collected in a shallow water habitat characterized by both fixed and flotant green algae. A clear space separation was not noted, nor was a particular fidelity to a confined portion of the lake rather than to the canals. The algal species and their relationship with the substratum (floating or fixed) seemed to be irrelevant, supporting the hypothesis of a wide ecological niche overlap.

4. Discussion

Cephalaspidea fauna inhabiting Faro Lake was investigated and all the *Haminoea* species collected were analyzed using morphological, anatomical and molecular approaches. This integrative approach confirmed the presence of species previously reported in the study area only based on morphological identification and also identified an additional

species that had not been reported before. In fact, the recent inventory of the "Opisthobranch" fauna from the Faro Lake, made by [20], where original data were integrated with information from a bibliographic review, reported four *Haminoea* species (one of them currently moved to *Lamprohaminoea* genus). This paper documented the first record of *Lamprohaminoea ovalis* (Pease, 1868) (previously identified as *Haminoea cyanomarginata* Heller and T. E. Thompson, 1983), although it was considered an occasional occurrence [20]. This introduced Red Sea species, widely spreading in Mediterranean and firstly reported by [47] for the Strait of Messina area (between the Tyrrhenian and Ionian Seas), was never found during our investigation. Similarly, *H. orteai* Talavera, Murillo and Templado, 1987, first reported in Faro Lake by [21], was not detected in our collected samples, although it occurs elsewhere in the Strait (as demonstrated by the specimen with voucher RM3_2222). By contrast, the occurrence of *H. hydatis* (Linnaeus, 1758) and *H. navicula* (da Costa, 1778), both cited by [22] as characterizing Faro Lake and Ganzirri Lake mollusk fauna, is confirmed here. Notably, the presence of *H. orbignyana* is reported here for the first time, adding an additional species to the list of Haminoeidae living in Faro Lake.

Both records of persistent and transient taxa agree with the high space-temporal and interannual variability of assemblages that characterizes the transitional systems, together with their intrinsic heterogeneity and homeostatic properties [48]. In this context, a further consideration might concern the possible introduction of the Indo-Pacific invasive *Haloa japonica* (Pilsbry, 1895), whose worldwide spread would have been unnoticed for a long time due to the difficult morphological identification characterizing the Haminoeidae species [13]. The present investigation, excluding the colonization of Faro Lake by *H. japonica*, opens interesting new questions on the limiting (biotic and abiotic) factors able to influence the spreading of this efficient invader even in a basin notoriously subjected to a high rate of a natural and anthropic introduction of non-indigenous species, and highlights the importance of identifying species with both morphological and molecular methods. This last point becomes even more necessary if species with an extremely variable and confounding phenotype are involved, like in the case of some Haminoeidae species. In fact, several taxonomic errors in mollusks have been revealed and resolved in recent decades thanks to integrative systematic studies [7,8,49–51] which, have highlighted the complexity of natural environments and of the interactions between species and the very complicated evolutionary patterns that can be generated.

Regarding possible reasons why an efficient invader such as *H. japonica* has not colonized Faro Lake, we can speculate that high densities of at least three indigenous species, in an environment not very prone to destabilizing events such as dystrophic crisis, can successfully contrast the settlement of taxonomically and functionally related non-indigenous taxa [52]. Even if this is lacking scientific experiments to support it, it would be interesting to explore further, as if confirmed it would be additional evidence reinforcing the idea that a good environmental status helps to protect the endemic biodiversity improving environmental resilience. Finally, even if the importance of Mediterranean biodiversity is already known, especially for Heterobranchia, the case of the Voucher NHMUK 20070177 deposited in GenBank as *H.* cf. *fusari* but here confirmed to belong to *H. hydatis*, is evidence of the confusion still existing around Mediterranean species. This should be considered in future studies along with the need to increase the sampling effort from the Mediterranean basin, which would improve the representative samples and species from this important marine basin.

Finally, results from molecular analyses carried out here confirm the morphological identifications of the species collected in Faro Lake and shed light on their phylogenetic relationships. *Haminoea hydatis* and *H. navicula* were confirmed to be close to each other, with *H. orteai* as sister to them and with *H. orbignyana* as basal. However, the molecular analyses performed here leave some open questions, as in the case of *H.* cf. *hydatis* and the two undetermined *Haminoea* species (*Haminoea* sp. 3 and sp. 4), that will need further in-depth studies.

5. Conclusions

In this paper, an integrative taxonomic approach involving morphological, anatomical and molecular methods has been carried out allowing the identification of *Haminoea* species living in Faro Lake. In contrast, determinations based only on macroscopic characters, such as shell shape and body color patterns, cannot be considered reliable in this group, leading to possible misidentifications and to a confounding taxonomy. The occurrence of three sympatric *Haminoea* species, of which two are considered native or long time naturalized, was ascertained in Faro Lake. However, other species might reflect some stochastic space-temporal dynamic that naturally occurs in transitional environments. The study of an area as interesting as a coastal lagoon with a high anthropic pressure represents an inexhaustible source of case studies and a great opportunity to investigate species interactions and ecological factors limiting alien species spreading.

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Data Availability Statement: Data available in a publicly accessible repository. The data presented in this study are openly available in National Center for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov/, accessed on 8 July 2023 [See Table 1].

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Conflicts of Interest: The authors declare no conflict of interest.

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