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***Aplysina aerophoba* (Nardo, 1833) (Porifera, Demospongiae): an unexpected miniaturised growth form from the tidal zone of Mediterranean caves: morphology and DNA barcoding**

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Abstract

In the Mediterranean Sea, the two sponges of the genus *Aplysina* (*A. aerophoba* and *A. cavernicola*) are identified on the basis of their external morphology and the environment in which they live. During a research program on the sponge fauna in semi-submerged caves of the Italian coasts, we have sampled an abundant very small yellow sponge, often living in the tidal zone, which were attributed to the genus *Aplysina*. Failing to assign the samples to a species through classical taxonomic methodologies (growth form and skeleton arrangement) and for the particular environment where this sponge lives, we have decided to use the COI analysis to solve the taxonomic problem offered by these miniaturized specimens. The analysis indicated that, in spite of the morphological differences, they belong to *A. aerophoba*. During old detailed surveys, conducted in the '60 years in some of the studied caves, this species was not recorded. It is possible that its abundant presence is related to the modifications occurred in the Mediterranean sponge communities occurred in the last decades in relation to global warming.

Keywords: *Mediterranean caves*, *Aplysina aerophoba*, *morphology*, *DNA*

Introduction

Due to the lack of mineral skeleton (siliceous or calcareous) and the presence of chitin-spongin fibres (Ehrlich et al. 2007) with very similar size and shape, identification of the species of the genera belonging to the order Verongiida Bergquist, 1978 is generally critic. The two Mediterranean species of the Aplysinidae family, *Aplysina aerophoba* (Nardo 1833) and *A. cavernicola* (Vacelet 1959), are separated on the base of the external morphology and, in particular, surface texture, shape of digitations, presence of lateral projections and pigmentation of living specimens. Moreover, these two species were always found in different habitats: shallow-waters lighted environments for *A. aerophoba* while *A. cavernicola* lives in deep coralligenous habitats or at the entrance of shadow caves. On the other hand, the distinction of the two taxa on the base of skeletal fibres is considered very uncertain (Vacelet 1959).

During a research program on the sponge fauna of semi-submerged marine caves present along the

Italian coasts, we have recorded abundant, very small specimens of a yellow sponge, often living in the tidal zone, that were attributed to the genus *Aplysina*; these miniaturised growth forms did not fit with the characteristics of the two known Mediterranean species of this genus (Costa et al. 2018). This taxonomic uncertainty leads us to collect specimens in different localities to solve the problem by means of morphological and genetic analysis.

Over the past decades, molecular analyses represented a powerful tool in the clarification of species boundaries in the marine realm. This was particularly useful in species whose separation on morphological base presents a high degree of uncertainty as sponges subjected to high intra-specific variation of shape and colour (Heim 2003): due to the incorporation of molecular techniques into the field of systematics, the discovery of morphologically cryptic but genetically divergent species has increased exponentially over the recent decades (Knowlton 1993; Bickford et al. 2006).

Heim et al. (2007) tested different molecular markers, as internal transcribed spacer-1 and -2 (ITS-1,

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ITS-2) rDNA, mitochondrial 12S, 16S, and cytochrome oxidase subunit I (COI), in the separation of the species of the genus *Aplysina* of the Mediterranean Sea. Authors found that a high degree of intra-individual polymorphism within the ITS-1 and ITS-2 while, in contrast, the mitochondrial 12S and 16S are highly conserved. Authors concluded that only the COI was suitable in the separation of *A. cavernicola* from specimens which are regarded as *A. aerophoba*. Nevertheless, this last taxon probably is not a single valid species, but a cluster of species.

On this base, we have decided to use the COI analysis to solve the taxonomic problem offered by the miniaturised sponge belonging to the genus *Aplysina* recorded in the tidal zone of 4 semi-submerged caves along the Italian coasts.

Materials and methods

Sampling localities

Sampling was performed by SCUBA diving in the summer 2017 and 2018 in four semi-submerged caves in different localities of the Italian coasts (Figure 1); two were located in the Eastern Ligurian Riviera: “Bonassola Cave” (44°10'24.60"N, 9°35'25.32"E) and “Zoagli Cave” (44°20'13.70"N, 9°15'28.87"E); one was located in the western Ligurian Riviera: “Grotta marina di Bergeggi” (44°14'33.25"N, 8°26'42.90"E). The last one was located in the south Apulia coast: “Matrona Cave” (39°54'22.55"N, 18°

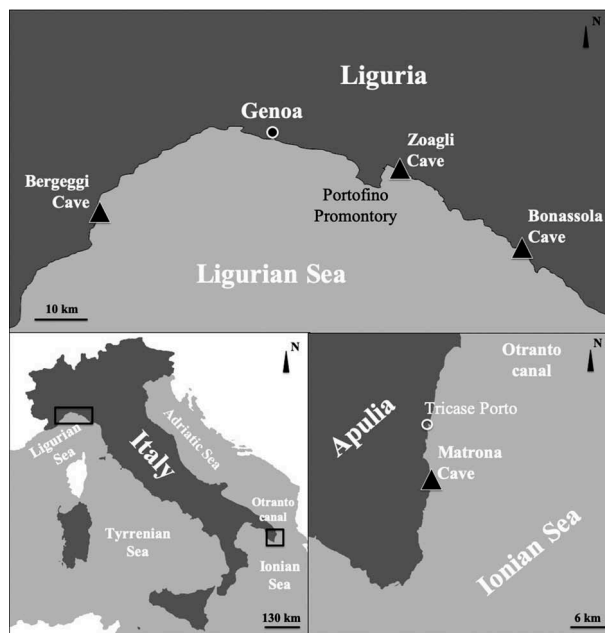


Figure 1. Maps with study area. The triangle indicates the semi-submerged caves sampled.

Table 1. Species and sequences downloaded from Genbank database with origin area and Genbank accession numbers. [§]Species chosen as outgroup. Accession numbers of our specimens of *Aplysina aerophoba* from Ligurian Sea and Ionian Sea are with asterisks (*).

Species	Number of sequences	Area	Genbank accession numbers
<i>Aplysina aerophoba</i>	11	Mediterranean Sea	EF043369, EF043370, EF043371, EF043372, HQ379407, JX999079, KY625183, KY625184, KY625185, MN020524*, MN020525*
<i>Aplysina</i> sp.	2	Mediterranean Sea	KX866737, KX866776
<i>Aplysina cavernicola</i>	3	Mediterranean Sea	EF043365, EF043366, EF043367
<i>Aplysina</i> sp.	4	Brazil, Saint Peter and Saint Paul Archipelago	KX034567, KX034568, KX034569, KX034570
<i>Aplysina fistularis</i>	3	Caribbean Sea	EF519570, EF519571, EF043376
<i>Aplysina cauliformis</i>	3	Caribbean Sea	EF519567, EF519569, EF043374
<i>Aplysina insularis</i>	1	Caribbean Sea	EF043373
<i>Aplysina fulea</i>	2	Caribbean Sea	JX999063, EU237476
<i>Aiolochoroia crassa</i> [§]	1	Caribbean Sea	KX034574

23°28.01"E). The sponge-fauna of the Bonassola and Zoagli caves were already deeply studied by Sarà (1964) and Costa et al. (2018).

The Bonassola Cave is a semi-submerged, horizontal cavity which opens on gabbro rocks about 1 km eastward of the homonymous village. The bottom of the cave reveals irregular boulders and pebbles. The cavity is accessed through a 4–5-m long passage, 2–2.5 m wide and with a ceiling 2 m above sea level. Numerous crevices and small galleries are present on the side walls. Light conditions create a semi-obscure environment, while total darkness is reached only in parts of the cave. The Zoagli Cave is a small blind-end (cul-de-sac) semi-submerged cave, which opens in a marly cliff about 1 km west of the Zoagli village. It is about 16 m long and 4 m wide at the entrance, with a height of the vault decreasing from about 3 m at the entrance to about 1 m at the end of the cave. The pebble floor steadily rises from a depth of 2 m to the end of the cave where it forms a small beach. The walls are mostly smooth. The opening is oriented to the east. The Bergeggi cave originated by karst corrosion in a dolomitic limestone of the middle Trias and its appearance was shaped by marine erosion. The main entrance, facing East, is about 10 m wide and 8 m high. The main cavity, which occupies about 350 m², is characterized by large boulders detached from the ceiling. The sea penetrates this room forming pools of variable width and depth. The benthic communities of the Bergeggi marine cave were deeply studied through the last three decades starting from the paper of Bianchi et al. (1988). The Matrona cave has a predominantly horizontal development, it is about 12 m long and about 7 m wide. It has a large collapsed semi-submerged cave. It opens with a mouth of about 7–8 m and a height varying between 1.5 and 3 m.

Morphological characterization

Sponge samples were preserved in 95% ethanol and processed by standard methods for sponge identification (Rützler 1978). Taxonomic decisions were taken according to the Systema Porifera (Hooper & Van Soest 2002) implemented by the Demosponge revision of Morrow and Cárdenas (2015) and the World Porifera Database (WPD) (van Soest et al. 2019). The skeleton was studied measuring the length and the total diameter and the core width of at least 30 fibers. Minimum, mean (in parentheses) and maximum values (µm) of fibres and axial pith dimensions are reported.

For SEM analysis, dried tissues and skeleton were coated with gold and observed by a scanning electron microscope Vega3 TESCAN type LMU.

Molecular investigations

For genetic analysis, small portions of sponge tissues were obtained from 2 specimens of about 5-mm size sampled in Bergeggi cave (Ligurian Sea) and Matrona Cave (Ionian Sea). Total DNA was isolated using a Genomic Tissue DNA Kit (Rabbit Bio-Tek) following the manufacturer's protocol and subsequently stored at –20°C. To perform species identification, the complete mitochondrial COI gene of 710 base pairs (bp) was amplified using two pairs of primers: PorCOI2fwd (5'-aatatgngggcncnggnatnac-3') and PorCOI2rev (5'-actgcccccatngataaaacat-3') developed by Xavier et al. (2010) and LCO1490 (5'-gggtcaacaatacataagatatattgg-3') and HC02198 (5'-taaaacttcagggtgacccaaaaaatca-3') developed by Folmer et al. (1994). Polymerase chain reactions (PCR) were carried out in a 20 µl volume containing 1 µl of extracted DNA, 0.4 µl of each primer (10 µmol), 0.4 µl of dNTP mix (10 µmol each, Promega, USA), 2.4 µl of MgCl₂ (25 mM), 4 µl of 5x Green GoTaq Flexi Buffer (Promega), 0.1 µl of GoTaq Taq DNA polymerase (Promega), and deionized water to volume. PCR reactions were run with the following parameters: 3 min of first denaturation at 95°C, each of 35 cycles consisted of denaturation for 30 sec at 94°C, annealing for 45 sec at 45°C, and extension at 72°C for 90 sec with a final 10 min extension at 72°C for Xavier et al. (2010) primers, and initial denaturation at 94°C, each of 35 cycles consisted of denaturation for 30 sec at 94°C, annealing for 55 sec at 40°C, and extension at 72°C for 1 min with a final extension for 7 min at 72°C for Folmer et al. (1994) primers. Amplification products were confirmed by using gel agarose electrophoresis and then purified using 2 µl of ExonucleaseI-Shrimp Alkaline Phosphatase (ExoSAP, USB, Cleveland, OH, USA) per 5 µl of PCR product at 37°C for 15 min, followed by deactivation at 80°C for 15 min. The run for DNA sequencing was performed with the "ABI 3730XL" Genetic Analyzer with the sanger technique. Sequences were aligned, edited, and trimmed to a common length using the DNA sequence assembly and analysis software Geneious Pro v. 9.0.4 (Biomatters, LTD, Auckland, NZ) and deposited in GenBank (accession numbers MN020524–MN020525; <http://www.ncbi.nlm.nih.gov/genbank/>). A similarity search (BLAST) was performed to exclude any possible contaminants such as symbionts and for species identification. For inclusion in the phylogenetic analyses, as described in (Table I), 27 COI sequences of 8 species from *Aplysina* genus plus a sequence of *Ailochroia crassa* (Hyatt, 1875) were downloaded from the public databases (Barcode of Life Data (BOLD) Systems, www.barcodinglife.org) (Ratnasingham & Hebert 2007).

and GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). All sequences were trimmed to a common length of 660 bp. A Maximum Likelihood (ML) tree was constructed using the program mega 7 (Kumar et al. 2016). The best fit general substitution model (as indicated by MEGA) was implemented (Tamura 3-parameter model) and all codon positions selected (Tamura 1992). Bootstrap support values were calculated using 1000 replicates.

Results

The studied specimens were small, cushion-shaped (2–10 mm in diameter; 1–3 mm thick) (Figure 2(a,b)). They were widely distributed in the semi-dark and dark portions of all the caves. Several specimens recorded in the tidal zone of the Zoagli and Bergeggi Caves remained completely emerged during low tides. Sometimes several small specimens fused together, forming large encrusting plates (Figure 2(b,c)). Most of the specimens had abundant thin branching processes even longer than 1 cm with an enlarged extremity (Figures 2(a–c) and 3(a,b)).

The colour in life was bright yellow (Figure 2) and fades to violet/black after alcohol preservation. Live specimens were soft, very fragile, with small elevated oscula (Figure 2(c,d)). The surface was conulose (Figure 3(c)), sometimes showing an evident fibrous network (Figure 2(d)).

The skeleton was fragile and showed the typical Aplysinidae structure. Primary and secondary fibres were indistinguishable and formed a regular three-dimensional framework (Figure 4(a–d)). The fibres ended inside the conules of the surface (Figure 4(c)). The fibres were laminar with a dark large axial pith (Figure 4(a,b)). The diameter of the fibres was 10 (47.7) 120 μ m and the axial pith was 7.5 (35) 100 μ m. Table II describes the main morphological and skeletal characteristics of the specimens collected in Ligurian Sea and Apulian Sea.

DNA investigations

For the selected gene, aligning sequences from both sets of primers, we obtained 1110 bp from both specimens. The alignment performed with 27 sequences from genus *Aplysina* has generated sequences of 660 bp in length. A BLAST search

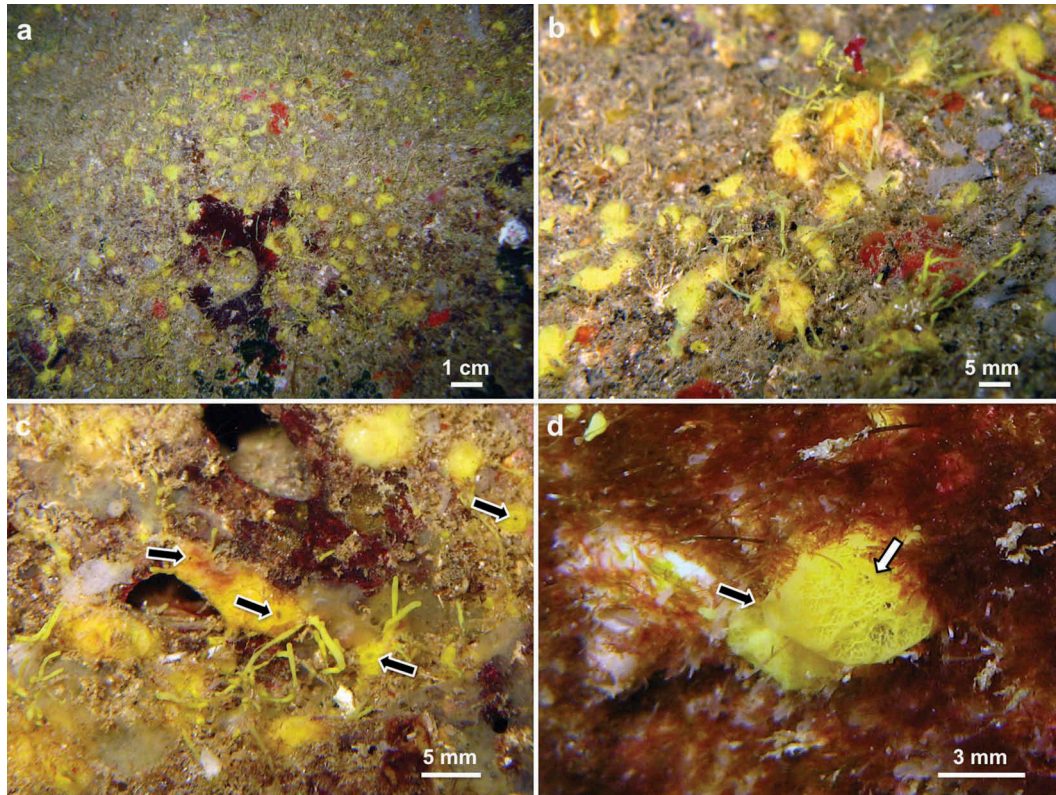


Figure 2. *Aplysina aerophoba* (a,b) Cushion-shaped specimens joined together by thin, sometimes branching processes. (c) Samples with evident oscula indicated by arrow. (d) Magnification of the conulose surface with oscula (black arrow) and fibrous network (white arrow).

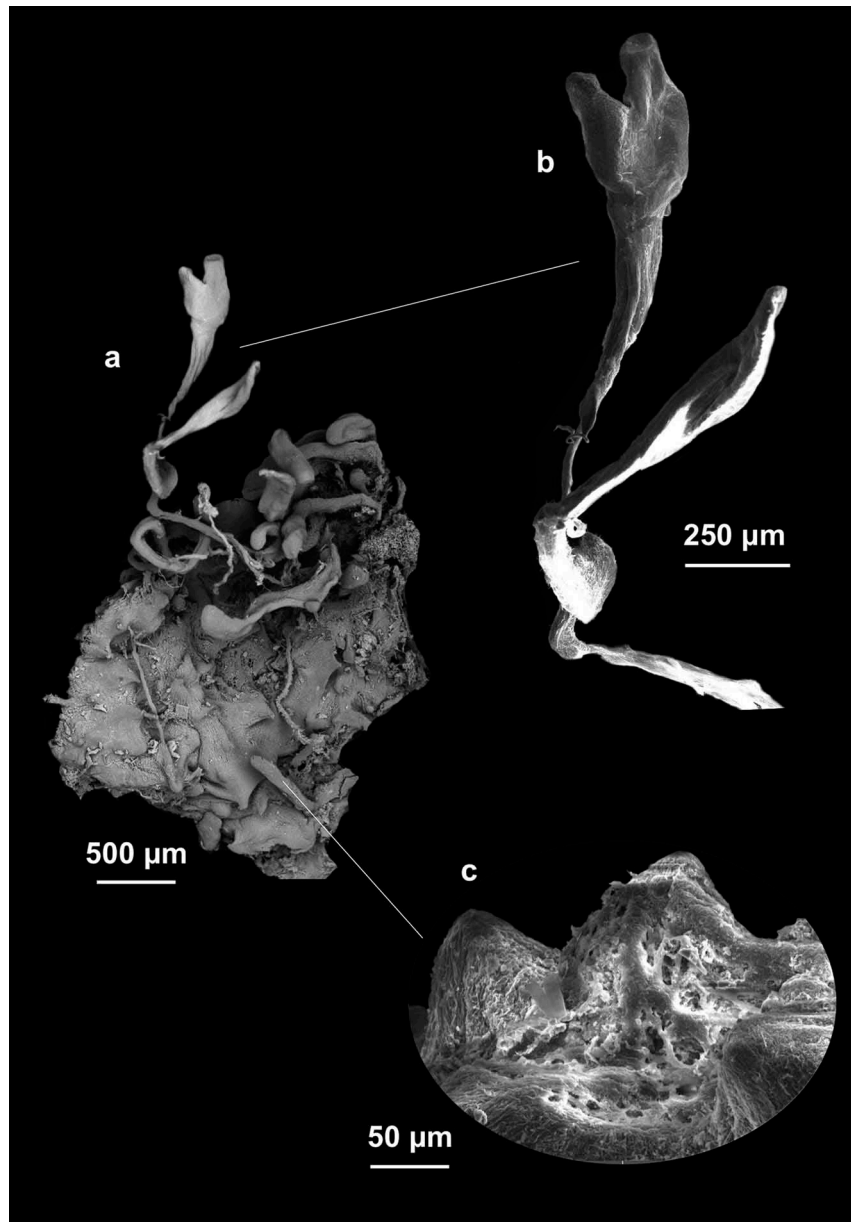


Figure 3. (a) Specimen with thin branching processes that they end up widening. (b) Magnification of the branching processes. (c) Conulose surface with evident fibrous network.

performed both in GenBank and BOLD System excluded any contamination event with symbionts for our specimens, that belong to *A. aerophoba* (100% identity). Analysis of nucleotide sequences within the COI gene among the 8 *Aplysina* species showed six phylogenetically informative sites. Five of these sites represent substitutions in the third position of the respective codon. In only one, the substitution in the first position reveals an amino acid substitution of aspartic acid with asparagine for the species *Aplysina* sp. (Table III). Considering these 5 sites, in the position 181st the

two lineages reveal a clear difference of Adenine and Guanine nucleotide, respectively, for Mediterranean and Atlantic. The percentage identities between our *A. aerophoba* samples and the others Mediterranean species *A. cavernicola* and *Aplysina* sp. (this from Levantine Sea studied by Idan et al. 2018), have been 99.8% respectively with 3 bp of exchanges and 2 bp of exchange. Identical sites percentages and number of bp exchanges with other Atlantic species have been, respectively, 99.7% and 2 bp with *A. cauliformis* (Carter, 1882), *A. fistularis* (Pallas, 1766) and *A. insularis* (Duchassaing &

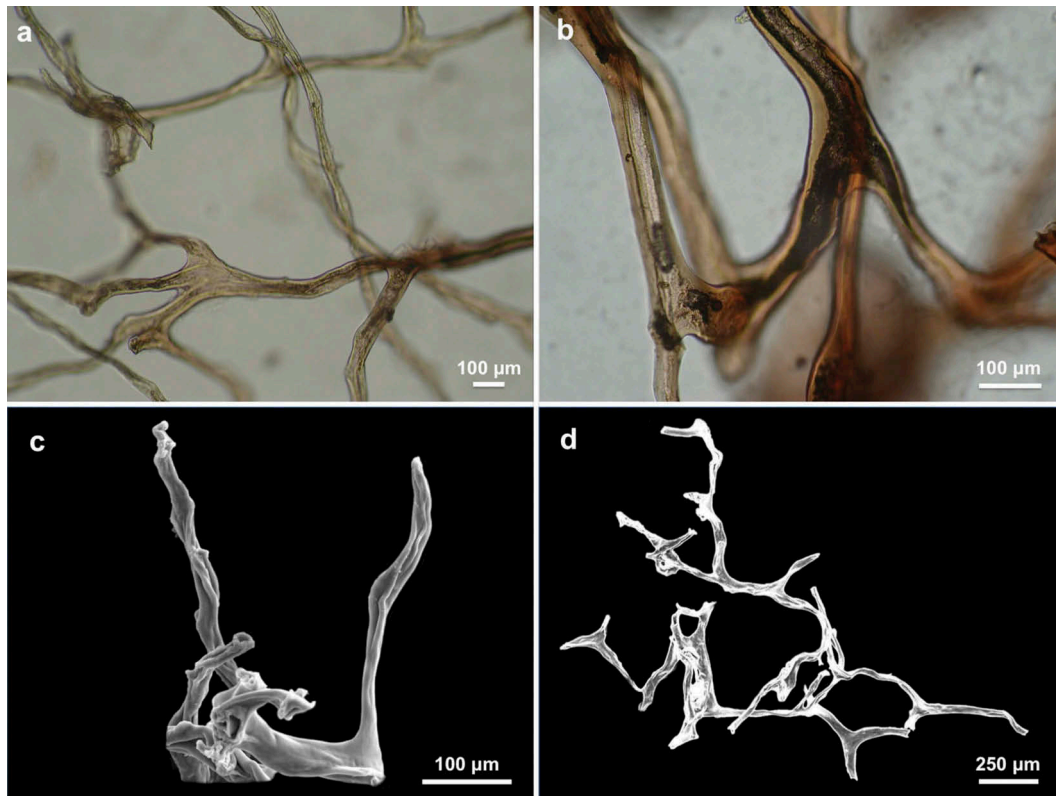


Figure 4. (a,d) Three-dimensional framework of fibres. (b) Laminar fibres with a large axial pith darker in colour. (c) Fibers end inside the conules of the surface.

Michelotti, 1864), 99.4% and 4 bp with *A. fulva* (Pallas, 1766) from Caribbean Sea, and 99.5% and 3 bp with *Aplysina* sp. from Brazilian coast. A maximum likelihood phylogenetic trees have been calculated with Tamura 3-parameter model (Figure 5). Our sequences cluster within *A. aerophoba* lineage. The two Mediterranean species *A. aerophoba* and *A. cavernicola* have shown the closest match and grouped together in a single clade separated from other Atlantic species (Figure 5). Two sequences of *Aplysina* sp. from Mediterranean Sea show an uncertain position. *Aiolochoira crassa* has been chosen as outgroups. *Aplysina fulva* with the other Caribbean species (*A. fistularis* and *A. cauliformis*) represents a basal branch and cluster within the genus. The species *Aplysina* sp. from Brazilian archipelago has shown a separated clade between Mediterranean and Caribbean lineages.

Discussion

In the described case only with COI analysis, it was possible to assign our samples to *Aplysina aerophoba* (100% identity) confirming the usefulness of gene

analysis for sponge species identification (Hebert et al. 2003).

A. aerophoba and *A. cavernicola* are confirmed to be clustered in a Mediterranean lineage clearly separated from a Caribbean and Brazilian lineages confirming the finding of Heim et al. (2007).

This specific identification is not obvious in the light of the miniaturised shape of the specimens found in semi-dark caves described here for the first time. This is particularly strange when we consider that the distinction between the two Mediterranean species of the genus is mainly based on the general morphology of the specimens (Vacelet 1959). On the other hand, Heim et al. (2007) suggested that the morphological taxon “*A. aerophoba*” is probably a cluster of related species. On this base and due to the peculiar morphological and ecological features of our specimens it is possible to hypothesise that, future more powerful analyses will allow to distinguish this species as a separate taxon.

However, a degree of morphological plasticity was already known both in *A. aerophoba* and *A. cavernicola*. In fact, transplantation experiments, indicated that the growth of both these species was affected by the level of solar radiation. Wilkinson and Vacelet (1979)

Table II. Morphological and skeletal characteristics of the specimens collected in Ligurian Sea and Apulian Sea.

	Body	Surface	Conules	Branching processes	Oscules	Fiber	Axiak pith	Meshes
Ligurian specimens	Bright yellow (fades to violet after alcohol preservation) - Cushion-shaped (2/10 mm diameter) - Encrusting plates (in some cases, it covers an area up to 100 cm ²)	- Conulose surface, in some specimens the fibrous network is visible - Finely conulose surface with visible oscules	0.1/0.3 mm high and apart	Mostly present in the cushion shaped forms, 0.2/1 cm long	1/2 mm	10 (47.7) 120 µm	7.5 (35) 100 µm	192 (299.5) 423 µm
Tricase specimens	Bright yellow (fades to violet after alcohol preservation) - Encrusting plates (in some cases it covers an area up to 100 cm ²)	Finely conulose surface with visible oscules	0.1/0.3 mm high and apart	Mostly present in the cushion shaped forms, 0.2/1 cm long	1/2 mm	15 (50) 110 µm	7 (25.5) 95 µm	185 (301) 430 µm

Table III. Amino acid (upper section) a nucleotide (lower section) substitutions of the COI between different Mediterranean species of *Aplysina*.

	<i>Aplysina aerophoba</i>	<i>Aplysina</i> sp.	<i>Aplysina cavernicola</i>	
<i>A. aerophoba</i>	*	1	0	Aminoacids substitutions
<i>A. sp.</i>	2	*	0	
<i>A. cavernicola</i>	3	4	*	
	Nucleotide substitutions			

transplanted specimens of *A. aerophoba* in habitats characterized by different conditions of light intensity. The growth of this species, characterized by the presence of symbiotic cyanobacteria, was enhanced when solar radiation increased. On the contrary, the growth of *A. cavernicola*, which do not contain cyanobacteria, was inhibited by light. Similar results were obtained by Klöppel et al. (2008) which reared *A. aerophoba* in aquaria in the aim to increase the production of bioactive compounds. When sponges are reared under lighted conditions the specimens maintained, after 150 days about the original volume. On the other hand, under dark conditions, the specimens experienced a significant reduction in size. This was not accompanied by any visible change in the structure or density of the skeleton.

The extreme reduction of the size of the specimens found in semi-submerged caves is therefore congruent with the low light intensity inside the caves. Regarding the color of the miniaturized specimens, *A. aerophoba* always express a dark yellow pigmentation, but our specimens show the bright yellow coloration typical of *A. cavernicola* that live in semi-dark zone. Very likely this is due to the loss of symbiotic cyanobacteria in dark as demonstrated by Klöppel et al. (2009) during rearing experiments.

Most of the specimens showed abundant thin branching processes, sometimes branched and enlarged at the extremity. These processes are considered budding element (Vacelet 1959; Gerçe et al. 2009) and their morphology was already described by Díaz et al. (2019) that suggested that their external morphology is individually genetically determined.

An open question remains the causes that induce a photophilic species to colonize a dark habitat. This aspect is particularly intriguing because, at least in the Bonassola and Zoagli caves the species was not surely present during surveys carefully conducted by Sarà (1964) about 50 years ago (Costa et al. 2018).

It was recently suggested that during the recent decades the sponge communities of marine caves

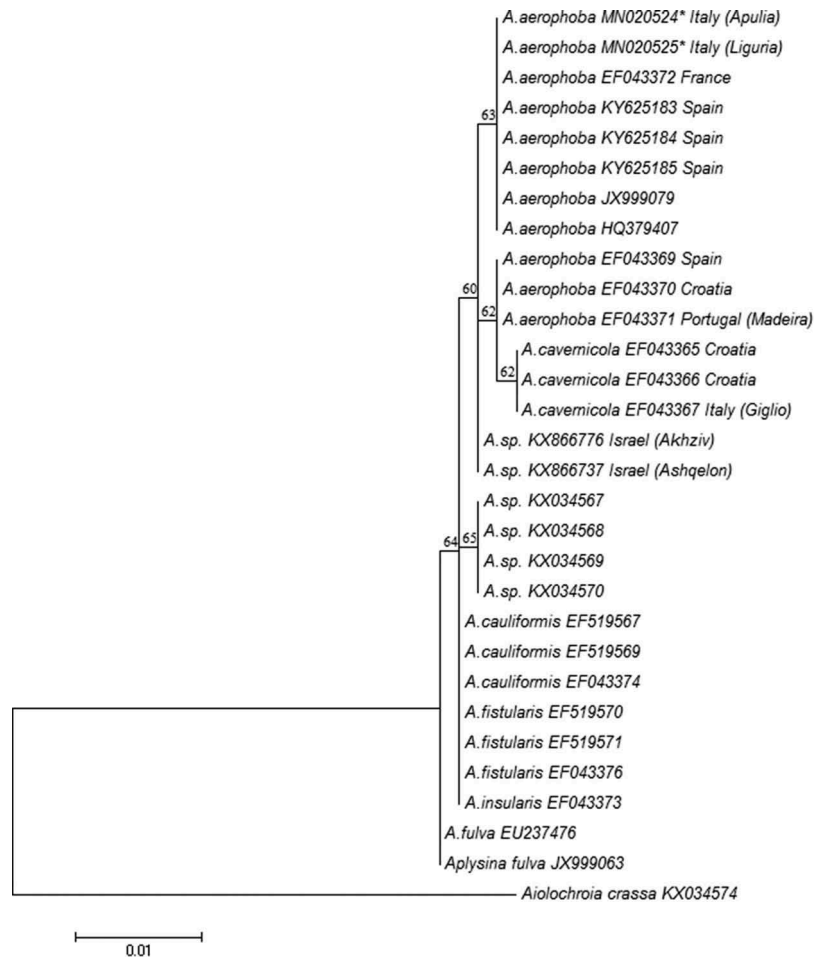


Figure 5. Maximum likelihood tree based on the Tamura-Nei model, calculated with MEGA 7. Bootstrap values higher than 50% are plotted above branches. Genbank accession numbers are listed. Our specimens from Ligurian Sea and Ionian Sea are with asterisks (*).

have experienced an increase in terms of specific richness and a significant change of the structural aspects. The three-dimensional growth forms decreased or remained quite similar, while numerous new two-dimensional ones were recorded (Costa et al. 2018; Montefalcone et al. 2018). We may, therefore, hypothesize that diffusion of this previously undescribed growth form of *A. aerophoba* is related to climatic events occurred which favoured some species and disadvantaged others.

Disclosure statement

No potential conflict of interest was reported by the authors.

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