

## Article

# How Many Abalone Species Live in the Mediterranean Sea?

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**Abstract:** Morphological traits in Haliotidae may be highly variable and not consistently diagnostic for species identification, highlighting the need for an integrative approach to the taxonomy of the family, including genetic data. Four species of the genus *Haliotis* are currently reported for the Mediterranean Sea and the neighboring Atlantic Ocean: *Haliotis tuberculata*, the common European abalone with the widest Atlanto-Mediterranean range; *Haliotis mykonosensis*, from the Aegean, the Tyrrhenian, and the Adriatic; *Haliotis stomatiaeformis*, from Malta, Lampedusa, and southeastern Sicily; and the Lessepsian *Haliotis pustulata*, only known on the basis of few samples from the Levant. However, their taxonomic status still relies only on shell morphology. Here, sequences of two fragments of the mitochondrial molecular marker COI were obtained from 84 abalone specimens collected in the Mediterranean Sea and the neighboring Atlantic and analyzed in order to provide for the first time a genetic framework for species delimitation. This study’s results prove that *H. mykonosensis* is genetically identical to *H. tuberculata*, whereas *H. stomatiaeformis* is a distinct species, endemic to a restricted area of the southern Mediterranean Sea. Finally, *Haliotis tuberculata coccinea* from Macaronesia may deserve its status as a subspecies of *H. tuberculata*, with genetic signature of a limited gene flow found in specimens of the nominal subspecies (*H. t. tuberculata*) in both the Atlantic and the Mediterranean Sea.

**Keywords:** *Haliotis*; haliotidae; COI; integrative taxonomy; species delimitation



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## 1. Introduction

Historically, gastropod taxonomy has largely been based on the study of shell morphology, also due to the convenience of the long-lasting nature of shells and the possibility of using the same methodological framework on fossils and extant taxa. As a result, most malacological taxonomic reviews are based on shell morphology, occasionally integrated with information on anatomy, ecology, and distribution and, only in more recent times, with genetic data [1,2]. Treating shell features as indicators of evolutionary divergence is a potential source of problems in groups whose morphological characters are plastic and change, sometimes dramatically, in response to local conditions. Consequently, the taxonomy of groups with highly variable shells is often confused, and species-delimitation analyses relying on molecular barcode data have been increasingly implemented to resolve discrepancies between the conflicting systematic frameworks proposed by different experts [1–8].

*Haliotis* Linnaeus, 1758 is the only genus currently recognized in the family Haliotidae Rafinesque, 1815, a group of vetigastropods with 57 accepted species distributed worldwide, from temperate to warm shallow waters [9,10]. They usually live on rocky substrata, where they graze on algae. Many species are highly appreciated as seafood and have a

remarkable economic value, being among the most expensive shellfish in the world [11]. The “abalones” or “sea ears” are easily recognizable from other Vetigastropoda for the presence of a distinctive arc of perforations (called tremata) on their ear-like and limpet-like shell. Morphological characters such as color, shape, size, and number of perforations can be extremely variable, even at the intraspecific level, and thus are now recognized as potentially not diagnostic for species identification and not phylogenetically useful for reconstructing evolutionary patterns [10,12–14].

Mostly due to their economic value, the ecology, recruitment, demography, population structure, and phylogeny have been studied in several species of this genus [15–19]. Nevertheless, the taxonomic assessment of the family Haliotidae remains vexed, mainly because of the plasticity of the shell. In the past, the sculpture and shape of the shell, together with the number of perforations, were treated as constant variables within species and therefore used as diagnostic characters for the description of numerous extant species and subspecies. However, as a greater number of specimens were taken into account, many of these taxa have been shown to have overlapping morphologies and ended up being synonymized as varieties of the same species [13,14,20,21]. The univocity of names is imperative in taxonomy, more than anything in case of commercial species, where stock management of specific biological entities can be crucial in conservation efforts. Species delimitation analyses using molecular data could be the proper tool to disentangle the confusing taxonomy of this genus, allowing a reliable definition of the actual number of species.

Several nominal taxa of *Haliotis* have been described from the Mediterranean Sea and the neighboring Atlantic Ocean. Of them, at least thirteen (*H. vulgaris* da Costa, 1778; *H. bistrinata* Gmelin, 1791; *H. pellucida* von Salis, 1793; *H. rugosa* Lamarck, 1822; *H. lamellosa* Lamarck, 1822; *H. marmorata* O. G. Costa, 1830; *H. speciosa* Reeve, 1846; *H. coccinea* Reeve, 1846; *H. incisa* Reeve, 1846; *H. reticulata* Reeve, 1846; *H. zealandica* Reeve, 1846; *H. adriatica* Nardo, 1847; *H. lucida* Requier, 1848; *H. canariensis* F. Nordsieck, 1975) are currently synonymized with *H. tuberculata* Linnaeus, 1758, also known as the European abalone, the main harvested haliotid species in Europe [22–24]. This species can be commonly found across the whole Mediterranean, and its distribution in the Atlantic ranges from the Channel Islands, throughout the European coast and Macaronesia, to the African coast in Senegal [25–27]. Throughout its range, an extreme morphological variability is observed, which explains the description of the numerous nominal taxa during the past centuries. Two of the most persistent ones in the literature are *H. lamellosa* Lamarck, 1822, from the central Mediterranean and *H. coccinea* Reeve, 1826, from the Canary Islands. However, following a molecular characterization, both these names are no longer considered as valid species [20,28]. Following this trend, all the *Haliotis* species described in the Mediterranean Sea and near the Atlantic could be destined to be ascribed to the omnipresent, multifaceted, *H. tuberculata*.

The two other haliotid nominal species native and endemic in the Mediterranean are *H. mykonosensis* Owen, Hanavan and Hall, 2001 and *H. stomatiaeformis* Reeve, 1846. The former was described from the Aegean Sea on the basis of morphological, ecological, and behavioral differences, such as the presence of forked tentacles in the epipodium, the preference for feeding on red algae, a higher light sensitivity, and the different spawning seasonality. The soft parts of *H. mykonosensis* are easily distinguishable from other Mediterranean abalones for their peculiar epipodium, but their shells are hardly diagnosed if not for subtle differences in size, color pattern, and shape [29–31]. There are recent reports of this species from Corsica (France), Procida Island (Italy), and several Croatian localities, where specimens were sampled together with typical *H. tuberculata* [31,32]. *Haliotis stomatiaeformis*, the senior synonym of *H. neglecta* Philippi, 1848, is also a very problematic taxon that has only recently resurfaced. It also lives in sympatry with *H. tuberculata*, from which it differs in size, color, and response to stimuli [33,34]. Finally, *H. pustulata* Reeve, 1846, is a Lessepsian immigrant, only reported from the easternmost Mediterranean.

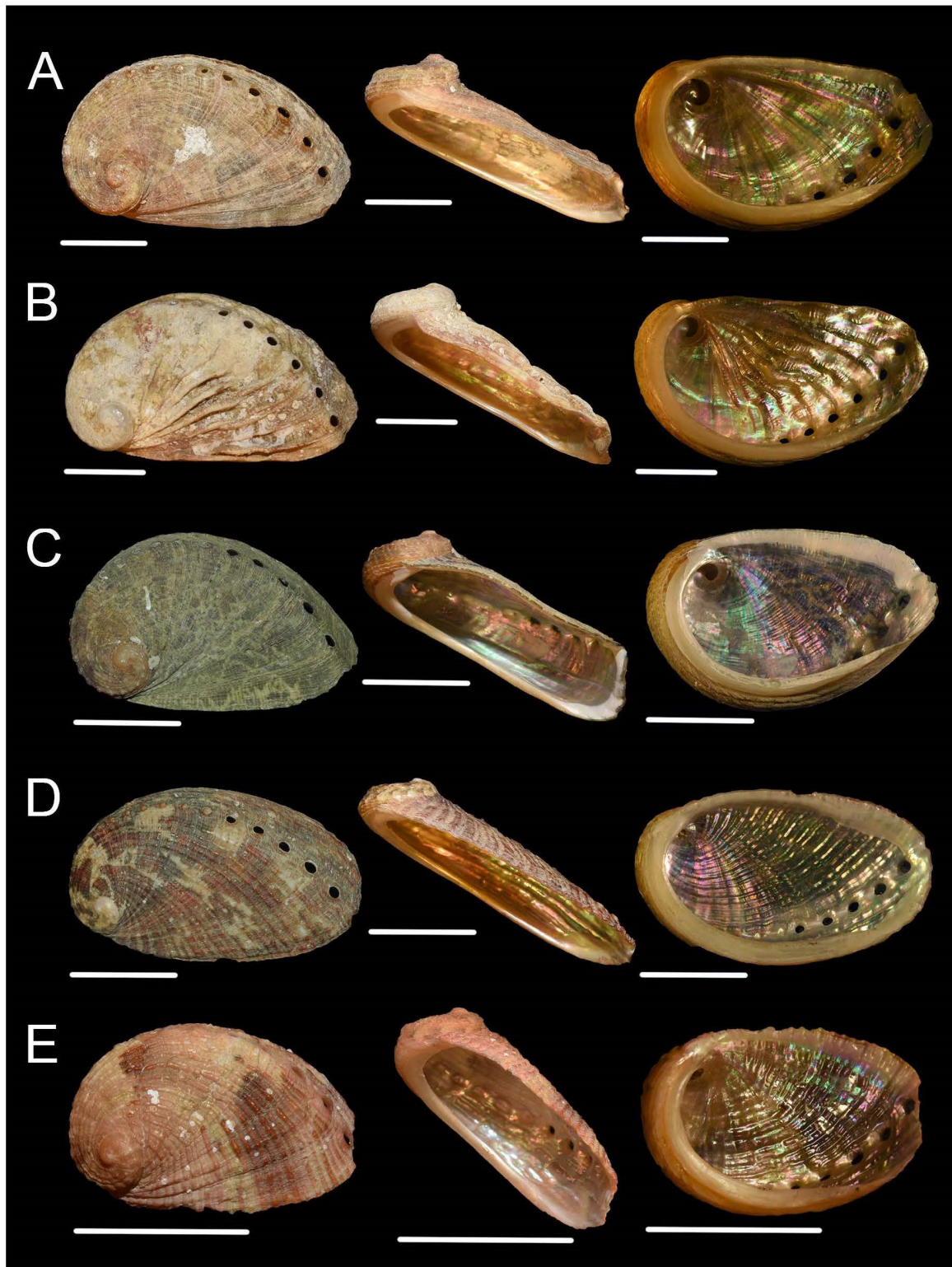
In this work, we have used a large molecular dataset, based on two fragments of the mitochondrial gene coding for the cytochrome C oxidase I (COI), in order to assess the taxonomic status of the various entities described in *Haliotis* for the Mediterranean Sea and the neighboring eastern Atlantic Ocean.

## 2. Materials and Methods

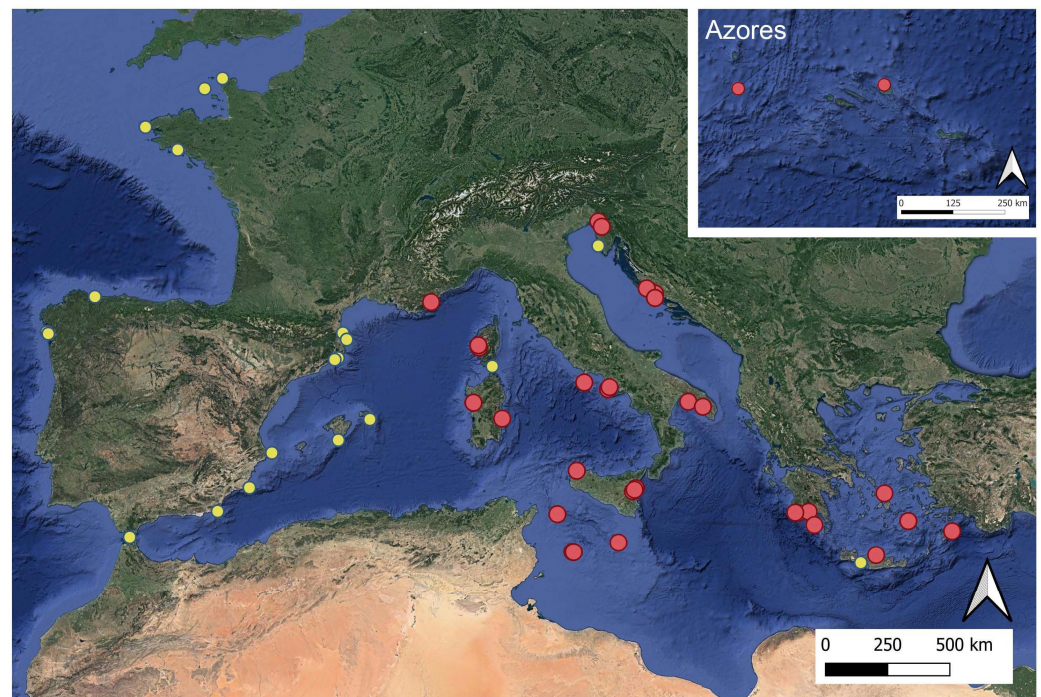
### 2.1. Dataset

Sequences of 84 abalones collected at 35 sites in the Mediterranean sites and in the Azores were included in this study (specimens per locality = 1–7). Samples were morphologically identified as *H. t. tuberculata* ( $n = 67$ ), *H. t. coccinea* ( $n = 4$ ), *H. mykonosensis* ( $n = 5$ ), and *H. stomatiaeformis* ( $n = 8$ ) (Figure 1). Some of the specimens of *H. t. tuberculata* (e.g., BAU 676.1, BAU 676.3, BAU 1777.1–3; BAU 1775, BAU 2617) with strong lamellae on their shells corresponded to the nominal taxon *H. lamellosa*. All newly processed specimens belong to the malacological collection of the Department of Biology and Biotechnologies “Charles Darwin”, Sapienza University of Rome (acronym BAU). Soft parts were preserved in ethanol 100°.

Two mitochondrial fragments for the COI gene were analyzed and will be hereafter referred to as “5'-COI” and “3'-COI”. For the phylogenetic analyses, all available sequences on the investigated species were downloaded from Genbank, and 73 sequences (5'-COI,  $n = 2$ ; 3'-COI,  $n = 73$ ) were selected to be included in the molecular dataset, retaining all the haplotypic and geographic diversity from recent studies (Figure 2). *Haliotis marmorata* Linnaeus, 1758, the sister species of *H. tuberculata* ([35]; NCBI accession number: FJ605487) was used as the outgroup in the phylogenetic analyses. For the species delimitation analyses, a total of 52 additional 5'-COI sequences of other *Haliotis* species were included (sources and NCBI accession numbers in Table S1).



**Figure 1.** Voucher shells of the *Haliotis* investigated in this work with their morphological identification. (A) *Haliotis tuberculata tuberculata* BAU 1391. (B) *Haliotis tuberculata tuberculata* with lamellae (formerly *Haliotis lamellosa*) BAU 676.3. (C) *Haliotis mykonosensis* BAU 657.3. (D) *Haliotis tuberculata coccinea* BAU 717.3. (E) *Haliotis stomatiaeformis*, juvenile BAU 699. All scale bars are 1 cm.



**Figure 2.** Sampling localities in the Mediterranean, northeastern Atlantic, and in the inlet Azores for this study. Red dots indicate new specimen localities from this study (Table 1), and yellow dots indicate specimens from other studies included in the dataset. Sources and NCBI accession numbers are in Table S1.

**Table 1.** Specimens from the BAU collection used in the present paper, with morphological identification, voucher codes, locality, coordinates, and gene fragments used (mb1 = minibarcode1; mb2 = minibarcode2). GenBank accession numbers are available in Table S1.

Species ID	Code	Locality	Coordinates	5'-COI	5'-COI mb1	5'-COI mb2	3'-COI
<i>Haliotis mykonosensis</i> Owen, Hanavan & Hall 2001	BAU 657.3	Italy, Procida	40.7691 N, 14.0196 E	X			X
	BAU 657.4	Italy, Procida	40.7691 N, 14.0196 E	X			X
	BAU 657.5	Italy, Procida	40.7691 N, 14.0196 E	X			X
	BAU 659	Greece, Mykonos	37.4088 N, 25.3465 E	X			X
	BAU 4230.2	Italy, Procida	40.7691 N, 14.0196 E	X			X
<i>Haliotis stomatiiformis</i> Reeve, 1846	BAU 667	Sicily, Acireale	37.6125 N, 15.1661 E				X
	BAU 669	Sicily, Acireale	37.6125 N, 15.1661 E				X
	BAU 673.1	Sicily, Lampedusa	35.5035 N, 12.5839 E			X	X
	BAU 673.2	Sicily, Lampedusa	35.5035 N, 12.5839 E	X			X
	BAU 680	Sicily, Lampedusa	35.5121 N, 12.5556 E		X	X	X
	BAU 4337.1	Sicily, Lampedusa	35.5011 N, 12.6072 E			X	X
	BAU 4337.2	Sicily, Lampedusa	35.5011 N, 12.6072 E		X	X	X
	BAU 4337.3	Sicily, Lampedusa	35.5011 N, 12.6072 E		X	X	X
BAU 4337.4	Sicily, Lampedusa	35.5011 N, 12.6072 E		X	X	X	
<i>Haliotis t. coccinea</i> Reeve, 1846	BAU 717.2	Azores, Flores	39.4581 N, −31.1251 W	X			X
	BAU 717.3	Azores, Flores	39.4581 N, −31.1251 W	X			X
	BAU 733	Azores, Terceira	38.8033 N, −27.2564 W	X			X
<i>Haliotis t. tuberculata</i> Linnaeus, 1758	BAU 658.1	Italy, Zannone Is.	40.9694 N, 13.0455 E	X			X
	BAU 658.2	Italy, Zannone Is.	40.9694 N, 13.0455 E	X			X
	BAU 663.1	Sicily, Acireale	37.6125 N, 15.1661 E				X
	BAU 668	Sicily, Acireale	37.6125 N, 15.1661 E	X			X
	BAU 670.9	Italy, Muggia	45.6056 N, 13.7216 E	X			X
	BAU 670.14	Italy, Muggia	45.6056 N, 13.7216 E	X			X
	BAU 670.30	Italy, Muggia	45.6056 N, 13.7216 E	X			X
	BAU 674	Sardinia, Ogliastra	39.8408 N, 9.6755 E	X			X
	BAU 676.1	Sicily, Lampedusa	35.5011 N, 12.6072 E	X			X
	BAU 676.2	Sicily, Lampedusa	35.5011 N, 12.6072 E	X			X

Table 1. Cont.

Species ID	Code	Locality	Coordinates	5'-COI	5'-COI mb1	5'-COI mb2	3'-COI
	BAU 676.3	Sicily, Lampedusa	35.5011 N, 12.6072 E	X			X
	BAU 676.4	Sicily, Lampedusa	35.5011 N, 12.6072 E	X			X
	BAU 676.5	Sicily, Lampedusa	35.5011 N, 12.6072 E	X			X
	BAU 677	Sicily, Pantelleria	36.7622 N, 11.9436 E				X
	BAU 678	Sicily, Pantelleria	36.7622 N, 11.9436 E	X			X
	BAU 682	Sicily, Lampedusa	35.5011 N, 12.6072 E				X
	BAU 878	Crete, Ligaria	35.3990 N, 25.0283 E		X	X	X
	BAU 879.1	Italy, Porto Pavone	40.7945 N, 14.1610 E		X	X	X
	BAU 883.1	Croatia, Zaboric	43.6659 N, 15.9388 E			X	X
	BAU 883.3	Croatia, Zaboric	43.6659 N, 15.9388 E	X			X
	BAU 883.4	Croatia, Zaboric	43.6659 N, 15.9388 E	X			X
	BAU 883.5	Croatia, Zaboric	43.6659 N, 15.9388 E		X	X	X
	BAU 883.7	Croatia, Zaboric	43.6659 N, 15.9388 E	X			X
	BAU 883.9	Croatia, Zaboric	43.6659 N, 15.9388 E		X	X	X
	BAU 883.10	Croatia, Zaboric	43.6659 N, 15.9388 E			X	X
	BAU 884	Italy, Leporano	40.3754 N, 17.3001 E			X	X
	BAU 885.1	Sardinia, Bosa	40.3145 N, 8.4619 E				X
	BAU 885.3	Sardinia, Bosa	40.3145 N, 8.4619 E			X	X
	BAU 885.4	Sardinia, Bosa	40.3145 N, 8.4619 E				X
	BAU 885.5	Sardinia, Bosa	40.3145 N, 8.4619 E		X	X	X
	BAU 885.6	Sardinia, Bosa	40.3145 N, 8.4619 E		X	X	X
	BAU 885.7	Sardinia, Bosa	40.3145 N, 8.4619 E	X			X
	BAU 886.13	Croatia, Murter Is.	43.7969 N, 15.6094 E				X
	BAU 886.19	Croatia, Murter Is.	43.7969 N, 15.6094 E				X
	BAU 1384	Greece, Astypalea Is.	36.5869 N, 26.4028 E				X
	BAU 1386	Greece, Astypalea Is.	36.5869 N, 26.4028 E				X
	BAU 1387	Greece, Astypalea Is.	36.5869 N, 26.4028 E				X
	BAU 1388	Greece, Astypalea Is.	36.5647 N, 26.3533 E				X
	BAU 1389	Greece, Astypalea Is.	36.5647 N, 26.3533 E				X
	BAU 1390	Greece, Astypalea Is.	36.5647 N, 26.3533 E				X
	BAU 1391	Greece, Astypalea Is.	36.5759 N, 26.3931 E			X	X
	BAU 1393	Greece, Astypalea Is.	36.5759 N, 26.3931 E			X	X
	BAU 1394	Greece, Astypalea Is.	36.5759 N, 26.3931 E			X	X
	BAU 1701.1	Corsica, Tour D'Ancone	42.0433 N, 8.7208 E	X			X
	BAU 1701.2	Corsica, Tour D'Ancone	42.0433 N, 8.7208 E	X			X
	BAU 1701.3	Corsica, Tour D'Ancone	42.0433 N, 8.7208 E	X			X
	BAU 1701.4	Corsica, Tour D'Ancone	42.0433 N, 8.7208 E	X			X
	BAU 1701.5	Corsica, Tour D'Ancone	42.0433 N, 8.7208 E	X			X
	BAU 1703	Corsica, Sagone	42.1049 N, 8.6800 E	X			X
	BAU 1703.1	Corsica, Sagone	42.1049 N, 8.6800 E	X			X
	BAU 1704	Sicily, San Gregorio	38.1586 N, 14.7602 E	X			X
	BAU 1760	Greece, Foinikounta Is.	36.8051 N, 21.8146 E	X			X
	BAU 1771.1	Italy, Sant'Isidoro	40.2176 N, 17.9212 E	X			X
	BAU 1773	Greece, Stoupa	36.8439 N, 22.2573 E	X			X
	BAU 1775	Greece, Methoni	36.8153 N, 21.7030 E	X			X
	BAU 1777.1	Greece, Archangelos	36.6293 N, 22.8798 E	X			X
	BAU 1777.2	Greece, Archangelos	36.6293 N, 22.8798 E	X			X
	BAU 1777.3	Greece, Archangelos	36.6293 N, 22.8798 E	X			X
	BAU 1780	Greece, Cape Matapan	36.4014 N, 22.4873 E	X			X
	BAU 1946.1	Sicily, San Vito Lo Capo	38.1897 N, 12.7347 E	X			X
	BAU 1946.2	Sicily, San Vito Lo Capo	38.1897 N, 12.7347 E		X	X	X
	BAU 1946.3	Sicily, San Vito Lo Capo	38.1897 N, 12.7347 E	X			X
	BAU 2031.2	Malta, Blue Grotto	35.8197 N, 14.4514 E		X	X	X
	BAU 2089.2	Sicily, Porto Palo di Menfi	37.5733 N, 12.9002 E		X	X	X
	BAU 2090	Italy, Sistani	45.7687 N, 13.6253 E			X	X
	BAU 2090.1	Italy, Sistani	45.7687 N, 13.6253 E			X	X
	BAU 2090.2	Italy, Sistani	45.7687 N, 13.6253 E				X
	BAU 2093	Italy, Muggia	45.6056 N, 13.7216 E	X			X
	BAU 2615.1	Croatia, Knin Rogoznica	43.5376 N, 15.9583 E			X	X
	BAU 2615.3	Croatia, Knin Rogoznica	43.5376 N, 15.9583 E		X	X	X
	BAU 2615.4	Croatia, Knin Rogoznica	43.5376 N, 15.9583 E		X	X	X
	BAU 2615.6	Croatia, Knin Rogoznica	43.5376 N, 15.9583 E				X
	BAU 2617	France, Saint Raphael	43.4112 N, 6.8461 E	X			X

## 2.2. Laboratory Analyses

DNA was extracted from a foot tissue fragment using a proteinase K/phenol–chloroform extraction protocol [36]. PCR were performed to amplify two fragments of the mitochondrial gene COI using the following primers: LCO and HCO [37] or COXAF and COXAR [38] for the standard barcode fragment (5'-COI fragment, 658 bp), and COIfw and COIrev [35] for the second fragment (3'-COI fragment, 631 bp). PCR reactions were performed by adding 1 µL of DNA to a 24 µL mix comprehensive of 16.55 µL H<sub>2</sub>O, 2.5 µL Reaction Buffer (10× NH<sub>4</sub>), 0.5 µL dNTP (10 pmol), 2.5 µL MgCl<sub>2</sub>, 1 µL BSA 10%, 0.4 µL of each primer (25 pmol), and 0.15 µL BioTAQ™ DNA Polymerase. PCR conditions were as follows: 5' at 94 °C, followed by 35 cycles with 30'' at 94 °C, 40'' at 40–48 °C, and 50'' at 72 °C, with a final elongation step at 72 °C for 7'. For some specimens, the amplification of the entire 5'-COI fragment was not achievable; therefore, the internal primers mICOLintR and mICOLintF [39] were used to amplify the two halves of the 5'-COI (hereafter called “minibarcodes”), using the protocol described in Leray et al. 2013. Primer sequences and PCR protocols can be found in Table S2.

PCR amplification success was checked by electrophoresis on 1.5% agarose gel stained with 3.5 µL of Midori Green Advance (Nippon Genetics Europe, Düren, Germany). Suitable PCR products were purified with 8 µL ExoSAP-IT (79 µL H<sub>2</sub>O, 20 µL rAPid Alkaline Phosphatase, 1 µL Exonuclease I), incubated at 37 °C for 15' and at 80 °C for 15', and finally sent to Macrogen Inc. (Amsterdam, The Netherlands) for Sanger sequencing.

## 2.3. Bioinformatic Analyses

Chromatograms were visually checked and trimmed, forward and reverse sequences were assembled, and the resulting consensus sequences were aligned using Geneious pro 4.8.5 ([www.geneious.com](http://www.geneious.com), accessed on 10 December 2022). Each alignment end was trimmed in order to reduce the overall quantity of missing data. The minibarcode sequences amplified using internal primers were assembled leaving a 28 bp gap in the central region. Alignments were then checked for stop codons.

A species delimitation analysis was carried out on ASAP ([bioinfo.mnhn.fr/abi/public/asap/](http://bioinfo.mnhn.fr/abi/public/asap/), accessed on 10 December 2022) [40], a hierarchical clustering program, with the K80 substitution model. Intraspecific and interspecific genetic divergence among hypothetical species delineated by the ASAP best partitions were calculated on the same fragment using MEGA 10.2.4 [41], selecting the Kimura 2-parameter nucleotide substitution model and the pairwise deletion option for missing data treatment.

The best fitting nucleotide substitution model for each COI fragment partitioned by codon was determined using jModelTest 2.1.6 [42,43] on the CIPRES portal ([www.phylo.org](http://www.phylo.org), accessed on 10 December 2022), following the Bayesian Information Criterion.

Phylogenetic reconstruction analyses were performed by Bayesian Inference on MrBayes 3.2.2 [44] on the CIPRES portal, running 4 independent MCMCs of 5 to 15 mln generations depending on the dataset size, with a 25% burnin. Trees were graphically edited using FigTree v1.4.4 [45] and GIMP 2.10.32. Convergence results were verified on Tracer v1.7.1 [46] by ensuring all ESS values were above >200.

All sequences were uploaded on GenBank (NCBI Accession Numbers: OP985208–OP985289).

## 3. Results

### 3.1. Dataset

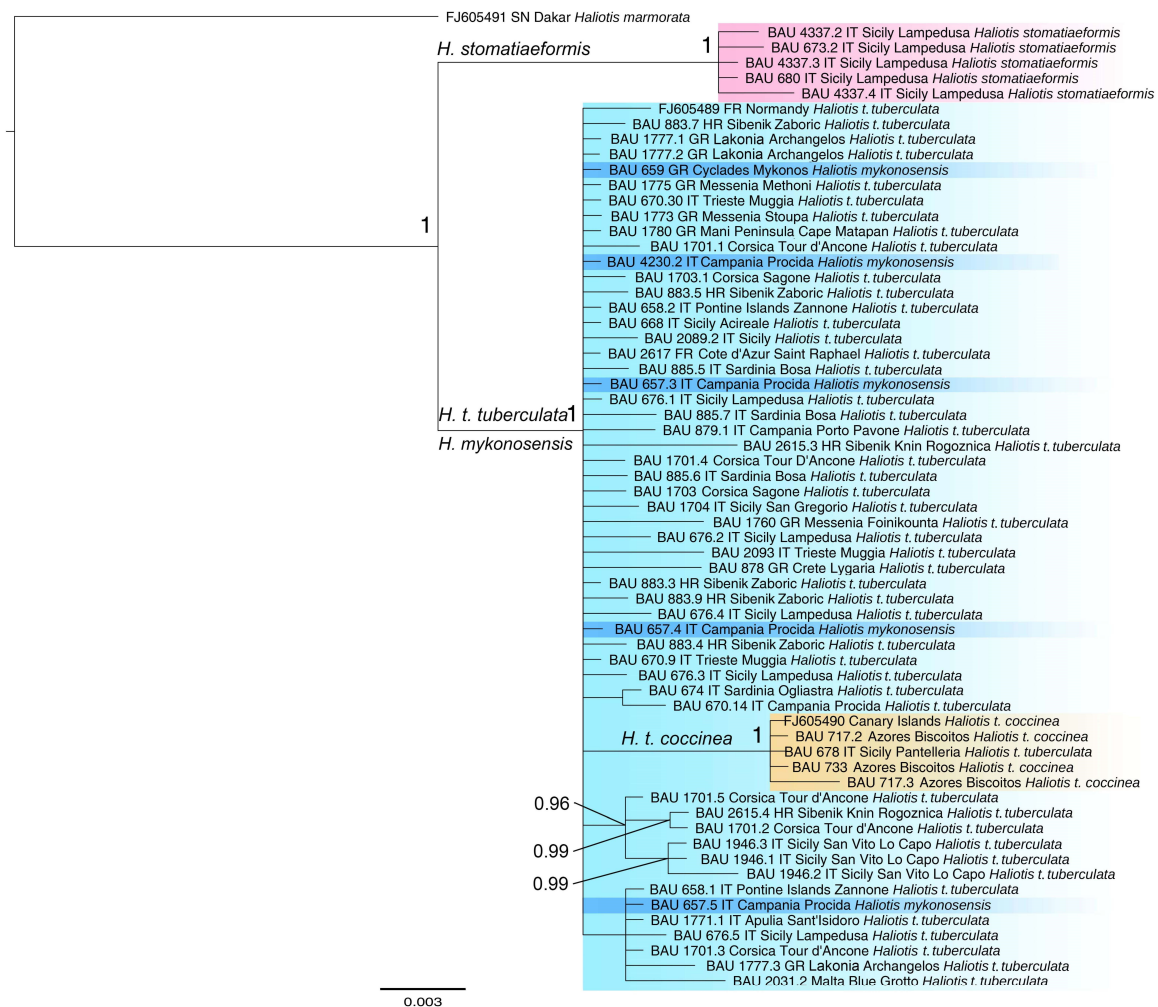
For this study, 61 sequences of the 5'-COI fragment (20 of which were obtained by an assemblage of the minibarcode sequences), 82 of the 3'-COI fragment, and 12 single minibarcode sequences were newly produced (Table 1). After integration with the publicly available data, the resulting alignment was partitioned into three datasets to be analyzed separately: the 5'-COI fragment (5'-COI:  $n = 64$ , 548 bp), the 3'-COI fragment (3'-COI:  $n = 157$ , 542 bp), and their concatenation (tot-COI:  $n = 76$ , 1090 bp), which included the minibarcode sequences. For the species-delimitation analyses, only 5'-COI sequences with

no missing nucleotides were retained ( $n = 51$ ) and aligned with the 52 sequences from Genbank (5'-COI-ASAP:  $n = 103$ , 548 bp).

### 3.2. Bayesian Inference

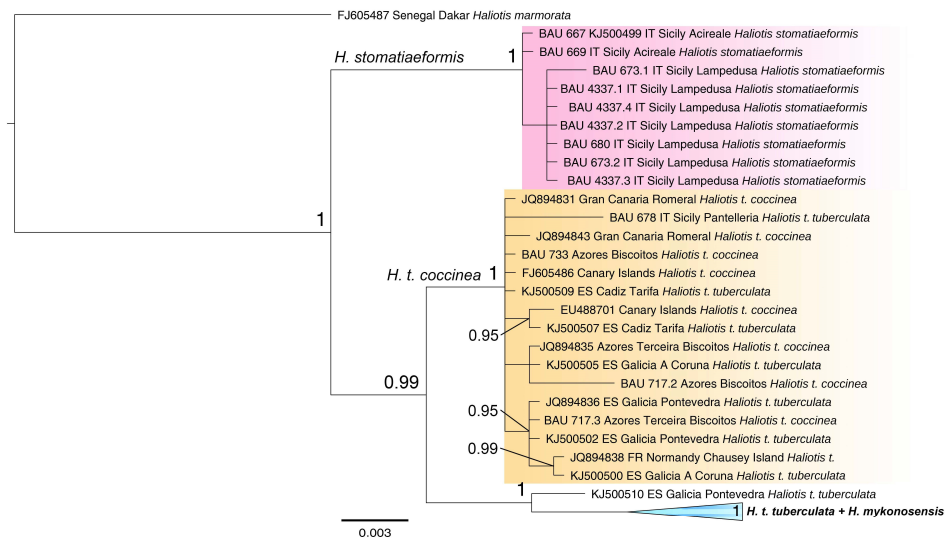
According to the Bayesian Information Criterion, the best substitution models for each codon position (first, second, and third), among those implemented in MrBayes, were as follows: JC, F81, and HKY for the 5'-COI fragment, and TrNef, F81, and TrNef + G for the 3'-COI fragment.

In all phylogenetic reconstructions *H. stomatiaeformis* was retrieved as monophyletic and sister to *H. tuberculata* with maximal support scores ( $PP = 1$ ), whereas *H. mykonosensis* was never retrieved as a clade separated from *H. tuberculata* (Figures 3–5). Specimens of *H. t. coccinea* were joined in a monophyletic clade in all trees, but they were nested inside the *H. t. tuberculata* clade in the 5'-COI analysis. In the 3'-COI analysis, a sequence from Fernandez et al. ([16]; NCBI accession number: KJ500510) was positioned between the *H. t. coccinea* and the *H. t. tuberculata* clades. This sequence is almost identical (99.82% identity, 100% query cover on BLAST) to a hybrid recombinant of the two subspecies sequenced by Van Wormhoudt et al. ([47]; NCBI accession number: FJ605488).

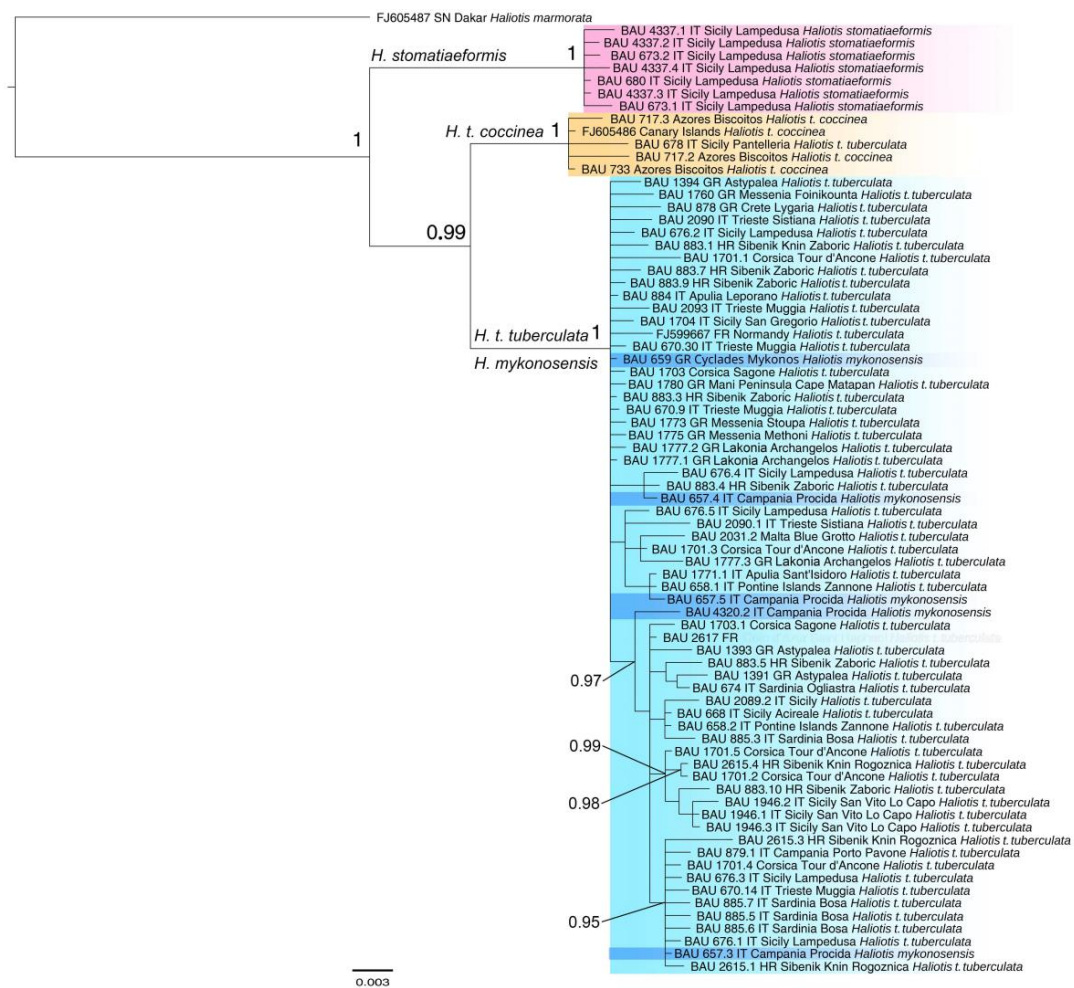


**Figure 3.** Phylogenetic relationships among northeast Atlantic and Mediterranean haliotids. Tree derived after a Bayesian analysis of the 5'-COI fragment alignment. Numbers at nodes are posterior probabilities, values shown only when  $PP > 0.95$ . Names at tips are morphological identifications.





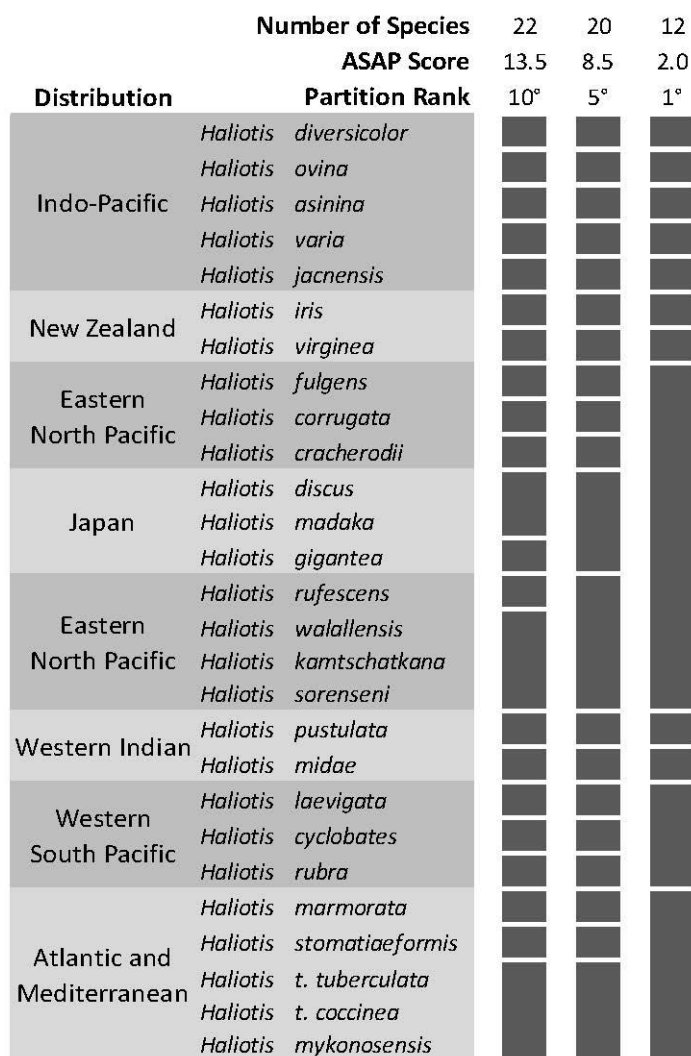
**Figure 4.** Phylogenetic relationships among northeast Atlantic and Mediterranean haliotids. Trees derived after a Bayesian analysis of the 3'-COI fragment alignment. Numbers at nodes are posterior probabilities; values shown only when  $PP > 0.95$ . Names at tips are morphological identifications.



**Figure 5.** Phylogenetic relationships among northeast Atlantic and Mediterranean haliotids. Tree derived after a Bayesian analysis of the concatenated COI dataset. Numbers at nodes are posterior probabilities, values shown only when  $PP > 0.95$ . Names at tips are morphological identifications.

### 3.3. Species Delimitation Analyses

Among the partitions proposed by ASAP (Figure 6), the one with the highest ASAP score ( $n = 12$ ;  $p$ -value =  $1.79 \times 10^{-2}$ ,  $p$ -value rank = 3;  $W = 8.93 \times 10^{-4}$ ,  $W$  rank = 1; threshold distance = 12.53%) identified nine known haliotid species consistently with current taxonomy but clustered together three African species (*H. cyclobates* Péron, 1816, *H. laevigata* Donovan, 1808, *H. rubra*, Leach, 1814), all the Mediterranean–Atlantic nominal taxa (*H. marmorata*, *H. mykonosensis*, *H. stomatiaeformis*, *H. tuberculata*), and many north Pacific species (*H. fulgens* Philippi, 1845, *H. cracherodii* Leach, 1814, *H. rufescens* Swainson, 1822, *H. walallensis* Stearns, 1899, *H. kamtschatkana* Jonas, 1845, *H. sorenseni* Bartsch, 1940, *H. gigantea* Gmelin, 1791, *H. madaka* (Habe, 1977), *H. discus* Reeve, 1846, and *H. orrugate* W. Wood, 1828). The fifth partition by ASAP score ( $n = 20$ ;  $p$ -value =  $5.81 \times 10^{-1}$ ,  $p$ -value rank = 8;  $W = 1.90 \times 10^{-4}$ ,  $W$  rank = 9; threshold distance = 2.59%) identified 20 hypothetical species (mostly corresponding to the nominal species included in the dataset), separating *H. stomatiaeformis* from *H. tuberculata* (which in turn included *H. t. tuberculata*, *H. t. coccinea* and *H. mykonosensis*), in accordance with the phylogenetic analyses. In this partition, seven Pacific haliotid species clustered into two groups, namely, (i) *H. discus*, *H. madaka*, and *H. gigantea* and (ii) *H. rufescens*, *H. walallensis*, *H. kamtschatkana*, and *H. sorenseni*.



**Figure 6.** Summarized results of the ASAP analysis (available in Supplementary Figure S2). All specimens included are collapsed into taxonomic entities. Each column represents a partition proposed by the analysis, and it is subdivided into blocks, each representing a distinct hypothetical species-level group in that partition.

The 5'-COI genetic distances calculated between the species hypothesis identified by the fifth ASAP partition showed more than 3% interspecific distance from *H. stomatiaeformis* and the other Mediterranean abalones, whereas distances between *H. t. coccinea* and *H. t. tuberculata* ranged from 1.3% to 2.8%. Genetic distances among the Pacific species that clustered together showed interspecific distance values below 3%, down to 0.2–0.9% between *H. kamtschatkana* and *H. sorenseni*, and 0.6% between *H. madaka* and *H. discus* (Table 2).

**Table 2.** Genetic minimum and maximum interspecific and intraspecific distances (%) calculated on the 5'-COI-ASAP alignment.

	<i>H. marmorata</i>	<i>H. stomatiaeformis</i>	<i>H. tuberculata</i>	<i>H. coccinea</i>	
<i>H. marmorata</i>	-				
<i>H. stomatiaeformis</i>	8–8.2	0.2–0.4			
<i>H. tuberculata</i>	6.7–8.2	3–4.4	0–1.9		
<i>H. coccinea</i>	7.4–7.8	3.4–3.8	1.3–2.8	0.2–0.6	
	<i>H. crackerodii</i>	<i>H. rufescens</i>	<i>H. walallensis</i>	<i>H. kamtschatkana</i>	<i>H. sorenseni</i>
<i>H. crackerodii</i>	0.2				
<i>H. rufescens</i>	8.1–8.5	0.2–0.7			
<i>H. walallensis</i>	7.4–8.2	2.2–2.8	0.2–0.5		
<i>H. kamtschatkana</i>	7.2–7.8	2.4–3.4	1.5–2.4	0.2–0.6	
<i>H. sorenseni</i>	7.2–7.6	2.2–2.8	1.3–1.9	0.2–0.9	0.2
	<i>H. corrugata</i>	<i>H. discus</i>	<i>H. madaka</i>	<i>H. gigantea</i>	
<i>H. corrugata</i>	0.2				
<i>H. discus</i>	8.4–9.3	0.7			
<i>H. madaka</i>	8.9–9.1	0.6	-		
<i>H. gigantea</i>	8.9–9.3	2.1–3.0	2.2–2.8	0.6	

#### 4. Discussion

Among the numerous nominal taxa that have been synonymized with *H. tuberculata* in recent decades, the most remarkable is probably *H. lamellosa* Lamarck, 1822, corresponding to a peculiar phenotype occurring mostly in the central Mediterranean with a smaller and slightly more elevated shell, adorned with a number of dorsal lamellar ribs, first resurrected as a variety [25] and then as a subspecies [48] of *H. tuberculata* [24]. The actual status of this nominal taxon has been a matter of debate for decades [49,50], with authors discussing whether *H. lamellosa* should be addressed as a valid species or subspecies of *H. tuberculata* [14] or as a phenotypic variety [20,51]. This framework is further confused by the fact that, in several Mediterranean checklists, *H. lamellosa* is the name used for the European abalone [52–54]. Molecular data using the 3'-COI fragment, the sperm lysin gene, and microsatellite loci have confirmed that *H. t. tuberculata* and *H. t. lamellosa* cannot be separated at any taxonomic level [55], as initially suggested by a study on the sperm lysin gene only [28]. We provide here additional confirmation of this correspondence, as none of the specimens with a phenotype attributable to *H. t. lamellosa* (e.g., BAU 676.1, BAU 676.3, BAU 1777.1–3; BAU 1775, BAU 2617) clustered separately from the European abalone either in the phylogenetic or in the species delimitation analyses.

Originally described from Cabo Verde Islands, *H. coccinea* has been a frequently used name for the haliotids in the Macaronesia region, recognizable for their peculiar bright coloration [14]. Sometimes, *H. coccinea* was considered endemic to the Canaries and isolated from other species [20,56], with occasional findings in Madeira and the Selvagens Islands [14]. However, the same chromatic phenotype was also found in *H. t. tuberculata* along the Western African coast, so this taxon was reevaluated as a variety [14] or

a subspecies [20] of *H. tuberculata*. In addition to that, a recent molecular survey in the Atlantic recorded the same haplotypes of *H. t. coccinea* from the Azores and also in Brittany and Normandy, in sympatry with *H. tuberculata* s.s. [55]. Finally, there are three historical records [10,57] of *H. t. coccinea* in the Mediterranean Sea (based on morphological identification), but those specimens could not be checked morphologically nor genetically [32]. Contrary to *H. t. lamellosa*, specimens morphologically ascribed to *H. t. coccinea* proved to be monophyletic in all analyses, yet nested inside a larger *H. tuberculata* clade in the 5'-COI tree. Furthermore, the genetic distance calculated with *H. t. tuberculata* (1.3–2.8%) is too low to be considered as a clear-cut indication of a distinct species. These results confirm the taxonomic status of *H. t. coccinea* as a subspecies of *H. tuberculata*. The sequence of one specimen from Sicily (BAU 678) clustered within the *H. t. coccinea* clade, providing the first confirmed record of a “*coccinea*” haplotype in the Mediterranean Sea.

*Haliotis mykonosensis* is a recently described species that was originally discovered in the Aegean Sea. The external features of the animal are undoubtedly unique and several ecological differences from the typical European abalone were presented to validate this species [29,33]. Some specimens showing the characteristic morphological and ecological features of *H. mykonosensis* were recently reported from Italy, France, and Croatia well outside the original range in the Aegean Sea [31,32]. We have assayed a topotypical specimen along with samples from the Italian locality (Procida Island), and none of the five specimens in the dataset showed any significant genetic difference with *H. tuberculata*, nor did they constitute any monophyletic group. This phenotype thus corresponds to one of the multiple forms of the European abalone and should be synonymized with *H. tuberculata*.

The history of the discovery of *Haliotis stomatiaeformis* from the central Mediterranean is quite remarkable. First described by Reeve (1846) and later by Philippi (1854) from Malta with the name *H. neglecta*, this species was then forgotten for almost 150 years because of the erroneous type locality of Reeve’s original description (New Zealand) and the loss of the type material of Philippi. After recent findings of some peculiar haliotids in Malta, this taxon was resurrected by Geiger [20] and has since waited for genetic validation [30,33,34]. In all phylogenetic reconstructions, *H. stomatiaeformis* was retrieved as the sister species to *H. tuberculata*. The genetic distance between *H. stomatiaeformis* and *H. tuberculata* ranged from 3% to 4.4%, congruently with observed minimum interspecific distances among gastropod taxa, usually between 2% and 4% [1,6–8,58,59]. The fifth ASAP partition hypothesis, which considers *H. stomatiaeformis* as a different species, is also the most congruent with the current taxonomy of abalone on a global scale and the levels of genetic divergence measured among haliotid clades.

Similarly to the Mediterranean area, the haliotids from both sides of the Pacific count a large number of described species, subspecies, and varieties, most of them being important commercialized abalones in the international shellfish market. Despite many of these species being highly studied for conservation purposes, some of them remain doubtful from an integrative taxonomy perspective, as the genetic patterns do not match the biological and ecological data. For example, a remarkably low maximum COI genetic distance of 0.74% was found between the Western Pacific *H. discus* and *H. madaka* [60], and the Pacific endangered species *H. kamtschatkana*, *H. walallensis*, and *H. sorenseni* cannot be adequately separated on a genetic base using COI [5,61,62]. These results are confirmed by the K2P distance analyses, and according to the most coherent ASAP partition, *H. rufescens*, *H. walallensis*, *H. kamtschatkana*, and *H. sorenseni* would actually belong to the same cluster with a ~3% threshold, and the same holds true for *H. discus*, *H. madaka*, and *H. gigantea*. Even if we lower this threshold below 2%, only *H. rufescens* and *H. gigantea* would split from the others. It is not within the scope of this paper to revise the Pacific abalone based solely on the molecular information currently available, yet we stress the need for a solid molecular framework for these species.

The European abalone is currently classified as vulnerable because of past overexploitation of fisheries [15,24,63], and precautionary measures are still urged in order to preserve this species, including the subspecies *H. t. coccinea*. However, there is not any

estimate of the conservation status of the “neglected” *H. stomatiaeformis*, which is regrettable, given its restricted range and being the only endemic abalone of the Mediterranean. Finally, the taxonomic assessment of several Pacific species (including some that suffer from overexploitation and are currently being the focus of conservation efforts [61]) needs an integrated approach with a solid genetic framework. This may have effects on the conservation assessment of those taxa.

## 5. Conclusions

Shell morphology, anatomy, and ecology were not proven to be effective indicators of evolutionary divergence in northeastern Atlantic and Mediterranean *Haliotis*. In fact, abalones’ surprising capacity to adapt to local factors has been underestimated by taxonomists and the number of recent species of the genus has long been in need of confirmation. Taxa with highly variable morphological traits should not be defined without the support of genetic data. With this work, samples representing all the recently recognized nominal taxa from the Mediterranean and the neighboring Atlantic have received a first molecular assessment, and among them, only two, namely *Haliotis tuberculata* and *Haliotis stomatiaeformis*, proved recognizable at a species level. *Haliotis tuberculata coccinea* is confirmed as a monophyletic lineage within the European abalone with evidence of gene flow with the nominotypical subspecies. In addition, the results of this revision suggest that several currently accepted species of *Haliotis* in the Pacific region are in need of revision as their interspecific genetic divergences are remarkably small and their status needs to be assessed through an integrative taxonomy approach.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/d14121107/s1>, Figure S1: Bayesian Inference 3'-COI complete tree; Figure S2: ASAP ten best partitions; Table S1: Sources and NCBI accession numbers. Table S2: primers list and PCR protocols. Table S3: K2P COI distances. References [64–73] are cited in the Supplementary Materials.

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