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Caves Biodiversity in the Marine Area of *Riviera d'Ulisse* Regional Park, Italy: *Grotta del Maresciallo* Overview

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Abstract

The caves have a significant economic importance in their role as tourist attraction and are of great importance for the presence of some rare species. The fauna identification in the "*Grotta del Maresciallo*" cave, situated in the *Riviera di Ulisse* Regional Park, started in march 2013 by a visual census and molecular approach. The studies allowed to detect 12 classes, 46 families, 47 species with their ecological niches, and the percentage contribution of each group to the Mediterranean marine cave diversity. Furthermore, we report the presence of two thermophilic species, as the star coral, *Astroides calycularis* (Pallas, 1766) and the goldblotch grouper, *Epinephelus costae* (Steindachner, 1878); the tropical species, the ringneck blenny *Parablennius pilicornis* (Cuvier, 1829); the endangered species, the dusky grouper *Epinephelus marginatus* (Lowe, 1834) included in the IUCN Red List; the rare species, the black brotula *Grammonus ater* (Risso, 1810), and some uncommon species such as the golden coral shrimp, *Stenopus spinosus* (Risso, 1826) and the spotted bumblebee shrimp, *Gnatophillum elegans* (Risso, 1816). Species of economic and medical interest were also recorded. In a DNA barcoding approach, Neighbour Joining (NJ) phylogenetic tree of 25 mitochondrial cytochrome oxidase subunit I species sequences, indicates that COI gene is suitable for an unambiguous identification. This first geological and biological attempt at the Marine Area of the "*Grotta del Maresciallo*" provides useful indications to focus future investigations, and may become a potential management tool for local administrations to protect these habitats.

Keywords: *Grotta del Maresciallo cave*; Marine areas; Preservation; Visual census; COI barcodes; Marine drug; *Epinephelus marginatus*

Introduction

The marine areas in a Park are geographically individuated and defined, legally protected due to their environmental, cultural and economic value [1]. In presence of natural monuments, such as caves inside the Park, the use or the public entrance in such areas are regulated by the Park plan, which provides limitations and constraints, to protect the particular sensible habitats and most vulnerable species [2]. The cave system, in fact, could be highly affected by external pollutants, such as ballast water, bilge water, wastewater discharges, and by hydrocarbons and poisonous substances present in antifouling paints, used to treat the hulls of the ships, or by the global warming [2-4]. Still there can be a biological pollution also, due to new thermophilic and/or tropical species [5-7]. For these reasons a continuous monitoring action is necessary to provide information about the costal structure, sea level changes, spatio-temporal species in the caves performed by a census made and supported by professional divers and molecular techniques [8-12]. In this optic the census and the identification represent an important step to establish the abundance/rarity degree of the marinea species [13,14], as well to detect possibly cryptic species and even to describe new species [15-17]. The newest EU political actions regarding maritime strategic objectives, such as the Marine Strategy Framework Directive (MSFD), and the European Strategy for Marine and Maritime Research [18], represent a push to draw up an inventory of the alien species in the Mediterranean sea. The biodiversity and community structure in the north-western and central Mediterranean caves have been widely studied, while still few information are available for the 738 marine caves in the eastern Mediterranean [7,19,20]. However, the marine cave biodiversity list was also checked for the presence of alien species [12] in relation to their worst effect [21]. As reported in the inventory by Darling and Blum [22], organism morphology for micro- and meio-fauna can require the expertise of multiple taxonomists for complex communities, thus significantly elevating costs. In addition, depending on the taxa under investigation, availability of taxonomic expertise may be limited or altogether absent [23,24]. Typically, such considerations force researchers to base biodiversity estimates on identifications to family level or to "morphospecies". Furthermore, the accuracy of morphological identification is severely attenuated by the requirements of invasive species monitoring: the difficulty of identifying early life history stages (eggs and larvae) by morphological criteria is well known, and yet recognition of these stages is crucially important to the task of tracking invasions [25]. To help non-experts

in the identification process, developing a unifying identification system for animal species, an universal marker called "DNA barcode" has been used in the last decade [26,27]. This DNA barcode is the sequence of the "Folmer fragment" [28], a polymorphic part of the mitochondrial Cytochrome Oxidase subunit I gene (COI), which can be used to identify closely related species as well as higher taxa in many animal phyla. Additional studies have shown that genetic identification by "COI barcodes" can provide a useful tool to detect possibly cryptic species, to describe new species and to discuss evolutionary implications [15]. The "*Grotta del Maresciallo*" cave, even if it's less famous than the neighboring ones (such as the "*Grotta del Turco*"), presents a high variety of species, that's why the objectives of this study have been to describe relative spatial patterns of quantitative distribution and relative abundance of species; to calculate the percentage contribution of a single group to the total Mediterranean marine cave diversity, to verify the possibility to identify species by using barcoding as complementary taxonomy. This census, will serve as baseline data for future comparisons.

Material and Methods

Study area

Marine area of *Riviera di Ulisse* Regional Park and the "*Grotta del Maresciallo*" cave: Underwater surveys were carried out from March 2013 in the Marine Area of *Riviera di Ulisse* caves (central Italy, LT Italy; Figure 1). The regional Park *Riviera di Ulisse* has been established by the Lazio Region with the law no. 2 of February 6, 2003 as an instrumental entity with the task of manage three regional protected areas, included in the cities of Minturno, Formia, Gaeta and Sperlonga: the Gianola Park and Mountain of Scauri, the natural monument 'Promontorio Villa di Tiberio and Costa Torre Capovento-Punta Cetarola', and the Regional Urban Park of Monte Orlando. By applying zonation parameters [29], the marine protected area of Monte Orlando was separated in four sectors: the Piloni, the Cigliatina, the Franata at the "Montagna Spaccata". The "*Grotta del Maresciallo*" cave (lat. 41° 12' 17"N; lon.13° 34' 17" E), placed close to the cliff of the Montagna Spaccata, is a linear cave, hidden in the rock and parallel to the Grotta del Turco cave. The entrance of the cave is visible on the surface as a thin creek which, going under and straight to the bottom, widen in a large chamber (Figure 1a-c).

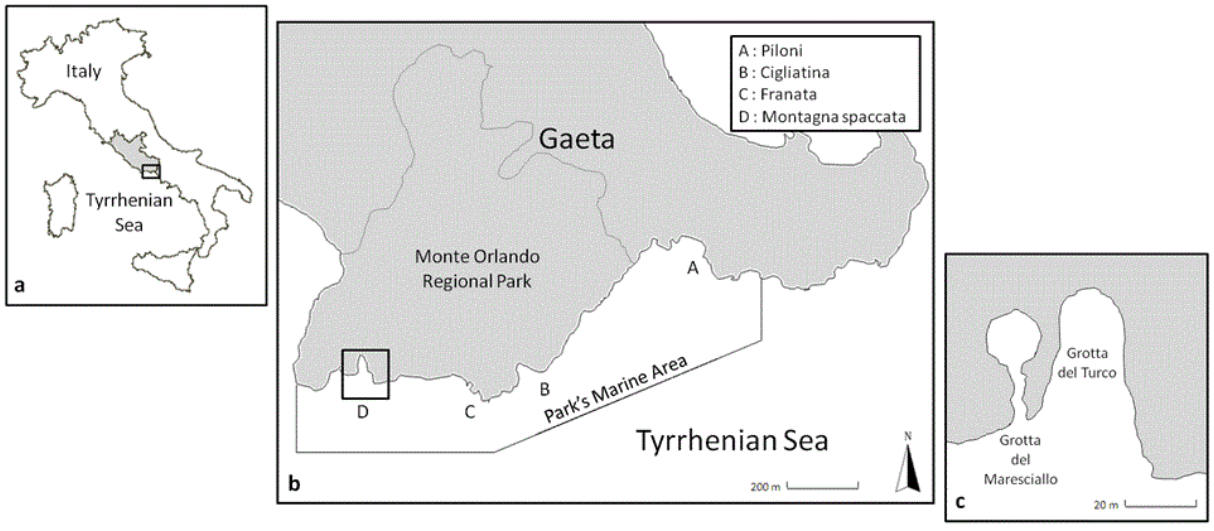

Figure 1: Geographical framework of the Marine Area of Riviera di Ulisse Regional Park, at the Latium Italian Region (a); its areas in the Tyrrhenian Sea (b); site of caves: *Grotta del Maresciallo* and *Grotta del Turco* (c).

Field studies

Cave exploration: The investigated cave was blind or with several openings and varied remarkably in terms of morphology and length, presence/absence of the ceiling and characteristics of the bottom (e.g. rocky vs muddy); data were reported by specialized cave diving techniques before census and sampling.

Census and Sampling: A detailed survey of the distribution and abundance of species in a range of habitats and locations using visual census techniques was done. The followed morphological classification was in agreement with that proposed in Systema Porifera [30], the World Porifera Database, WPD [31], the World Register of Marine Species, WoRMS [32] and the Integrated Taxonomic Information System (http://www.itis.gov) and in numerous and specific text and manuscripts Table 1 e.g. for Bryozoan, Chimenz Gusso et al., [33].

Data analyses: Analysis of biodiversity index was used to assess quantitative distribution and relative abundance of species. Species richness was expressed by considering the number of species (D), and species diversity, but homogeneity were determined using the Shannon-Wiener (SW) diversity index (H') and the Evenness index (J') [34]. The presence of Porifera, Cnidaria and Bryozoa was reported in square meters (0.5 to 3 mq), and the colonies counted as single individuals (see Table 1, relative abundance) following Loya [35].

These parameters were calculated for each site (outside and inside the cave) by pooling data from the sample replicates. The percentage contribution of each group to the total Mediterranean marine cave diversity was calculated following Gerovasileiou & Voultsiadou [36].

Molecular identification

Sampling and DNA Extraction: In order to examine the resolution power of the molecular markers in species delineation and to account for intraspecific sequence variation, avoiding any misleading results, DNA barcoding studies in samples (100mg of each specie) whose COI sequence of which is already reported sequence in GenBank (see Table 1) were performed thanks to SCUBA divers specimens collection through non-destructive methods. For the species belonging to the Actinopterygii family (Pisces) the DNA extraction was performed as reported in Di Finizio et al., [37] with a phenol/chloroform standard method by using autoclaved glassware and equipment. About 100 mg of ground freeze dried tissues were mixed in a DNA extraction buffer (50 mM NaCl, 10 mM EDTA and 10 mM Tris base) and the cells were lysed by adding 2% sodium dodecyl sulfate. The RNA was removed by adding RNAse (10 mg/mL) followed by incubation at 37°C for 30min. Proteinase K was added (0,5 mg/mL) to remove protein and the samples were incubated for 1h 37°C in a shaking water bath. The extracts were further purified by extracting twice with phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and by centrifuging at 10,000×g for 15 min at 4°C. The upper aqueous layer was transferred into a new micro-centrifuge tube and the DNA was precipitate by adding 1/10th volume of 3 M sodium acetate at pH 5.2 and two volumes of 100% chilled ethanol to each sample and mixed centrifuged at 15,000×g for 30 min at 4°C. The pellet was washed with 70% ethanol, air dried, and finally re-suspended in 50 µl sterilized deionized water. Optical density (OD) of each sample was measured at 260 and 280 nm respectively, by UV-spectrophotometer (Biochrom Libra S12), and the purity of DNA was measured by the OD260/OD280 ratio (ideal ratio = 1.7–2.0), and the quality by electrophoresis on a 0.8% agarose gel and visualized under UV light [37].

Total DNA of samples belonging to all other taxa was extracted using Qiagen DNeasy mini kits according to the manufacturer's instructions. For the initial tissue homogenization, a cube of tissue approximately 3 mm3 in size was ground. After initial digestion a centrifugation step was added e.g. to remove spicules, in case of sponges, prior to using the lilac Qiashredder Mini Spin column.

DNA Barcoding: DNA Barcoding Sequences of 25 species were obtained to compare the applicability of the COI genes as markers for DNA barcoding. PCR amplification was performed as previously published [37] using the following primers: COI_UP (5'-ACTTCAGGGTGACCGAAGAATCAGAA-3') and COI_DW (5'-ATCTTTGGTGCATGAGCAGGAATAGT-3') [38] for the species belonging to the Actinopterygii family. For all other species standard primers [28] were used. PCR reaction was performed in a Techgene Thermal Cycler (Thecne Ltd., Cambridge, UK). Thirty-five cycles of amplification were carried out in a reaction buffer containing 50 mM KCl, 10 mM Tris/HCl, pH 9.0; 10 mM NaCl; 0.01 mM EDTA; 2.5 mM of each dNTP; 1 µM of each primer; 10 ng of template DNA; 0.5 unit of Taq DNA polymerase (Invitrogen, Milan, Italy). PCR amplification for COI Actinopterygii species conditions were as follows: denaturation for 50 seconds at 94°C; annealing for 50 seconds at 54°C, and extension for 1 minute at 72°C; while for other species amplification condition were: denaturation for 1 minute at 95°C; annealing for 1 minute at 40°C, and extension for 1 and a half minutes at 72°C, followed by a final extension step at 72°C for seven minutes. At the end of the incubation 5 µl of PCR products were separated by electrophoresis through 2% agarose gel and visualized under UV light. A 100 bp ladder (Invitrogen, Milan, Italy) was used to estimate the fragment size of the amplicons generated. Amplified DNA was desalted with Microcon 100 spin columns (Millipore-Amicon, Belford, MA, USA) according to the manufacturer's instructions and sequenced using Big Dye TM Terminator Cycle Sequencing Chemistry (Applied Biosystems, Foster City, CA, USA) in an automatic capillarity sequencer (ABI 310 Genetic Analizer; Applied Biosystems).

Multiple alignments of these orthologous sequences were performed with the programme Clustal W [39] as implemented in BioEdit (version 7.0.4.1), [40] to ensure that all sequences of COI marker gene provide a homologous fragment. COI sequences were translated into amino acids with the online program ExPASy translation tool (http://web.expasy.org/translate/) in order to exclude sequencing errors and to avoid the inclusion of pseudogene sequences in the datasets. Neighbour Joining (NJ) trees were constructed and genetic distance was calculated within species [41]. Since the aim of this task was to identify species using barcodes, phylogenetic trees were constructed without selecting a priori an evolutionary model appropriate for the dataset.

Results

General description of study area

The "*Grotta del Maresciallo*" cave is a completely submerged tunnel, 32 meters long and 2.5 meters wide, and culminates in a circular chamber completely dark, with little stalactites and stalagmites, which can form only in air (data not shown). The water depth varies depending on the state of erosion of the sediment: typically is around 7 meters, however, is reduced in the vicinity of the chamber, which features on the bottom sediment and rock bass. Light and hydro-dynamism are reported in Figure 2 and in Table 1 in relation to the important parameters that affect the biocenosis of the cave.

General distribution, abundance of species and taxa Mediterranean biodiversity percentage: Common name of fish species in the entrance and inside the "*Grotta del Maresciallo*", their distribution as the site physical conditions detected are reported in Tab.1. Furthermore, the morphological reference and GenBank accession number of species barcode sequences, informative for discrimination, scientific name and taxonomy of the *Grotta del Maresciallo* fauna are also included. The total number of species detected is 47; they belong to 46 families and 12 different classes. Abundance and richness of species change from the semidark zone, zone 1, to the completely dark one, zone 2 (Table 2). The determinations show that in zone 1 there is an intermediate situation regarding the species richness; a fair distribution of individuals in the community is valuable, thus not one species is dominant compared to the others; even if the biodiversity is low there is a wide variety of species in relation to the small size of the area considered.

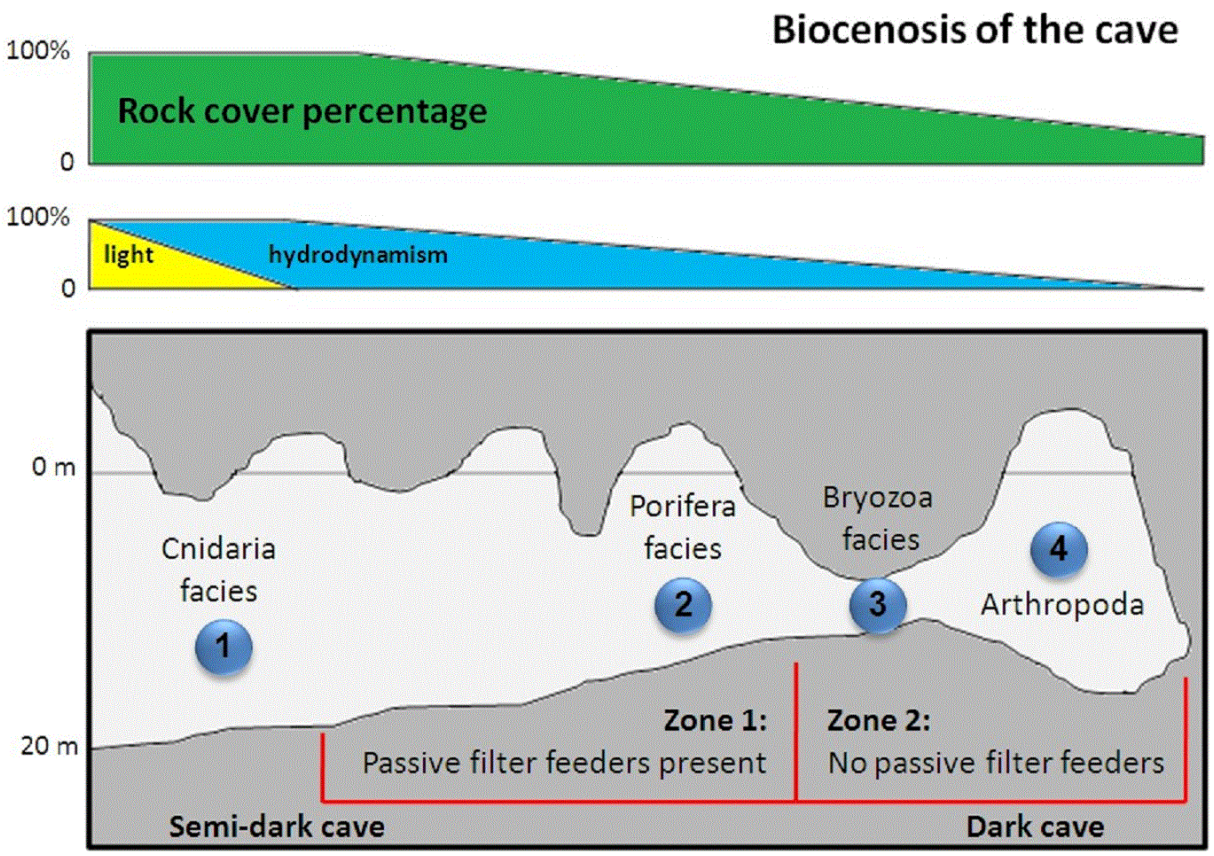

Figure 2: Drawing of "*Grotta del Maresciallo*": two parameters, light and hydro-dynamism affecting the biocenosis of the cave (zone 1 and 2). In green is evidenced the rock cover, which is maximum in the zone 1, composed mainly of sessile organisms. The light, such as the hydrodynamism, are maximum at the zone 1, to completely disappear in zone 2. Schematic indication of fauna detected.

Location	Luminosity	Species Common Name	R.a.	Number of Specimens Detected	Taxonomical Reference	COI GenBank Accession Number	Identified Species	Specie Taxonomy (Phylum, Subphylum, Class, Order, Family)
Grotta del Maresciallo zone 1 (a)	d	Bluish encrusting sponge	h	1 (2 mq)	Cerrano, 2004	n.d.	*Phorbas tenacior* (Topsent, 1925)	Porifera,Cellularia, Demospongiae, Poecilosclerida, Hymedesmiidae
	d	Yellow network sponge	h	15	Hooper, 2002	n.d.	*Clathrina clathrus* (Schmidt, 1864)	Porifera, Celluaria, Calcarea, Clathrinida, Clathrinidae
	d	Orange lumpy sponge	h	5	Díaz, 2015	HQ379408	*Acanthella acuta* (Schmidt, 1862)	Porifera, Celluaria, Demospongiae, Halichondrida, Dictyonellidae
	d	Orange lobed sponge	h	5	Cerrano, 2004	JX999060	*Agelas oroides* (Schmidt, 1864)	Porifera, Celluaria, Demospongiae, Agelasida, Agelasidae
	d	Orange-red encrusting sponge	h	7	Mojetta, 2003	JX999091	*Crambe crambe* (Schmidt, 1862)	Porifera, Celluaria, Demospongiae, Poecilosclerida, Crambeidae

	d	Kidney-shaped sponge	h	25	Cerrano, 2004	JX999074	*Chondrosia reniformis* (Nardo, 1847)	Porifera, Celluaria, Demospongiae, Chondrosida, Chondrillidae
	d	Stony sponge	h	1 (3 mq)	Cerrano, 2004	JX999088	*Petrosia ficiformis* (Poiret, 1789)	Porifera, Celluaria, Demospongiae, Haplosclerida, Petrosiidae
	d	Encrusting orange sponge	h	20	Mojetta, 2003	n.d.	*Spirastrella cunctatrix* (Schmidt, 1868)	Porifera, Celluaria, Demospongiae, Hadromerida, Spirastrellidae
	d	Bath Sponge	h	15	Cerrano, 2004	HQ830364	*Spongia officinalis* (Linnaeus, 1759)	Porifera, Celluaria, Demospongiae, Dictyoceratida, Spongiidae
	d	Carnaccia	h	20	Mojetta, 2003	JQ082796	*Scalarispongia scalaris* (Schmidt, 1862)	Porifera, Celluaria, Demospongiae, Dictyoceratida, Thorectidae
	d	Star Coral	l	1 (1 mq)	Trainito, 2004	JQ343192	*Astroides calycularis* (Pallas, 1766)	Cnidaria, Anthozoaria, Anthozoa, Scleractinia, Dendrophylliidae
	d	Yellow encrusting anemone	m	1 (2 mq)	Trainito, 2004	EF672659	*Parazoanthus axinellae* (Schmidt, 1862)	Cnidaria, Anthozoaria, Anthozoa, Zoantharia, Parazoanthidae
	d	False coral	h	1 (2 mq)	Chimenz, 2004	n.d.	*Myriapora truncata* (Pallas, 1766)	Bryozoa, Gymnolaemata, Cheilostomatida, Myriozoidae
	d	Ross coral	m	1 (0,5m)	Chimenz, 2004	n.d.	*Pentapora fascialis* (Pallas, 1766)	Bryozoa, Gymnolaemata, Cheilostomatida, Bitectiporidae
	d	Neptune's lace	m	10	Chimenz, 2004	FJ196084	*Reteporella grimaldii* (Jullien, 1903)	Bryozoa, Gymnolaemata, Cheilostomatida, Phidoloporidae
	d – t.d	Leopard sea slug	a	15	Trainito, 2005	AF120637	*Discodoris atromaculata* (Bergh, 1880)	Mollusca, Conchifera, Gastropoda, Nudibranchia, Discodoridinae
	d – t.d	Giant doris	l	5	Trainito, 2005	LN715204	*Felimare picta* (Schultz in Philippi, 1836)	Mollusca, Conchifera, Gastropoda, Nudibranchia, Chromodorididae
	d – t.d	Mediterranean violet aeolid	a	5	Trainito, 2005	HQ616753	*Flabellina affinis* (Gmelin, 1791)	Mollusca, Conchifera, Gastropoda, Nudibranchia, Flabellinidae
	d – t.d	Red-mounthed rock shell	m	25	Gofas, 2001	FR695839	*Stramonita haemastoma* (Linnaeus, 1767)	Mollusca, Conchifera, Gastropoda, Neogastropoda, Muricidae
	d – t.d	Lurid cowry	l	5	Repetto, 2005	AY161695	*Luria lurida* (Linnaeus, 1758)	Mollusca, Conchifera, Gastropoda, Littorinimorpha, Cypraeidae

	d	Noah ark shell	h	50	Doneddu, 2005	n.d.	*Arca noae* (Linnaeus, 1758)	Mollusca, Conchifera, Bivalvia, Arcoida, Arcidae
	d – t.d	Broad lobster	l	20	Trainito, 2004	KC789473	*Scyllarus arctus* (Linnaeus, 1758)	Arthropoda, Crustacea, Malacostraca, Decapoda, Scyllaridae
	d	Red starfish	h	10	Trainito, 2004	GU330217	*Echinaster sepositus* (Retzius, 1783)	Echinodermata, Eleutherozoa, Asteroidea, Spinulosida, Echinasteridae
	d	Purple seastar	h	10	Mojetta, 2003	n.d.	*Ophidiaster ophidianus* (Lamarck, 1816)	Echinodermata, Eleutherozoa, Asteroidea, Valvatida, Ophidiasteridae
	d	Spiny starfish	h	7	Hansson, 2001	n.d.	*Marthasterias glacialis* (Linnaeus, 1758)	Echinodermata, Eleutherozoa, Asteroidea, Forcipulatida, Asteriidae
	d	Brown brittle star	h	5	Stöhr, 2009	n.d.	*Ophioderma longicauda* (Bruzelius, 1805)	Echinodermata, Eleutherozoa, Ophiuroidea, Ophiurida, Ophiodermatidae
	d	Feather star	h	3	Mojetta, 2003	KC626517	*Antedon mediterranea* (Lamarck, 1816)	Echinodermata, Crinozoa, Crinoidea, Comatulida, Antedonidae
	d	Black sea urchin	h	20	Hansson, 2001	JQ745256	*Arbacia lixula* (Linnaeus, 1758)	Echinodermata, Echinozoa, Echinoidea, Arbacioida, Arbaciidae
	d	Common sea urchin	h	20	Trainito, 2004	EF462949	*Paracentrotus lividus* (Lamarck, 1816)	Echinodermata, Echinozoa, Echinoidea, Camarodonta, Parechinidae
	d	Light bulb sea squirt	m	7	Cerrano, 2004	AY603104	*Clavelina lepadiformis* (Müller, 1776)	Chordata, Tunicata, Ascidiacea, Enterogona, Clavelinidae
	d	Red sea-squirt	h	10	Cerrano, 2004	n.d.	*Halocynthia papillosa* (Gunnerus, 1765)	Chordata, Tunicata, Ascidiacea, Pleurogona, Pyuridae
	d – t.d	Red scorpionfish	h	15	Louisy, 2006	KJ768308	*Scorpaena notate* (Linnaeus, 1758)	Chordata, Vertebrata, Actinopterygii, Scorpaeniformes, Scorpaenidae
	d	Ringneck blenny	m	6	Louisy, 2006	n.d.	*Parablennius pilicornis* (Cuvier, 1829)	Chordata, Vertebrata, Actinopterygii, Perciformes, Blenniidae
	d	Mediterranean moray	h	5	Mojetta, 2003	KJ768264	*Muraena helena* (Linnaeus, 1758)	Chordata, Vertebrata, Actinopterygii, Anguilliformes, Muraenidae
Grotta del Maresciallo zone 2 (b)	t.d	Common prawn	h	50	Howson, 1997	n.d.	*Palaemon serratus* (Pennant, 1777)	Arthropoda, Crustacea, Malacostraca, Decapoda, Palaemonidae

	t.d	Narwal shrimp	m	50	Holthius, 1980	n.d.	*Parapandalus narval* (Fabricius, 1787)	Arthropoda, Crustacea, Malacostraca, Decapoda, Pandaloidea
	t.d	Spotted bumblebee shrimp	l	3	Trainito, 2004	n.d.	*Gnathophyllum elegans* (Risso, 1816)	Arthropoda, Crustacea, Malacostraca, Decapoda, Gnathophyllidae
	t.d	Golden coral shrimp	l	20	Trainito, 2004	n.d.	*Stenopus spinosus* (Risso, 1826)	Arthropoda, Crustacea, Malacostraca, Decapoda, Stenopodidae
	t.d	Spider crab	m	10	Türkay, 2001	n.d.	*Herbstia condyliata* (Fabricius, 1787)	Arthropoda, Crustacea, Malacostraca, Decapoda, Epialtidae
	t.d	Sponge crab	l	4	Peter, 2008	n.d.	*Dromia personata* (Linnaeus, 1758)	Arthropoda, Crustacea, Malacostraca, Decapoda, Dromiidae
	d – t.d	Cardinal fish	h	35	Louisy, 2006	n.d.	*Apogon imberbis* (Linnaeus, 1758)	Chordata, Vertebrata, Actinopterygii, Perciformes, Apogonidae
	t.d	Forkbeard	m	10	Louisy, 2006	KJ768279	*Phycis phycis* (Linnaeus, 1766)	Chordata, Vertebrata, Actinopterygii, Gadiformes, Phycidae
	t.d	Black brotula	l+	3	Louisy, 2006	n.d.	*Grammonus ater* (Risso, 1810)	Chordata, Vertebrata, Actinopterygii, Ophidiiformes, Bythitidae
	t.d	European conger	m	3	Louisy, 2006	KJ709742	*Conger conger* (Linnaeus, 1758)	Chordata, Vertebrata, Actinopterygii, Anguilliformes, Congridae
	d – t.d	Brown meagre	m	10	Louisy, 2006	n.d.	*Sciaena umbra* (Linnaeus, 1758)	Chordata, Vertebrata, Actinopterygii, Perciformes, Sciaenidae
	d – t.d	Dusky grouper	l	1	Louisy, 2006	KC500692	*Epinephelus marginatus* (Lowe, 1834)	Chordata, Vertebrata, Actinopterygii, Perciformes, Serranidae
	d – t.d	Goldblotch grouper	l	1	Louisy, 2006	JX456389	*Epinephelus costae* (Steindachner, 1878)	Chordata, Vertebrata, Actinopterygii, Perciformes, Serranidae

Table 1: Site physical conditions and species with their abundance, morphological reference and GenBank accession number of their barcode sequences for species discrimination, scientific name and taxonomy of the Grotta del Maresciallo fauna. a. idrodynamisms present, clear or muddy water, depending on the conditions of the sea; b. idrodynamisms absent, water is generally clear in calm sea conditions; t.d. total darkness; d. dimness; r.a., relative abundance: h, high; m, medium; l, lower; n.d., not detected.

Inside the cave, zone 2, the indexes show that a slight inequality abundance of some species respect to others can be registered, that's why there is a little dominance of some species than others in the community; low values of the SW index mean that the division within the community is biased in favor of one species. The biodiversity is low as in zone 1 but there is less variety of species too due to the special conditions inside the cave, as the absence of light and recycling water, so that few species are able to adapt and survive. Furthermore the data obtained allowed to calculate the taxa percentage contribution of the "*Grotta del Maresciallo*" biodiversity (Figure 3). The data of "*Grotta del Maresciallo*" cave (shown in yellow) results to be greater for some taxa (Chordata, Echinodermata, Arthropoda) in relation to the biodiversity found in caves of the central Mediterranean sea (shown in green), and in general in all the caves of the Mediterranean.

"*Grotta DEL MARESCIALLO*" **Biodiversity index ES**		
	zone 1	zone 2
Tot. number of species S	34	13
Tot. number of specimens N	371	200
Shannon-Wiener index H'	3.161	1.997
Simpson index D	0.948	0.829
Evenness index J	0.534	0.377
Berger-Parker d	0.134	0.250
Maraglef Dm	5.577	2.264

Table 2: "*Grotta del Maresciallo*" (zone 1 and 2) biodiversity indexes.

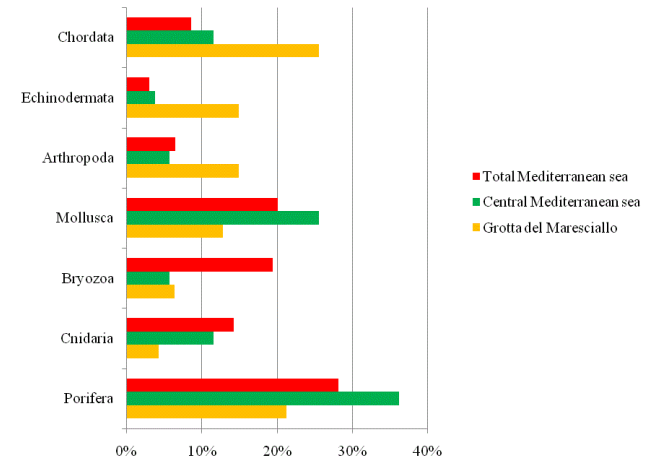

Figure 3: Contribution (%) of marine Mediterranean cave biodiversity including data from "Grotta del Maresciallo" (modified from ref. 12).

Habitat distribution and species characteristics: Immediately before the entrance, on the left, there is a wall that descends vertically onto the bottom, filled with an abundant "sessile" fauna, such as sponges, madrepores and briozoa, organisms with a plagiotropic growth. Following Tab. 1 we can see the many species of sponges, which completely cover the bedrock, gives home to some shellfish nudibranchs, in particular the Mediterranean violet aeolid, *Flabellina affinis* (Gmelin, 1791) and the leopard sea slug, *Discodoris atromaculata* (Bergh, 1880), the latter permanently attached to the stony sponge, *Petrosia ficiformis* (Poiret, 1789) (Figure 4 c). The organisms that live at the entrance of the "*Grotta del Maresciallo*" cave, zone 1, have availability to a sufficient amount of food, since the sea currents carry large amounts of plankton and organic matter. Moreover, as the rate of low irradiance light decrease, there is no competition to win brighter areas, because all organisms prefer the semi-darkness. The competition is established primarily for the conquest of space on the substrate. The sponges, in particular those encrusting as *Agelas oroides* (Schmidt, 1864), have maximum success. They expand horizontally forming large stains. Other sponges, instead, use sessile organisms of different species as a substrate on which taking root and grow, thus becoming epibiontic organisms (e.g. *Parazoanthus axinellae*, Schmidt, 1862) on encrusting sponges. In this area are not observed photophilous algae, due to the low rate of light, but sciaphilous algae are present, such as red algae, including those belonging to the genus *Peyssonelia* and to the family of coralline known for the calcified thallus (containing calcium carbonate), and some species of green algae such as *Halimeda tuna* and *Palmophyllum crissum*, (data not shown). A few species pictures indicative of the representative taxa of the cave examined (for site location, see Tab. 1) are reported in Figure 4 (a-m) the orange lumpy sponge, *Acanthella acuta*, which colonies rocky walls at the entrance of the cave; the star coral *Astroides calycularis*, gathering of several individuals; the leopard sea slug, *Discodoris atromaculata*, lying on the stony sponge *Petrosia ficiformis*; the common prawn, *Palaemon serratus*, present in large populations on the seabed of the dark cave; the spotted bumblebee shrimp, *Gnatophillum elegans*, hidden among the algae; the golden coral shrimp *Stenopus spinosus*, placed at the bottom of the cave, in a completely dark site; the narwal shrimp, *Parapandalus narval*, swimming in group; the goldblotch grouper, *Epinephelus costae*, hidden on the seabed, between the rocks; the dusky grouper, *Epinephelus marginatus*, always present in the caves; the european conger, *Conger conger*, holed up in a crevice of the rock at the entrance of the chamber; the brown meagre, *Sciaena umbra*, cave-dwelling and solitary species; the black brotula, *Grammonus ater*, a rare species living in the darkest part of the cave. For about a third of its length, the cave makes a curve that leads to a circular chamber, zone 2, where there is total darkness. The water here form a kind of 'pond', while on the right wall, some faint ray of light penetrates through two slits communicating with the outside, which also make the air breathable. The bottom of the cave consists of sediment and rocks. Among them, some croakers and musdee hide, fish that are known to live in dark environment. There are also plenty of shrimp, including the very common bait shrimp, *Palaemon serratus* (Pennant, 1777), shrimp saw *Parapandalus narval* (Fabricius, 1787) and coral shrimp, *Stenopus spinosus* (Risso, 1826). A higher number of species was usually associated to cave walls compared to ceilings or bottoms. The complete darkness in the innermost cave portions host the strictly cave-dwelling fish, *Grammonus ater* (Risso, 1810) rare and typically benthic species.

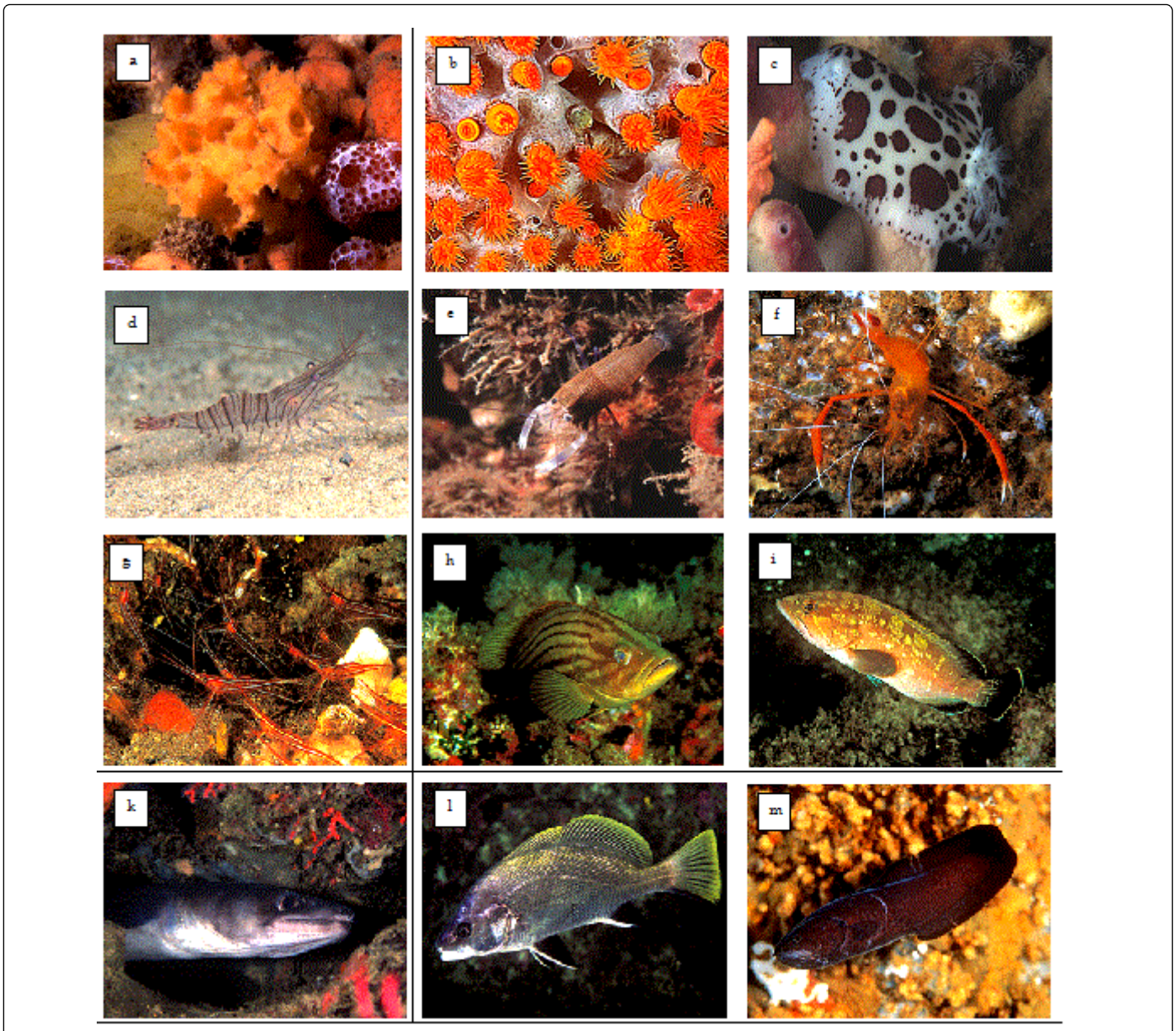

Figure 4: The main species in the "Grotta del Maresciallo". a) *Acanthella acuta*; b) *Astroides calycularis*; c) *Discodoris atromaculata*; d) *Palaemon serratus*; e) *Gnatophillum elegans*; f) *Stenopus spinosus*; g) Parapandalus narval; h) *Epinephelus costae*; i) *Epinephelus marginatus*; k) *Conger conger*; l) *Sciaena umbra*; m) *Grammonus ater*.

A total of 25 species collected were analyzed, thus a corresponding set of COI (532-659bp) sequences was obtained, and confirm the sequences reported in Tab. 1, available at the GenBank data base (www.ncbi.nlm.nih.gov/genbank/). No stop codons, insertions, and deletions were observed in the mitochondrial cytochrome oxidase subunit I (COI) gene of species from the "*Grotta del Maresciallo*" cave, indicating that they represent fragments of functional mitochondrial genes and not nuclear mitochondrial pseudogenes (data not shown). The data were then used for the construction of a phylogenetic tree reported in Figure 5, that estimate the divergence between the several species. In our study results evident that the "*Grotta del Maresciallo*" species examined have genetic distance each other and tight cluster for all samples belonging to the class Actinopterygii, as for Arthropoda, Mollusca and Cnidaria Phyla. See e.g. in the three Mollusca *Felimare picta*, *Discodoris atromaculata* and *Flabellina affinis* that formed a close cluster, such as Porifera, with the Cnidaria *Astroides calycularis* and *Parazoanthus axinellae*, which exhibited similar close phylogenetic relationship between *Agelas oroides, Petrosia ficiformis* and *Acanthella acuta*.

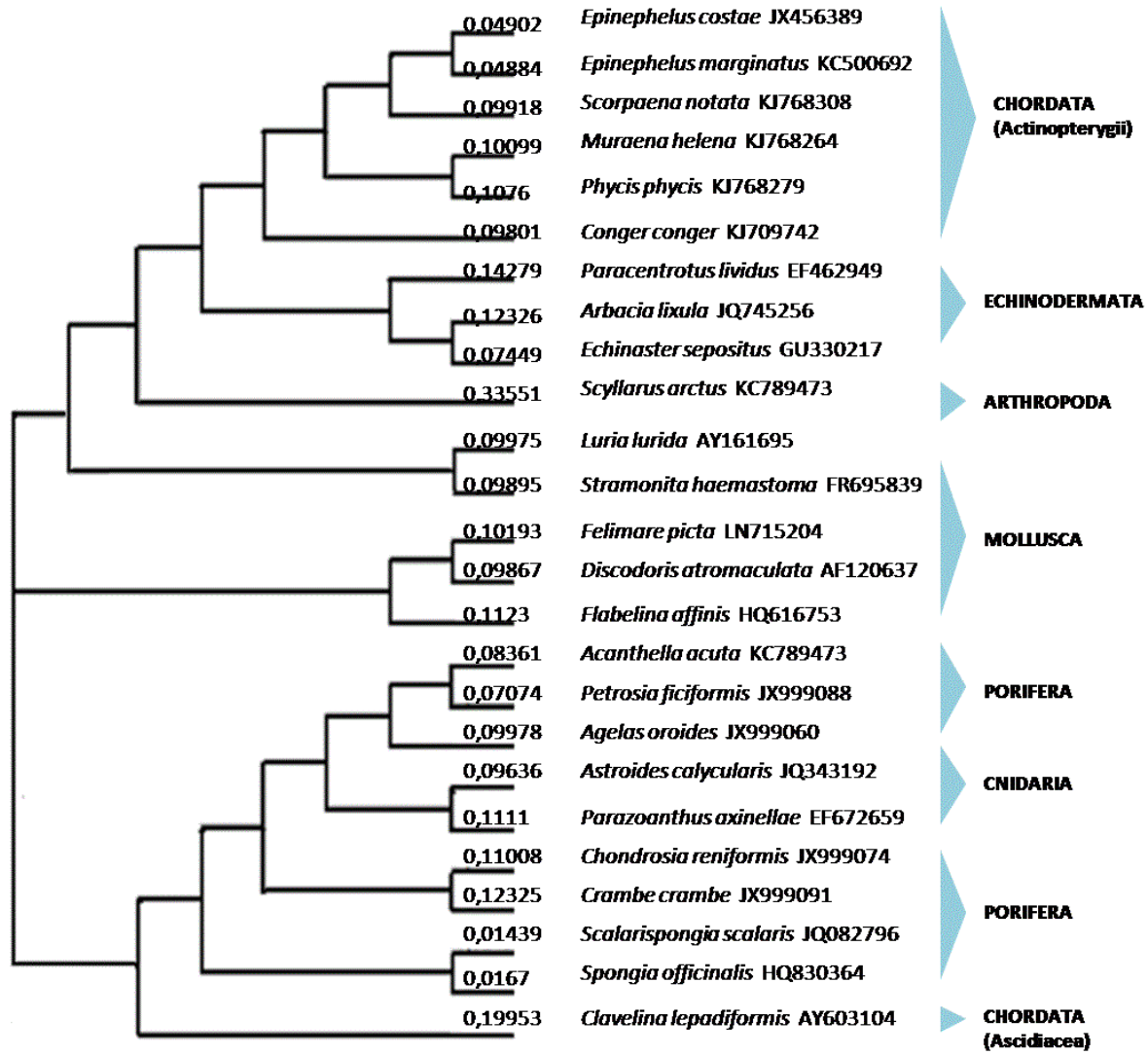

Figure 5: Neighbour Joining tree for partial sequences of the mitochondrial cytochrome oxidase subunit I (COI)gene of species from the "Grotta del Maresciallo" cave. Species names and GenBank accession numbers are given at branch tips. Numbers indicates genetic distances.

Discussion

During the course of the current biological studies, several significant geological data (depth, light and hydrodynamism) and Mediterranean comparative phenomena too have been recorded by scuba divers of our equipè for the cave examined named "*Grotta del Maresciallo*", one of the eight cave at Marine Area of *Riviera di Ulisse* in Italy. See, for example the component of the rocky bottoms that are carbonate rocks, as reported in other marine caves in many coastal marine areas of the Mediterranean environment for karstic processes [8] and submerged little stalactites and stalagmites, which are known to form only in air. The development of specialized cave diving techniques and equipment used has provided a mean to gain access to the submerged cave as our small cave examined. The visual census technique carried out was focus on the identification of marine cave species for their protection, according to the current Habitat Directive including the Marine Strategy Framework Directive and the Barcelona Convention, the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) as the Intergovernmental Panel on Climate Change (IPCC) [1,2,19]. The used approach and methods have proved to be the most efficient to assess species abundance and diversity overview, because of their minimal impact. To get a better description of species diversity, a measure of diversity indices, species richness and evenness of their distribution at the entrance and inside the "*Grotta del Maresciallo*" cave were undertaken too. Examination of the fauna from the cave recorded 47 species. They belong to 46 families and 12 different classes and no one species is dominant but the Margalef index indicates that the number of species is very high compared to the specimens examined. The low evenness and the variation in richness between zone 1:semi-dark and zone 2: dark cave (Table 1) could reflect the not negligible variability among the investigated cave in terms e.g. of morphology, length and presence/absence of light in the innermost portions. Indeed, the calculated taxa

percentage contribution of the "*Grotta del Maresciallo*" biodiversity integrates the Mediterranean's evaluation already calculated and reported in literature [36], showing the taxa of "*Grotta del Maresciallo*" result to be copius for Chordata, Echinodermata, Arthropoda in relation to the biodiversity found in caves of the central Mediterranean sea (shown in green), and in general in all the caves of the Mediterranean [12]. The presence of abundant sponges (see Table 1) on overhanging ledges in these caves, but not observed elsewhere, indicates that even in deep waters, caves offer a preferred biological niche for some animals. Moreover, marine caves as the zone 1 and 2 of the "*Grotta del Maresciallo*" might be used for sponge culture, due to their limited sediment deposition and illumination [20]. Considering the importance to follow the biodiversity [42,43], the limitation due to organism morphology for micro- and meiofauna that require the high expertise and costs [23,24] and that differentiation of closely related species constitutes a challenging task not only for morphological, but genetic methods as well, the DNA barcoding studies are also included. In our study, a data set of 25 COI sequences from the "*Grotta del Maresciallo*" species was obtained and compared with ones already present in GenBank data. Our analyses indicate that the individuals examined, that were reliably assigned binomial names a priori, possessed distinct COI sequences. The use of molecular markers (such as COI) to identify cases of termophilic or alien invasions is a promising method that may resolve the status of some issues recorded in literature [26,27], especially thanks the genetic distance that can be present in cave's species, as in our case, where distinct clusters for vertebrate and invertebrate are evident. Our data confirmed the taxonomy of a widely distributed species, present in Marine Mediterranean cave e.g. the fish *Apogon imberbis*; the sponges *Agelas oroides*, *Petrosia ficiformis*, *Chondrosia reniformis* [9,12] and permits to identify species with great interest for their edibility as the fishes, *Scorpaena notate*, *Phycis phycis*, *Conger conger*, *Sciaena umbra*, *Epinephelus marginatus*, *Epinephelus costae*; the mollusca, *Stramonita haemastoma*, *Arca noae*; the *crustacea Scyllarus arctus*, *Palaemon serratus* and *Parapandalus narval*. Furthermore, we detected in the "*Grotta del Maresciallo*" cave a lot of species with various economic utility: against human breast cancer (MCF-7) and human neuroblastoma (SH-SY5Y) cell lines as for the spiny starfish, *Marthasterias glacialis* [44]; potential unexploited collagen source, easily obtainable as a food industry waste product, as for the edible sea urchin *Paracentrotus lividus* [45]; source of original natural substances belonging to two families of guanidine alkaloids, namely crambescins and crambescidins, which exhibit cytotoxic and antiviral activities, as for the orange-red encrusting sponge, *Crambe crambe* [46] and natural source of nanoparticles for the transdermal drug delivery of 17beta-estradiol-hemihydrate in hormone replacement therapy, as for the kidney-shaped sponge, *Chondrosia reniformis* [47]. Furthermore, in the "*Grotta del Maresciallo*" can be found species where were isolated actinobacterial strains and screened for antagonistic activity against various bacterial and fungal pathogens, as for the bath sponge, *Spongia officinalis* [48] and were isolated and purified acetylcholinesterase inhibitor isolated, as from the yellow encrusting anemone, *Parazoanthus axinellae* [49]. Known are the biological activities of the bacterial community associated with the bluish encrusting sponge, *Phorbas tenacior*; the antimicrobial 2-aminoimidazole alkaloid produced by the calcareous sponge, yellow network sponge, *Clathrina clathrus*; the antifungal effects of secondary metabolites isolated from the orange lumpy sponge, *Achantella acuta*; the effects of the orange lobed sponge, *Agelas oroides* and of the stony sponge *Petrosia ficiformis* crude extracts on human neuroblastoma cell survival; the tryptophan derivatives from the anthozoan, the star coral, *Astroides calycularis*. So, the combination of morphological and molecular data could be essential for accurately assessing the world's biodiversity indicating useful diagnostic morphological traits, informing needful revision, and flagging unseen species. Moreover, the BOLD system, which deposits barcodes, morphological, geographical and other data, has the potential as a convenient taxonomic platform [23] rapid, and accurate means of identifying specimens and assessing biodiversity and permitted to demonstrate the presence of two established thermophilic species, as *Astroides calycularis* and *Epinephelus costae* as the consequence of sea water temperature anomalies on a Mediterranean submarine cave ecosystem [4,50]; the tropical species *Parablennius pilicornis*, as biological pollution effect, the arrival already evidenced in the Mediterranean [18,51] and by the authors also in the Gaeta Gulf [17]; the rare species, *Grammonus ater*, and some uncommon species such as *Stenopus spinosus* and *Gnatophillum elegans* and the endangered species *Epinephelus marginatus*, included in the IUCN Red List (**http://www.iucnredlist.org**), encountered in several different marine caves review by [review in ref. 12,52,53] in the eastern Mediterranean. The use of DNA barcoding method is hindered by the fact that only a lowest percentage of the phyla have an associated barcode. So, for their richness, the caves can offer this opportunity to add new data in the panorama of mitochondrial sequence too (see our contribution at http://www.ncbi.nlm.nih.gov/genbank/, accession number KM606627, for *Sciaena umbra*; and KJ499110 for *Conger conger* from gametes and muscle specimens of the "*Grotta del Maresciallo*"). This first attempt to assess the state of knowledge on the submarine caves of *Riviera di Ulisse* marine area evidenced that the most important gap that should be filled in the near future concerns the biology and ecology of cave-dwelling communities. A detailed and updated knowledge on the submarine caves would provide the basic information for protection measures, to strengthen the science-policy interface for biodiversity and ecosystem services for the conservation and sustainable use of biodiversity, long-term human well-being and sustainable development especially for those caves that today are still outside the boundaries of marine protected areas and of sites of community interest.

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