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Conservation of marine biodiversity: biology, taxonomy, biogeography and sustainable development of target sponges

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INDEX

Summary (English/Italian) 3

Introduction 5

Chapter 1 13

Paper 1 14
Aquatic fauna and water parameters in an estuarine cave of eastern Sardinia

Abstract 1 59
How many sponges live in anchialine caves?

Abstract 2 63
Fauna bentonica di una grotta d'estuario: il caso della Grotta del Bue Marino (Golfo di Orosei)

Chapter 2 66

Paper 2 67
The hidden diversity of Mediterranean marine caves: A new species of *Protosuberites* (Porifera: Demospongiae: Suberitidae) and a first contribution to the phylogenetic relationships of the genus

Chapter 3 94

Paper 3 95
Spicular morpho-traits of the Mediterranean palaeoendemic genus *Petrobiona* Vacelet and Lévi, 1958 (Porifera: Calcarea) by traditional and geometric morphometric approach

Abstract 1 145
Petrobiona massiliana (Porifera, Calcarea): distribuzione e analisi morfometrica di alcune popolazioni del Mar Mediterraneo

Abstract 2 148
La Grotta Azzurra (Capri): Primi dati sul fouling dei Nymphaea

Abstract 3 151
Checklist of cave-dwelling Porifera from the Ligurian Sea.

Other abstract

Maricoltura di *Crambe crambe* nel Mar di Sardegna 156

Aknowledgements 159

Summary

The assessment of biodiversity is the basis to plan any activity of conservation.

This doctoral thesis aims to contribute to sponges global diversity assessment focusing on cryptic environments i.e. caves. A multidisciplinary approach, combining morphological, molecular, ecological and statistics techniques, attempts to solve current issues in taxonomy, biogeography and ecology of some taxa of Mediterranean Porifera with high conservation value. Other aims are to clarify the main adaptive strategies of some target taxa to colonize caves.

The geographic range and variation in size and shape of skeletal spicules of two cave-dweller species belonging to the Mediterranean endemic genus *Petrobiona* (Calcarea: Lithonida: Petrobionidae) are investigated using traditional and geometric morphometric approaches.

A checklist of sponge fauna from anchialine environments represents a contribution to the biological knowledge of extreme caves at global scale, and a multi-year study of cave-dwelling faunal assemblages (composition, structure and topographic distribution) focus on the Bue Marino Cave (Sardinia, Orosei Gulf) as model in relation to the physico-chemical water parameters, with notes on the adaptive strategies of sponges.

Integrative taxonomy by morphological and genetic approaches is performed to describe a new species from the Bue Marino Cave and to attempt a phylogenetic reconstruction of the genus *Protosuberites* using three different markers (COI, 18S rRNA, and 28S rRNA).

Key words: *cave-dwelling sponges, barcoding, coastal and marine caves, spatial distribution, adaptive strategies.*

Sommario

Valutare la biodiversità è la base per pianificare qualsiasi attività di conservazione.

Questa tesi di dottorato si propone di contribuire alla valutazione della diversità globale della fauna a poriferi, concentrandosi su ambienti criptici, quali le grotte. Tramite un approccio multidisciplinare, che unisce metodologie ecologiche, statistiche, morfologiche e molecolari, abbiamo tentato di risolvere problematiche attuali in campo tassonomico, biogeografico ed ecologico, per poriferi ad elevato valore conservazionistico. Altri obbiettivi sono stati quelli di chiarire le principali strategie adattative e di colonizzazione delle grotte, da parte di alcuni taxa.

Abbiamo analizzato la distribuzione geografica e le variazioni di forma e dimensioni delle spicole di due specie di grotta appartenenti al genere endemico del Mediterraneo *Petrobiona* (Lithonida: Petrobionidae), utilizzando un approccio morfometrico tradizionale e morfometrico geometrico.

Presentiamo una checklist mondiale della fauna a poriferi degli ambienti anchialini, per contribuire alla conoscenza biologica delle grotte, e uno studio pluriennale delle associazioni faunistiche (composizione, struttura e distribuzione topografica), focalizzato sulla grotta del Bue Marino, in relazione ai parametri fisico-chimici dell'acqua, con note sulle strategie adattative delle spugne.

Abbiamo utilizzato un approccio morfologico e genetico per descrivere una nuova specie presente nella Grotta del Bue Marino e per ricostruire la filogenesi del genere *Protosuberites,* attraverso l'utilizzo di tre diversi *markers* molecolari (COI, 18S rRNA, e 28S rRNA).

Parole chiave: *spugne di grotta, barcoding, grotte marine e costiere, distribuzione spaziale, strategie adattative*

Introduction

Biodiversity loss is one of the most concerning issues worldwide (Wilson, 1992).

It has been estimated that in marine ecosystems around 30% of the species will become extinct in the next 30 years (Butler, 2000) and according to the IUCN Red List (2014) a higher number of species is characterized by some level of threat.

The dimensions of this phenomenon make it difficult the recovery of useful resources for programming actions of conservation. The highest priority to perform a strategic planning to optimize conservation efforts is, before study, to program activities aimed at assessing biodiversity at all spatial scales (Myers et al., 2000).

At present conservation planning focuses on conservation strategies cost-effectively based, centred to biodiversity hotspots (e.g. Balmford and Long, 1996; Myers et al., 2000) now widely used as a strategy to identify marine areas where focus conservation efforts.

Each hotspot is identified on the basis of taxonomic richness and endemicity patterns,

their dynamics and interdependencies, their interactions and responses to environmental factors (including impacts of invasive species), and investigations conducted at appropriate spatial scales (Jensen et al.,1999).

The focused conservation hotspots, as well as other conservation strategies, raises the problem of biodiversity assessment in relation to a specific or at different spatial scales.

In this context, the study of the sponge fauna is crucial for a comprehensive assessment of marine biodiversity levels. Porifera can be used as model taxon to act as 'surrogate' of diversity levels of other marine taxa for the following reasons:

- sponges are dominant in many Mediterranean hard bottom biocenosis as coralligenous formations and are typical to the cryptic environments (Cognetti et al., 2002; Manconi et al., 2008);

- sponges are centres of biological associations, representing selective micro-habitats for several key invertebrate taxa;
- in some cases, strong interspecific relations of symbiosis, commensalism, and highly specialized predation occur.

The diversity levels of sponge fauna, however, seem to be underestimated (Hooper, 2002) thus predestined to increase with future studies.

Currently (2014), there are 8,595 valid species (marine and non-marine) in the World Porifera Database, among almost 20,000 taxon names (van Soest et al., 2014), but are around 15,000 living species (Hooper and Van Soest, 2002).

The causes of this underestimation are insufficient and fragmentary data on:

- taxa distributed over a wide geographic range (cosmopolitan species)
- paleoendemic taxa characterized by a wide but strongly disjoined (spot-like) distribution range
- taxa present in cryptic and characteristic habitats as anchialine environments, marine caves and estuarine caves.

A further reason of biodiversity underestimation may result by the presence of a small, but significant portion of morpho-species considered cosmopolitan or widely distributed but in reality composed by a complex of cryptic or sisters species. The supposed cosmopolitanism condition of these morpho-species requires widespread connectivity inside the distribution range.

In contrast it is known that sponges have a low power of dispersal (e.g. Vacelet, 1999; Maldonado and Bergquist 2002; Borchiellini et al., 2004; Nichols and Barnes, 2005) being equipped with lecithotrophic larvae.

In sponges, characterized by a wide but spot-like distribution, a conspecific condition has been rarely tested with a non-morphological approach (e.g. micro-satellites, allozymes, mtDNA, DNA barcoding, etc.).

There is an increasing number of cases in which allopatric populations of sponges rather consist of complexes of cryptic species, genetically distinct (e.g., Solé-Cava et al., 1991 1992) as it has been highlighted in other phyla (e.g., Meyer and Paulay, 2000).

Sponges are genetically, as well as morphologically, ultraconservative, therefore genetic differences are not always expressed at the morphological level (Klautau et al., 1994).

Moreover, the taxonomy of Porifera is largely based on morphometric data, which are highly variable (influence of environmental factors) (Xavier et al., 2010), and that are subjected to a subjective interpretation (e.g., Klautau et al., 1999).

These are some issues that challenge our ability to define a reliable evaluation of sponge fauna diversity, obtaining estimates that, for the reasons mentioned above, vary depending on the spatial scale of investigation.

The most recent diversity assessment of sponges fauna in the Mediterranean has proposed a richness value of more than 600 species with an endemism rate higher than 30% (Coll et al., 2010; Pansini et al., 2011; Pronzato et al., 2012). These data support the Mediterranean Sea as a biodiversity hotspot.

The Mediterranean sponges taxonomic richness is constantly threatened because of climate change, epidemics, commercial sponges over-fishing, water pollution and alteration of ecosystems since ancient times.

The existing international agreements on conservation of marine fauna have focused on 15 sponges species in the Mediterranean region, considered as 'protected species' and found in Appendix II and III of the Barcelona Convention (Relini and Tunesi, 2009).

Marine caves as well as being precious laboratories for the study of life in atypical conditions, play a key role in the conservation of taxa of marine and freshwater origin because are important refuge for many marine taxa with faunal assemblages characterized by both true cave dwellers and peculiar representatives survived to the geological and climatic vicissitudes of the Mediterranean area, and are therefore biodiversity reservoirs and centres of endemism.

In addition, the caves, as the islands are isolated and discontinuous habitats that limit gene flow between populations, making the cave faunas ideal subjects for speciation analysis.

Marine caves matching the category of midlittoral caves, semi-dark caves and dark caves harbour protected biocoenosis registered as Habitat II.4.3, Habitat IV.3.2 and Habitat V.3.2 (Relini and Giaccone, 2009; Relini and Tunesi, 2009).

Caves are one of the listed endangered habitats (code 8330) under the CORINE Biotopes Classification by the European Commission.

Appropriate conservation planning of these faunistic elements represents one of the challenges of the western Mediterranean protected areas following the European Union directives (Natura 2000, Habitats Directive, Council Directive 92/43/EEC), but to make it possible, it is necessary to carry out a biodiversity assessment of these particular environments.

In this context, this doctoral thesis aims to contribute to the sponges global diversity assessment with regard to cryptic environments, through a multidisciplinary approach, combining morphological, molecular, ecological and statistics techniques of investigation, to solve current issues in taxonomic, biogeographic and ecological fields of sponges species with high conservation value, and to clarify the main adaptation strategies displayed by some taxa to colonize particular extreme habitats e.g. anchialine environments.

In the **Chapter 1** we contribute to the biological knowledge of Anchialine caves and to the global biodiversity assessment, by a checklist of sponge fauna from anchialine environments at global scaleand a multi-year study of fauna assemblage and distribution in the Bue Marino cave in relation to the physico-chemical water parameters with notes on the adaptive strategies of sponges.

In the **Chapter 2** we used a multidisciplinary approach (morphological and genetic) to describe a new species of the genus *Protosuberites* from the Bue Marino Cave (Orosei Goulf, Eastern Sardinia) and to a phylogenetic reconstruction of the genus *Protosuberites* using three different markers (COI, 18S rRNA, and 28S rRNA). This paper contributes to the biodiversity assessment and it increases the information available on sponge fauna from anchialine caves.

The experimental planning of the thesis together with field and lab analyses were carried out in Sardinia at the Department of Science for Nature and Enviornmental resources of the Sassary University. Genetic analyses was carried out during an eight months LLP Erasmus - Student Mobility for Placement grant 2013/2014, at the laboratories of molecular biology to the Department of Animal Biology and Biodiversity Research Institute (IrBIO) of the University of Barcelona, under the supervision of Dr. Ana Gil Riesgo.

In the **Chapter 3** the geographic range and variation in size and shape of skeletal spicules of two cave-dweller species belonging to the Mediterranean paleoendemic genus *Petrobiona* (Lithonida: Petrobionidae) are investigated.

A multidisciplinary approach by traditional and geometric morphometrics is applied.
Main objectives are i) to clarify the problematic taxonomic status of *P. incrustans* Sarà, 1963 ii) to investigate in spicule's shape and size variation of *P. massiliana* Vacelet and Lévi, 1958 from distant cave populations. In addition new records of *P. massiliana* contribute to better define the geographic range of the genus in the Mediterranean Sea.

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CHAPTER 1

Paper 1

- Manuscript in preparation -

Aquatic fauna and water parameters in an estuarine cave of eastern Sardinia

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Aquatic fauna and water parameters in an estuarine cave of eastern Sardinia

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Abstract

To contribute to the biological knowledge of coastal caves and their biodiversity assessment, we present here the results of a multi-year study of fauna assemblages in the Bue Marino Cave. Faunistic data are discussed in relation to the physico-chemical water parameters with notes on the adaptive strategies of sponges. The results show low levels of taxonomic richness linked to highly variable physico-chemical conditions typical of extreme environments. The composition of the benthic community, unusual for a totally dark coastal cave seems to be related to the peculiar status of river-lake-sea inside the karst system.

Key-words: *biodiversity, spatial distribution, physico-chemical water parameters, model-analysis*

Introduction

Subterranean estuaries scattered along the continental coasts are centres of endemism for taxa of marine origin (Humphreys, 2006; Humphreys et al., 2009). The coastal caves harbouring estuaries in karst areas match the traits of marginal caves or anchialine caves (Riedl, 1966; Riedl and Ozretić, 1969; Sket 1996).

These coastal groundwater systems are characterized by low energy input, absence of light, mixoaline water, hypoxic or even anoxic water, absence of connections with the sea surface, limited access of marine organisms, and presence of stigobiontic fauna with low dispersal capacity. Moreover, the absence of turbulence in such environments would prevent the mixing of sea and karst water with formation of vertical gradients of temperature, salinity and concentration of dissolved oxygen (Pesce, 1997).

In relation to the nature of these ecotone and extreme environments, the degree of biodiversity is often limited to a few marine taxa that could represent the first step in the process of colonization of continental aquatic habitat by marine organisms (Riedl, 1966; Sket, 1996; Manconi, 2009; Manconi et al., 2010, 2012; Melis et al., 2013).

The knowledge of biodiversity levels in these subterranean estuaries is extremely poor, especially for Mediterranean cave and in particular for the benthic communities (Chevaldonné et al., 2005; Oertel and Patzner, 2007).

Although the Sardinian karst harbours more than 3000 terrestrial and marine caves recorded in the Regional Speleological Register, there are few data on aquatic invertebrates living in caves of this Mediterranean island (Segre, 1956; Grafitti, 1990, 2002; Jaume and Boxshall, 1995, 1996a, 1996b; Schmalfuss, 2005; Manconi et al., 2006, 2009; Oertel and Patzner, 2007; Martinez-Ansemil and

Sambugar, 2008; Manconi and Serusi, 2008; Stocchino et al., 2008; Cadeddu, 2012).

Several complex cave systems are present in the central-east Sardinia karst with faunal assemblages characterised by both true cave dwellers and peculiar representatives survived to the geological and climatic vicissitudes of the Mediterranean area. This area represents a hot spot of biodiversity being one of the main centres of endemism in the island for terrestrial and freshwater fauna (Grafitti, 2002; Casale et al., 2008).

In the framework of a biodiversity assessment in Sardinian coastal caves, we report the discovery of a peculiar benthic assemblage from the Bue Marino Cave in eastern Sardinia. Data on distribution in time and space in function of the main physico-chemical water parameters that characterize this particular environment are reported.

Materials and Methods

Study area

The Bue Marino Cave (Cala Gonone, Dorgali, Nuoro, 40°14’51”N, 9°37’29”E Sardinian Speleological Register 12 SA/NU) is located along the Mesozoic coastal limestone of the Gulf of Orosei (Fig. 1). Karst processes, started in this area after emersion in the Upper Eocene (Carobene, 1972, 1978; Carobene and Pasini, 1982; De Waele, 2004) are related to a well-developed underground drainage and ancient estuary (Codula Ilune karstic complex) (De Waele, 2004). The periodical occurrence in this system of mixing between fresh and salt water determined the synergic action of erosion and corrosion with hyperkarst phenomena (Forti and Rossi, 1991; Forti, 1993; De Waele et al., 2001; De Waele and Forti, 2003). The mixing corrosion has resulted in the development along the Orosei Gulf of wide coastal cave systems which extend to several kilometres (De Waele and Forti, 2003).

There has been extensive documentation of exploration and biological surveys of the Bue Marino Cave (Riedl, 1966; Furreddu and Maxia, 1964; Zerbini, 1970; Casale, 1972; Furreddu, 1973ab; Cassola, 1982; Sbordoni et al., 1982; Forti and Rossi, 1991; Hovorka and Benysek, 1991; Slezak, 1992; Fancello and Mucedda, 1993; De Waele and Grafitti, 2000; Grafitti, 2002; Fancello et al., 1994; Casale et al., 2008; Fancello, 2009; Cukrov et al., 2010; Manconi, 2009; Manconi et al., 2010, 2012; Demurtas, 2012; Melis et al., 2013).

The Bue Marino Cave, with a large opening onto the Tyrrhenian Sea characterised by a developed underground drainage, presents an almost horizontal profile and is divided in three branches (northern, central and southern). The cave system is estimated to have a total length of more than 20 km (Fancello, 2009). We here consider the surveyed southern branch of the system that is characterised by the presence of a subterranean aquifer intermittently flowing through a series of large shallow freshwater pools. This branch (Fig. 2) shows impressive columnar speleothems along large subaerial tunnels (10-15 m in diameter) eroded during marine ingressions by the aggressive waters of the mixing zone.

In the southern branch, exploited in part as show cave, the largest freshwater pool is Lago Lungo (ca. 300 m in length) closed downstream by a calcitic dam with a small fall (*ca*. 900 m from the entrance). The last downstream pool with mouth at the sea level, is a large mixohaline water body (Lago delle Canne d'Organo and Lago degli Specchi, 900 m in length) subjected to flowing freshwater only during high flow phases (rain season) and to marine water intrusion that can occur during low flow phases, storms and tidal fluctuations (0.3 - 0.5 m) through the subaerial cave entrance, and from hypothetical lateral crevices.

Environmental parameters sampling

Time-lapse (hourly) measurements of water temperature and light intensity were carried out *in situ*, using 10 dataloggers Hobo mod. Pendant® Temperature/Light 8K - UA-002-08, positioned along a longitudinal transect (freshwater pool – mixohaline pool – marine entrance) (Fig. 3), in some places (Lago degli Specchi) at different depths (Tab. 1).

Temperature and brightness data of 2 years of measurements (from September 2012 to September 2014) were analyzed using statistical exploratory techniques, and included as explanatory variables in a model analysis.

Salinity, pH and conductivity were estimated in the laboratory by performing periodic water sampling. These samples were carried out, during faunistical surveys, in five fixed stations (from WSSO in marine entrance to WSS4 at the end of transect), positioned along a longitudinal transect (freshwater pool, mixohaline pool, entrance) (Fig. 3).

Salinity, pH and conductivity data of more than 2 years of measurements (from May 2012 to September 2014) were analysed using statistical exploratory techniques, to highlight spatial and temporal trends, then included as explanatory variables in a model analysis.

Sampling procedures and design

Faunistic surveys were performed in the southern branch of the cave, along the "show section", a part that leaving from the marine entrance, proceeds into the cave for about 900 m (Fig. 3).

This sampled area is located along a dark zones encompassing large mixohaline pools like Lago delle Canne d'Organo and Lago degli Specchi.

In this area, mixing between sea water and fresh water is evident for the turbulent opalescent water (from June to September).

The sampling was carried out by snorkelling in June 2008 and August 2009, 2010 and 2011, from May to September 2012, from April to September 2013 and from June to September 2014, for a total 28 days of sampling effort.

A target sampling was carried out by surface scraping to collect sessile benthic organisms or by fish trap with bait to capture benthic fauna.

Particular attention was focused to sponges sampling. Samples were fixed in absolute ethanol or preserved dry on the collected substratum. Sampled invertebrates are deposited in the CAVEBUEMAR benthos collection at the Dipartimento di Scienze della Natura e del Territorio (DIPNET), University of Sassari.

Each sample was examined in the laboratory using binocular microscopes (Zeiss Stemi 2000).

Sponges were dissected under a stereo microscope to observe macro-traits and to obtain representative fragments of the skeleton and gemmules. Spicules processed by dissolution of organic matter in boiling 65% nitric acid were suspended in alcohol and dropped onto slides for Light Microscopy (LM) following standard methods (Manconi and Pronzato, 2000). Gemmules and skeleton fragments were dehydrated for Light Microscopy (LM) or processed also for Scanning Electron Microscopy (SEM, ZEISS DSM 962) (see Manconi and Pronzato, 2000).

Growth form, architecture of the ectosomal and choanosomal skeleton, spicular morphology and dimensions, the topographic localization of spicular types have been considered as diagnostic characters for identification at the species level together with the topographic distribution of gemmules and their morphotraits. Spicular micrometries were performed for both species on almost 100 spicules for each spicular type. The taxonomic status of cited sponge taxa was validated on the base of the World Porifera Database (van Soest et al., 2014).

To test the hypothesis of a spatial and temporal variation in the distribution of sessile benthos communities present in the cave, and to evaluate the influence of biotic and abiotic factors on this distribution have been using a multifactorial sampling design following Bussotti et al., 2006.

The study area (south branch, tourist section, 900 m about) was divided into 5 sections of equal length, ranging from the marine entrance of the cave (station 0) to the waterfall placed at the end of the tourist branch (station 4) (Fig. 3).

A photographic sampling in the three central stations (station 1, 2, and 3) was performed, and 30 random replicas were carried out in each station using a photocamea Canon Power Shoot G10 with diving suit and 20x20 cm PVC photo-quadrat as a reference (Bohnsack, 1979; Pringle, 1984; Pansini and Pronzato, 1990; Benedetti-Cecchi et al., 1996; Bianchi et al., 2004; Preskitt, 2004).

Photo-squares were placed randomly and photographic samples were taken on rocky submerged walls of the caves.

The sampling design consisted of 2 factors: year (2012, 2013, 2014) and sector (ST1, ST2, ST3).

Sampling was repeated on randomly selected occasions in July and September 2012; April, June, July, August and September 2013; June and August 2014.

Each day of sampling 90 photograph samples (30 for each sector) were taken with a sampling effort of 10 days (total 900 photo).

The abundance of each taxon was determined visually (Pansini and Pronzato, 1990; Dethier et al. 1993, Benedetti-Cecchi et al. 1996) for each photo-quadrat by counting organism and estimating the density (specimens / 20 cm^2).

Data was placed in organized matrix using software Excel.

To monitoring life cycle of sponges belonging to the species *Chalinula* sp. and understand adaptation strategies of this species to sudden changes in environmental conditions, typical of this cave, we have carried out a photo sampling using three fixes photo-quadrats replicas, 1 m^2 each.

These three replicas was located non-random in hard-bottom cave wall in Lago degli Specchi pool, because of the high concentration of sponges present in this area (Fig. 3).

Replicas quadrats was photographed in different occasions in July and September 2012; April, June, July, August and September 2013; June and August 2014.

Sponges were counted in each replica for every day of sampling, to calculate sponge density (specimens/m^2) and area covering by applying a 100x100 grid on each photo, using the software Photoshop CS6. Data was placed in organized matrix using software Excel.

Uni- and multi-variate statistical analyses

For all statistical analysis we used R software version 3.1 (R Core Team, 2014) and RStudio software (R Core Team, 2012).

We choose Shannon–Weaver index (Shannon and Weaver, 1949) to calculate species diversity among cave's sectors (sector 1, 2, 3) and among year of sampling (2012, 2013, 2014) using the followings formula:

$$H' = -\sum_{j=1}^{s} p_j \log p_j$$

where *pj* is the proportion of the *j*-th species (*Σjpj* = 1) and s is the number of species (e.g. Poole, 1974; Spellerberg, 2003; Niklaus et al., 2001).

This index expresses the degree of relative uniformity in the numbers of all the species in the sample. *H'* takes values increased with increasing species richness and uniformity of relative abundance of the different species.

In addition, on the same data, we used Simpson's index to have a further estimation of species diversity, which takes into account the effect of an eventual dominant species.

$$\tilde{D} = 1 - \sum_{i=1}^{S} p_i^2,$$

Where *pj* is the proportional abundance of *ith* species (*ni / N), ni* represent the number of individuals of the *ith* species, *N* is the number of total individuals in sample and S is the number of species in the sample (e.g. Hunter, 1988; Keylock, 2005).

This index, unlike the previous one, shows the probability that two individuals randomly drawn from a sample belong to the same species, is therefore strongly influenced by the abundance of the dominant species.

Shannon-Weaver and Simpson index were calculated on photoquadrats monitoring data, among sectors (sectors 1, sector 2 and sector 3) and among year (2012, 2013, 2014) using data collected in September 2012; April, June, July, August and September 2013; June and August 2014.

Moreover, we aimed to explain the observed variation in the estimated densities of *Ficopomatus enigmaticus* (FD) and *Chalinula* sp. (CD) using generalised linear models (GLM), by the following geographic, temporal, spatial, physico-chemical and environmental factors as fixed effects:

- sector 1, 2 and 3 to the cave (ST1, ST2, ST3)
- data of sampling (DAY, YEAR)
- density values of *Protosuberites* sp. (PD)
- density values of *Chalinula* sp. (CD)
- density values of *Gibbula rarilineata* (GD)
- density values of *Sabella* sp. (SD)
- density values of *Calcarea* sp. 1 and sp.2 (SY1, SY2)
- density values of Perophoridae gen. sp. (PED)
- density values of *Barentsia gracilis* (BD)
- water temperature at the point of each density estimation (TEM)
- water salinity at the point of each density estimation (SAL)
- water conductivity at the depth of each density measurement (COND)
- water pH at the depth of each density measurement (pH)

Initially we considered FD as the dependent variable.

Considering the nature of our dependent variable (a non-negative integer), we had two main possibilities to model its distribution, i.e., to use a Poisson GLM or a negative binomial GLM.

An initial data exploration was carried out, in order to evaluate the possible relationships among the variables, and which pairs of variables were highly correlated. In case two variables were highly correlated (correlation >0.7), we retained one of them only for subsequent analyses.

The correlation between salinity and conductivity only turned out to be higher than 0.7 (r 0.953). All the remaining variables were then included in an initial 'full model' (FM1).

FM1 had FD as dependent variable and ST1, ST2, ST3, DAY, YEAR, PD, CD, GD, SD, SY1, SY2 PED, BD, TEM, SAL, COND and pH as explaining factors.

We assumed a Poisson distribution for the dependent variable Yi. The model uses a logarithmic link (i.e., relationship) between the mean of Yi and the predictor function, which deals with two aspects of count data (heterogeneous and non-negative), as pointed out by Zuur et al., 2009.

We then used the function "dredge" included in the MuMln package (Barton, 2012), that helped us in the process of model selection. This function, starting from FD, fits all the possible models (using all the possible combinations of fixed factors), and uses the Information-Theoretic approach based on Akaike information criterion corrected for small sample size (AICc) and Akaike weights (AICc weights, w_i) to compare the relative performance of these competitive models.

We considered the best models (i.e., the model with the lowest AIC and all the models with ΔAIC<2 respect to the best model). Among them, we selected simplest, (i.e., the model having the lowest number of independent variables, in agreement with the parsimony criterion (Berlocher and Swofford, 1997)). We verify if this model meet the assumptions, by checking the residuals plots. Then, we evaluated the overdispersion (ϕ) of the model, (i.e., if the variance is larger than the mean), by calculating the dispersion parameter whit the following formula:

$$\hat{\phi} = \frac{D}{n - p}$$

Since there was evidence of high overdispersion ($\phi > 10$), we repeated the entire process using a negative binomial GLM. A second full model (FM2, including all the explaining variables) was fitted using the glm.nb function in the R package MASS, and the dredge function was applied to FM2. The best model was selected in the abovementioned way.

This model was validated analyzing the residual plots. Then, we calculated type-II analysis of variance tables using the R-package *car* (Fox and Weisberg 2010) to evaluate the relative importance of each variable.

The same procedure was repeated using the density of *Chalinula* sp. as dependent variable and all other variables, including *Ficopomatus* density as explaining factors and assuming a Poisson distribution for the dependent variable.

Results

Taxonomic composition

Faunistical surveys conducted in the years 2008, 2010, 2011, 2012, 2013, 2014 show the presence of 21 taxa belonging to 9 phyla (Tab. 2); 17 taxa are invertebrates and 4 taxa are vertebrates.

Determination to species and genus level of some taxa is still in progress and they are reported as higher taxonomic groups.

The sessile benthos present along the transect includes several taxa such as foraminiferans (*Planulina* sp.), sponges (*Protosuberites* sp. (see chapter 2 present thesis); *Chalinula* sp.; Calcarea Fam. Gen. sp. 1; Calcarea Fam. Gen. sp. 2; Calcarea Fam. Gen. sp. 3; Chalinidae gen. sp.), flatworms (Proseriata fam. gen. sp.), kamptozoans (*Barentsia gracilis* M. Sars, 1835), bivalves (*Mytilus galloprovincialis* Lamarck, 1819; *Mytilaster lineatus* (Gmelin, 1791); *Manupecten pesfelis* (Linnaeus, 1758)), gasteropods (*Gibbula rarilineata* (Michaud, 1829)), annelids (*Ficopomatus enigmaticus* Fauvel, 1923; *Sabella* sp.), and tunicates (Perophoridae gen. sp.).

Among the vagile fauna are found decapod crustaceans (*Palaemon serratus* Pennant, 1777) and fishes (*Diplodus vulgaris* Geoffroy Saint-Hilaire, 1817; *Anguilla anguilla*, Linnaeus 1758; *Conger conger* Linneaus, 1758; and *Scyliorhinus canicula* Linnaeus, 1758).

The higher taxonomic diversity was recorded in the intermediate zone of the cave, namely sector 1 (n = 15 taxa) and sector 2 (n = 13 taxa) roughly corresponding to the Lago degli Specchi. Lower values were found downstream (sector 0; n = 3 taxa) and upstream (sector 4; n = 3 taxa), along the transect. Intermediate values characterize the Sala delle Canne d'Organo (sector 3; n = 5 taxa). Shannon-Weaver and Simpson's indices resulted in similar and mutually consistent values (Tab. 3).

The comparison between the sectors, considering data related to three years (2012, 2013, 2014), shows higher diversity values with H' of 0.888 and a D value equal to 0.462 in the sector 2 (Lago degli Specchi, innermost part), while lowest values in sector 1 where H' is equal to 0.555 and D equal to 0.257.

The comparison between years, considering data related to three sectors (sector 1, sector 2, sector 3) highlighted higher values with H' of 0.969 and D equal to 0.5551 in the year 2014, while 2012 show the lowest values for both indices with H’ equal to 0.010 and D equal to 0.002.

Lago degli Specchi (Fig. 3) showed the higher level of biodiversity, harbouring 20 taxa out of total recorded taxa (n = 21 taxa). The benthic assemblage composed by forams, sponges, polychaetes, bivalves, kamptozoans, crustaceans, tunicates (Tab. 2) colonized almost bare hard substrata that included rocky walls of the tunnel, submerged parts of speleothems, fragments of stalactites/stalagmites on the sandy bottom, and man-made substrata (cement, bricks, plastic objects, and wooden boards). Throughout the tourist branch, dominant taxa were sponges and serpulids. The phylum Porifera shows the highest taxonomic richness (Tab. 2).

The most frequent sponge species is the massive white haplosclerid *Chalinula* sp. living in a *facies ca*. 1 m in height and 4 m in length; it is represented for most of the year exclusively by resting bodies (gemmules) on the substrata, and as active sponges (vegetative phase) from from May to September.

Chalinula sp. was present in almost all years of observation in Lago degli Specchi (sector 1, 2 and partially 3) with considerable biomass fluctuations between years. Another massive, erected, brown haplosclerid also belonging to the family Chalinidae was found in Lago degli Specchi in April 2013. It is currently in phase of determination at genus and species level.

The large yellow encrusting suberitid sponges *Protosuberites* sp. was found in facies *ca*. 5 m in length on the rocky wall, associated with kamptozoans, in cave sectors 0, 1, 2 and 3. This sponge species is represented on the substrata exclusively by resting bodies for most of the year, displaying the active phase from June to August. It was recorded only in year 2008, 2013 and 2014, with a high variation of abundance in years.

Syntopic haplosclerid and hadromerid sponges were also found on speleothems fragments scattered on the sandy bottom.

Some specimens belonging to the class Calcarea were found in the sectors 2 and 3 of the cave from June to September 2014 (determination is still in progress).

The most abundant species is the serpulid *Ficopomatus enigmaticus,* always distributed in *facies* of about 1 m in height, extended from the water surface to 1.5 m in depth, present in all areas to the cave on all substrates.

Serpulids and forams were found also syntopic on the rocky wall with *Chalinula* sp. and *Protosuberites* sp..

All taxa are characterized by a patchy distribution.

Adaptive strategies of Porifera

Haplosclerid and hadromerid sponges (Demospongiae) of the Bue Marino Cave are characterised by the presence of typical resting bodies. Resting bodies were not found in sponges belonging to the class Calcarea.

Faunistic surveys conducted since 2008 showed that the population of *Chalinula* sp. was composed from May to September by massive to encrusting whitish specimens with abundant resting bodies (gemmule-like). Only detailed observations of substrates *in situ* revealed, however, the presence of large carpets of gemmules in a dormant phase in other months.

Monitoring on fixed square has partly confirmed these initial observations. The photo-monitoring of fixed square show that the population of *Chalinula* sp., although conspicuous in 2013, apparently disappeared in 2012 and 2014 surveys.

The maximum density was in July 2013 with a value of 64 sponges/m^2 while the maximum area covering was 2.875 % in the same month (Fig. 3). In July 2013, in proximity of fixed square, the average temperature detected was 22.132°C, conductivity 56.8 mS, pH 7.99 and salinity of 36.66 ‰.

The biological cycle of *Chalinula* sp. in the Bue Marino Cave is characterized by a cyclic alternation between criptobiotic phase, which lasts from October to May, in which the sponge regresses to dormant gemmules characterized by the presence of totipotent cells, and a vegetative phase that lasts from May to October.

The population of *Protosuberites* sp. was represented from June until the first half of August by large light yellow encrusting specimens in wide patches, with a few scattered resting bodies at the basal portion. It was scattered in the dark zone of the cave (0-2 m depth), in typical *facies* on the limestone vertical walls in the mixohaline pool. It was in association with *Ficopomatus enigmaticus* and sometimes with other sponges. The presence of only small patchy encrusting vegetative forms, with dormant resting bodies was observed on the substratum in August.

Environmental parameters

Conductivity, salinity, temperature and pH values show, between stations and years, very variable patterns.

In years of heavy rainfall, the cave is invaded by running fresh water from the underground aquifer system. In seasons of aquifer system minimum flow, a consistent stratification of the water column was evident and higher values of salinity and temperature are observed at greater depths. The range of water temperature throughout the year is between 18°C in the upstream part of the transect to 27°C in the marine entrance of the cave. In summer, average circadian variation of temperatura was, about 2°C at each station, with fluctuation typical of tide trend. Salinity shows highly variable values depending on the sector and sampling period, with a range between 22.7‰ and 39.12 ‰.

We observe a negative gradient of salinity along transect, proceeding from cave sea entrance (WSS0) at the end of the tourist branch (WSS4). In the Lago degli Specchi pool, at 1 m in depth, the average salinity varies in year: 35.2 ‰ in 2012, 36.32 ‰, in 2013 and 35.33 ‰ in 2014.

Statistical multivariate analysis

A correlation analysis between all data set variables showed a high-level correlation between water salinity and conductivity, with an *r-value* of 0.953.

According to this result, we excluded the conductivity to further analysis and added all the other variables in a full model.

Then we used the function "dredge" included in the MuMln package (Barton, 2012), starting from FD, to carry out a model selection.

Relying on this function we looked at the first 4 models, i.e. those with ΔAIC <2 and of these, we have considered only the first, the one that is with lowest AIC (Tab. 4).

The final model selected (n°254), using a negative binomial distribution, fits the FD as the dependent variable and use as explanatory variables the year in which have estimate FD, the sector cave, PD and pH, salinity and temperature relating to the place where FD densities were estimated.

This model explains 21% of variance.

The residuals plots do not shows particulars pattern.

The ANOVA performed on the final model and reported in Table 5 shows how all variables included are significant on *F. enigmaticus* densities and found to be highly significant variables as year and days of sampling, sector to the cave and *P. mereui* densities.

The summary of the selected model reported in Table 6 shows as densities of *F. enigmaticus* (FD) varies significantly across sector to the cave, but across ST1 and ST2 are not significantly different. Instead ST3 presents densities values significantly greater than the other two sectors.

FD density varies among year of monitoring, in particular there is a significant difference between the three years of observation (2012, 2013, 2014) characterized by a consistently negative trend.

In addition, density of FD is significantly dependent by water physic-chemical parameters, in particular is negatively related to the pH and positively to salinity and temperature, but the effect of this variables, is very slight.

FD is slightly negatively affected by *P. mereui* densities.

We used the function "dredge" included in the MuMln package (Barton 2012), starting from *Chalinula* sp. density (CD), to carry out a model selection.

Relying on this function we looked at the first 10 models, i.e. those with ΔAIC <2 and of these, we have considered the most simple (Tab. 7).

The final model selected (n°42), using a Poisson distribution, fits the CD as the dependent variable and use as explanatory variables the year in which have estimate CD, pH and salinity relating to the place where the CD density was estimate.

The residuals plots are good because do not shows particulars pattern.

The ANOVA performed on the final model and reported in Table 8 shows how all variables included are significant on *Chalinula* sp. densities and found to be highly significant variables as year of sampling, pH and salinity.

The summary of the selected model reported in the Table 10, shows as density of CD varies significantly among year of monitoring, in particular there is a significant difference between the three years of observation (2012, 2013, 2014) characterized by a consistently positive trend.

In addition, density of CD is significantly dependent by water physic-chemical parameters, in particular is negatively related to the pH and positively to salinity and temperature.

The effect of these physico-chemical variables, is slight.

Discussion

Benthic community and environmental conditions

The faunistic census on benthos established the presence of a simple but peculiar community in the darkest terminal area of the mixohaline zone of the southern branch in the Bue Marino Cave. The taxonomic richness (Tab. 2) is low and fits the concept of truncated biodiversity sensu Gibert and Deharveng (2002). The sparse benthic assemblage, dwelling the hard bottoms of the mixohaline pool seems to be based on species requiring similar water temperature, salinity, food availability and habitat morphology characteristics.

The relative low density and patchy distribution of the sessile benthic invertebrates, together the highly transparency of water, suggest low nutrients in the system. All taxa share the same trophic role impacting on the organic matter of the water column as both active (sponges, kamptozoans, bivalves, tunicates) and passive (polychaetes, foraminifers) filter-feeders that diverges for the size of captured material. The dominance of this trophic group in this cave sector indicates their ability to exploit a low availability of allochtonous food sources eventually transported by the flow from inland water and/or from the sea by storms and tides. Their success in the fluctuating trophic condition of the cave could be due to preadaptation to exploit a wide array of food ranging from bacteria to dissolved (DOM) and particulate organic matter (POM). We can argue that in this condition the food shortage, considered typical in marine caves (Fichez, 1990), seems to be not the rule in the dark innermost part of estuarine caves, that receiving freshwater input as supported also by Morri (2003) and Moscatello and Belmonte (2007).

Extreme and intermittent hydrodynamic fluctuations, related to rainfalls, typical of the central-east karst of Sardinia (Cossu et al., 2007) could represent other environmental constraints. In the Bue Marino Cave they ranges from extensive flash flooding by the aquifer (with water level of the tunnels increasing up to 3-4 m in the heavy rainy season) to ingression from the sea (see De Waele and Forti, 2003) and could have determined the almost exclusive presence of the spare sessile benthos strictly adhering to hard substrata.

Our water quality records along the cline seawater-freshwater are more or less in line with the ranges previously reported (Zerbini, 1970) according with the fluctuation of the freshwater input

from the aquifer and marine ingression. It has been verified the water column periodical stratification in the physico-chemical vertical profile as also reported by Zerbini (1970).

Long term analysis on salinity showed a high variability of values in both time and in space, with significant variations of average values of salinity in different years of monitoring, apparently associated with rains and tides trends. Every year it was observed a decrease in salinity in the direction ‘sea-inner cave’, together with a stratification in both salinity and temperature, that increasing at greater depths.

These traits of the southern branch of the Bue Marino Cave fit partially (Manconi et al., 2012; Melis et al., 2013) with the description of anchialine environments defined at the 1984 International Congress of Marine Biology Cave, Bermuda (Stock et al., 1986), focused on haline waters, water stratification with vertical gradients of temperature, salinity and dissolved oxygen concentration. The southern branch of the Bue Marino Cave does not match however the trait ‘restricted exposure to the open air’ and for this reason it is more correct to consider her an estuarine cave *sensu* De Waele and Forti (2003).

The recorded periodical shifts in salt content represent a strong environmental constraint for cave-dwelling invertebrates. Particularly osmotic stress could have played a key role in the selection of taxa, driving the community structure in the mixohaline zone and confirming that freshwater infiltrations could have a strong impact on communities of marine cave-dwelling invertebrates (Moscatello and Belmonte, 2007; Manconi et al., 2009).

As for the water temperature, pronounced variations during the year and between years characterize the southern branch of the Bue Marino Cave, together with a considerable decrease of temperature along the outer-inner axis of the cave.

Thermal tolerance could have also favoured these invertebrates to withstand water temperature change in the alternation of the constantly cold inland input (min. 13°C max. 18°C) and the more variable marine water temperature.

The total darkness could be a limit for some taxa usually living in well illuminated environments, although the sciophilic behaviour of sponges supports their dominance in the cave.

We found that levels of salinity, pH and temperature affect moderately the presence/absence and density values of organisms present in this cave. Therefore the particular patchy distribution, the alternation between active and cryptobiosis phases during the year, of sponges belonging to genus *Chalinula* and *Protosuberites*, could be attributed to a particular combination of salinity, pH and temperature values in the water column, occurring only at certain seasons and in some parts of cave.

This condition is also shared by other taxa such as the strong euryhaline worm *F. enigmaticus* (Obenat and Pezzani, 1994) able to colonize a wide variety of environments within the Bue Marino Cave.

In short the environmental constraints for colonization by benthic species are various and extreme. The presence of the benthic community at a long distance from the entrance of the Bue Marino Cave, in water subjected to unforeseeable variations according with the hydrological regimes, suggests the potential of these taxa as successful colonizers of the cave.

A high capability to adapt physiological performances to these fluctuations seems to represents a key prerequisite for the invasion of this extreme brackish pool system by a strategy of resistance.

The diversity index showed a marked diversification among cave sectors and between years of observation, confirming the high capacity of the taxa present in this cave to adapt to extreme and highly variable environmental conditions.

Comparative analysis on the community structure indicates that the taxonomic composition of the benthic community is consistent, in part, with those living in other Mediterranean estuarine caves, such as Port Miou (Corroy et al., 1958; Chevaldonné et al., 2005) and Grotta del Bel Torrente (zone 3, salinity 30-35‰, Oertel and Patzner, 2007). The pioneer taxa in the mixohaline pool of the Bue Marino Cave could have been favoured both by adaptive strategies (euryhalinity, dormancy to survive in harsh and variable habitats) and by low interspecific competition.

Our data on the peculiar faunal assemblage of the Bue Marino Cave seems indicative of a first phase of colonization of the subterranean estuary. Moreover the presence some metres above water level, on cave walls along the mixohaline zones, of subfossil oysters and rock-boring bivalves in the Bue Marino (Antonioli and Ferranti, 1992) and dead oysters in the Bel Torrente (Oertel and Patzner, 2007) suggest the long-term marine colonization processes in karst cavities of the study area. The latter processes would have occurred multiple times during the geological vicissitudes of the central-east Sardinian karst (Carobene, 1978) owing to various sea regressions and the consequent freshening of water in the caves and along the coast by several submerged springs (resurgences).

This area is indeed characterised by the coexistence of a wide spectrum of cave typologies harbouring subterranean estuaries with intermittent activity and ranging from semisubmerged (e.g. Bue Marino Cave) to almost totally submerged (e.g. Bel Torrente Cave and Utopia Cave).

These data suggest the key role of biodiversity assessment on subterranean estuaries at the local and global scale to test the hypotheses on the function of these anchialine habitats as the migration routes for marine species towards the subterranean water bodies (Riedl and Ozretić, 1969; Sket, 1996).

Additional work is necessary to investigate in depth these habitat characterised by a truncated biodiversity as a way of predicting the future of rapidly deteriorating ecosystems as suggested by Gibert and Deharveng (2002).

Sponge fauna from anchialine caves

Sponge fauna from anchialine caves are sparsely investigated as described in synopses (Vandel, 1964; van Soest and Velikonja, 1986; Vacelet, 1994) that highlight endemism, extreme rarity and relictual status of stygobiont/stygobious species. The few records from these biotopes refer to species endemic to a single cave or karstic system. The latter condition is valid for *Higginsia ciccaresei* (Pansini and Pesce, 1998) endemic to the Mediterranean Zinzulusa Cave, *Protosuberites geracei* (van Soest and Sass, 1981) and *Oceanapia penicilliformis* (van Soest and Sass, 1981) endemic to the Bahamian Dixon Hill Lighthouse Cave of San Salvador. Another reported species previously considered endemic to the Dixon Hill Lighthouse Cave is the widespread species *Cinachyrella apion* (Uliczka, 1929) (as *Cinachira subterranea* van Soest and Sass, 1981).

Some suberitid sponges recorded as dominant in anchialine lakes of the western Pacific (Arp et al., 2004; de Voogd et al., 2006; Becking and de Voogd, 2008; Azzini et al., 2007; Pisera et al., 2009; Becking and Lim, 2009). More recent data on diversity of sponges in anchialine caves are reported in Manconi et al. (2012) (see present chapter).

Adaptive strategies of sponges

Some suberitid sponges are known to produce resting bodies. However the morphology of the resting bodies produced by *Protosuberites* sp. n. in the Bue Marino Cave deeply diverges from those of *Suberites* sp. found by Pisera et al. (2009) but matches some traits with *Protosuberites* sp. recorded by Arp et al. (2004) and *P. aquaedulcioris* from the Chilka Lake (Annandale, 1915).

Protosuberites sp. n. from the Bue Marino is characterised by the total or partial degeneration and loss of functional tissue; indeed sponges are represented for long term exclusively by resting bodies on the substratum. This behaviour suggests a seasonal rhythm in the life cycle with an alternation of vegetative and cryptobiotic phases. The strategy of cryptobiosis typical of sponge resting bodies is characterised by low metabolic rate allowing long-term survival until the occurrence of a more favourable environmental conditions.

The cryptobiotic phase by dormant gemmules in the life cycle of sponges is suggestive of extreme environmental conditions in the Bue Marino Cave.

In conclusion data show low levels of taxonomic richness linked to highly variable physico-chemical conditions typical of extreme environments. The composition of the benthic community, unusual for a totally dark coastal cave seems to be related to the peculiar status of river-lake-sea inside the karst system. Data indicates the need of further investigations to define patterns and processes of colonization and persistence of benthic fauna in estuarine caves.

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TABLES

Table 1. Bue Marino Cave (E-Sardinia). Summary on investigated cave sectors/sampling stations for benthos distribution, life cycle of *Chalinula* sp., temperature monitoring (datalogger location/depth), water sampling, and average distance of each sector from cave entrance (seaside).

sector	Datalogger/depth	Water sample station/depth	Average distance from cave entrance	Benthos monitoring	Benthos monitoring with fixed squares
sector 0	ST0/1 m	WSSO/ 1.5 m	100 m	no	no
sector 1	ST1-1 / 50 cm ST1-2 / 1.5 m ST1-3 / 3 m ST1-4/ 1 m	WSS1-1 / 50 cm WSS1-2 / 2.5 m WSS1-3 / 5 m	200 m	yes	yes
sector 2	ST2-1 / 1 m ST2-2 / 50 cm ST2-3 / 50 cm	WSS2-1 / 50 cm WSS2-2 / 1.5 m WSS2-3 / 3 m	400 m	yes	no
sector 3	ST3 / 2 m	WSS3-1/ 50 cm WSS3-2/ 1.5 m	600 m	yes	no
sector 4	ST4/ 50 cm	WSS4 / 1 m	800 m	no	no

Table 2. Bue Marino Cave (E-Sardinia). Checklist of fauna from the anchialine southern branch.

Taxa	Sampling period	Habitus/Habitat	Sector
PROTOZOA FORAMINIFERIDA Fam. Planulinidae ***Planulina* sp**.	June – August 2008	Sessile Hard bottom Mixohaline pool	ST4
PORIFERA DEMOSPONGIAE HADROMERIDA Fam. Suberitidae ***Protosuberites* sp.**	June – August 2008, 2013, 2014	Sessile Hard bottom Mixohaline pool	ST0, ST1, ST2, ST3
HAPLOSCLERIDA Fam. Chalinidae ***Chalinula* sp**.	May – September 2008, 2012 2013, 2014	Sessile Hard bottom Mixohaline pool	ST1, ST2
HAPLOSCLERIDA **Chalinidae gen. Sp.**	April 2013	Sessile Hard bottom Mixohaline pool	ST2
CALCAREA **Fam. Gen. Sp. 1** CALCAREA **Fam. Gen. Sp. 2** CALCAREA **Fam. Gen. Sp. 3**	June – September 2013, 2014	Sessile Hard bottom Mixohaline pool	ST2, ST3
PLATYHELMINTHES PROSERIATA **Fam. Gen. Sp.**	July 2012	Soft bottom Freshwater pool	ST4
KAMPTOZOA Fam. Barentsidae ***Barentsia gracilis* M. Sars, 1835**	June – September 2013, 2014	Sessile Hard bottom Mixohaline pool	ST1
MOLLUSCA BIVALVIA Fam. Mytilidae ***Mytilus galloprovincialis* Lamarck, 1819** ***Mytilaster lineatus* (Gmelin, 1791)** Fam. Pectinidae ***Manupecten pesfelis* (Linnaeus, 1758)** *GASTEROPODA* Fam. Trochidae ***Gibbula rarilineata* (Michaud, 1829)**	April – September 2013, 2014	Sessile Hard bottom Mixohaline pool	ST1, ST2
ANNELIDA POLYCHAETA Fam. Serpulidae ***Ficopomatus enigmaticus* (Fauvel, 1923**) Fam. Sabellidae ***Sabella* sp.**	All year	Sessile Hard bottom Mixohaline pool	All sectors
CRUSTACEA DECAPODA Fam. Palaemonide ***Palaemon serratus* (Pennant, 1977)**	June – September 2012, 2013, 2014	Vagile Mixohaline pool	ST1, ST2
CHORDATA ASCIDIACEA Fam. Perophoridae **Gen. Sp.**	June – September 2012, 2013, 2014	Sessile Hard bottom Mixohaline pool	ST2, ST3

PISCES	June – September 2012, 2013, 2014	Vagile Mixohaline pool	ST0, ST1, ST2
Fam. Sparidae			
***Diplodus vulgaris* (Geoffroy Saint-Hilaire, 1817)**			
Fam. Anguillidae			
***Anguilla anguilla* Linnaeus, 1758**			
Fam. Congridae			
***Conger conger* Linneaus, 1758**			
Fam. Scyliorhinidae			
***Scyliorhinus canicula* Linnaeus, 1758**			

Table 3. Bue Marino Cave (E-Sardinia). Summary table of Shannon-Weaver's and Simpson's values among sections of the cave calculated considering all years of observation and between the years considering all sector.

Index	Sector 1	Sector 2	Sector 3	2012	2013	2014
Shannon-Weaver	0.555	0.888	-0.247	0.011	0.600	0.696
Simpson	0.257	0.462	-0.552	0.003	0.473	0.552

Table 4. Bue Marino Cave (E-Sardinia). First four model selected with model selection function, with *F. enigmaticus* density as a dependent variable.

Model n°	**254**	**94**	**246**	**86**
(Intrc)	0.3842	5.747	-1.195	3.742
Year	+	+	+	+
Chlnl				
Day	-1.53E-02	-5.98E-03	-1.32E-02	-5.93E-03
pH	-0.3465	-0.2495		
PD	-0.144	-0.1106	-0.1516	-0.1218
SAL	0.1272		0.1075	
ST	+	+	+	+
TEMP	0.1434		0.1047	
DF	11	9	10	8
logLIK	-1886.251	-1889.223	-1888.242	-1890.333
AICc	3795	3796.8	3796.9	3796.9
ΔAIC	0	1.8	1.91	1.96
WEIGHT	0.22	0.089	0.085	0.083

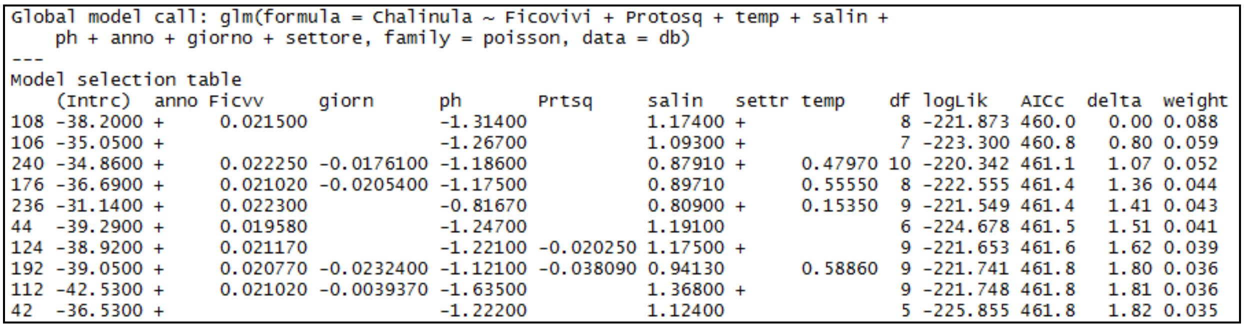
Table 5. Bue Marino Cave (E-Sardinia). ANOVA conducted on explanatory variables of selected model (254).

Variab	**LR**	**LR Chisq**	**Df**
PD	13.773	1	0.0002063
TEMP	4.202	1	0.0403705
SAL	5.598	1	0.017978
Ph	3.999	1	0.0455301
YEAR	90.215	2	< 2.2e-16
DAY	13.45	1	0.000245
SECTOR	45.054	2	1.65E-10

Table 6. Bue Marino Cave (E-Sardinia). Summary of the final model selected to explain *F. enigmaticus* estimated density in function to explanatory variables.

Variables	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
PD	-0.143983	0.036304	-3.966	7.31E-05
TEMP	0.143436	0.068613	2.09	0.036573
SAL	0.127224	0.047299	2.69	0.007149
pH	-0.346461	0.171502	-2.02	0.043367
Year 2013	-1.349758	0.177473	-7.605	2.84E-14
Year 2014	-1.600028	0.153724	-10.408	< 2e-16
Days	-0.015331	0.003997	-3.836	0.000125
ST2	0.008401	0.146725	0.057	0.95434
ST3	1.734897	0.26488	6.55	5.76E-11

Table 7. Bue Marino Cave (E-Sardinia). First ten model selected with model selection function, with *Chalinula* sp. density as a dependent variable.



```
Global model call: glm(formula = Chalinula ~ Ficovivi + Protosq + temp + salin +
    ph + anno + giorno + settore, family = poisson, data = db)
---
Model selection table
    (Intrc)  anno Ficvv    giorn      ph       Prtsq     salin   settr temp    df logLik   AICc  delta weight
108 -38.2000 +    0.021500            -1.31400           1.17400 +              8 -221.873 460.0  0.00 0.088
106 -35.0500 +                        -1.26700           1.09300 +              7 -223.300 460.8  0.80 0.059
240 -34.8600 +    0.022250 -0.0176100 -1.18600           0.87910 +     0.47970 10 -220.342 461.1  1.07 0.052
176 -36.6900 +    0.021020 -0.0205400 -1.17500           0.89710       0.55550  8 -222.555 461.4  1.36 0.044
236 -31.1400 +    0.022300            -0.81670           0.80900 +     0.15350  9 -221.549 461.4  1.41 0.043
44  -39.2900 +    0.019580            -1.24700           1.19100                6 -224.678 461.5  1.51 0.041
124 -38.9200 +    0.021170            -1.22100 -0.020250 1.17500 +              9 -221.653 461.6  1.62 0.039
192 -39.0500 +    0.020770 -0.0232400 -1.12100 -0.038090 0.94130       0.58860  9 -221.741 461.8  1.80 0.036
112 -42.5300 +    0.021020 -0.0039370 -1.63500           1.36800 +              9 -221.748 461.8  1.81 0.036
42  -36.5300 +                        -1.22200           1.12400                5 -225.855 461.8  1.82 0.035
```

Table 8. Bue Marino Cave (E-Sardinia). ANOVA conducted on explanatory variables of selected model (42).

Variab	**LR**	**LR Chisq**	**Df**
SAL	184.71	1	< 2.2e-16
pH	38.815	1	4.66E-10
YEAR	115.99	2	< 2.2e-16

Table 9. Bue Marino Cave (E-Sardinia). Summary of the final model selected to explain *Chalinula* sp. estimated density in function to explanatory variables.

Variables	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
SAL	1.1235	0.124	9.06	< 2e-16
pH	-1.2215	0.196	-6.231	4.63E-10
Year 2013	4.7555	0.5976	7.958	1.75E-15
Year 2014	5.1002	0.7303	6.983	2.88E-12

FIGURES

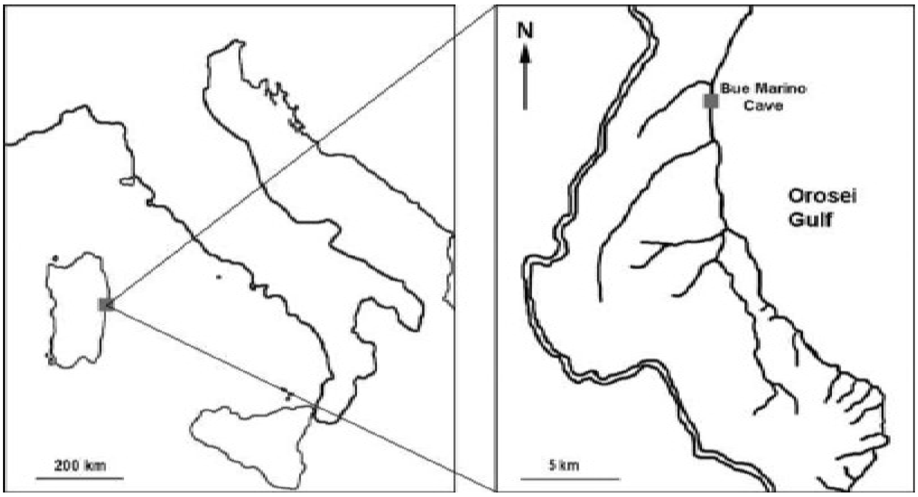


Figure 1. Bue Marino Cave (E-Sardinia) in the Orosei Gulf karstic region (Central Tyrrhenian Sea, Western Mediterranean).

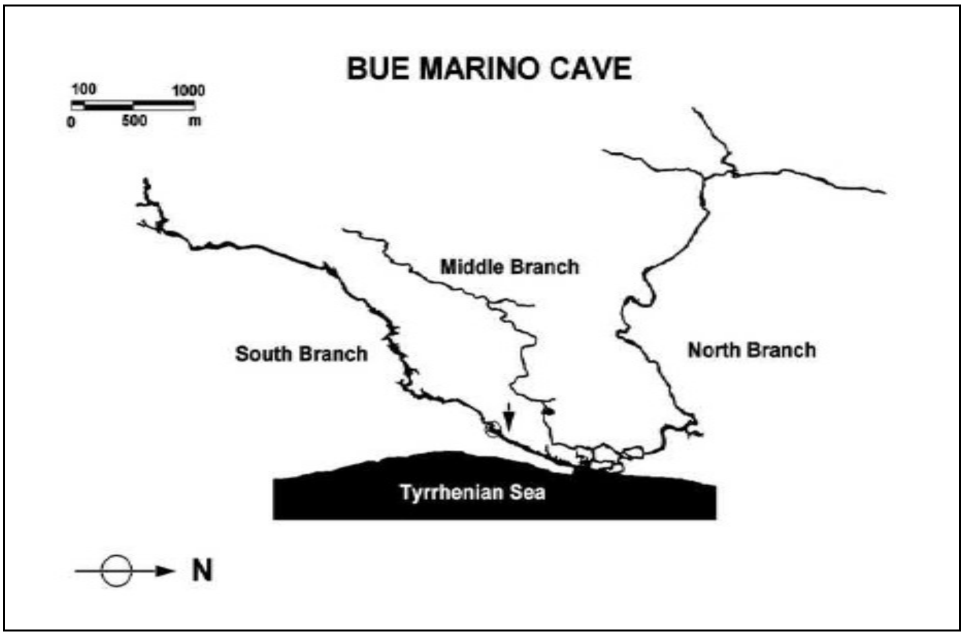


Figure 2. Bue Marino Cave (E-Sardinia). Plan-view map of the southern branch (40°14’51”N, 9°37’29”E Cala Gonone, Dorgali, Nuoro). Benthic community localization in the mixohaline Lago degli Specchi is shown (arrow). Waterfall between freshwater and mixohaline pools is indicated (circle).

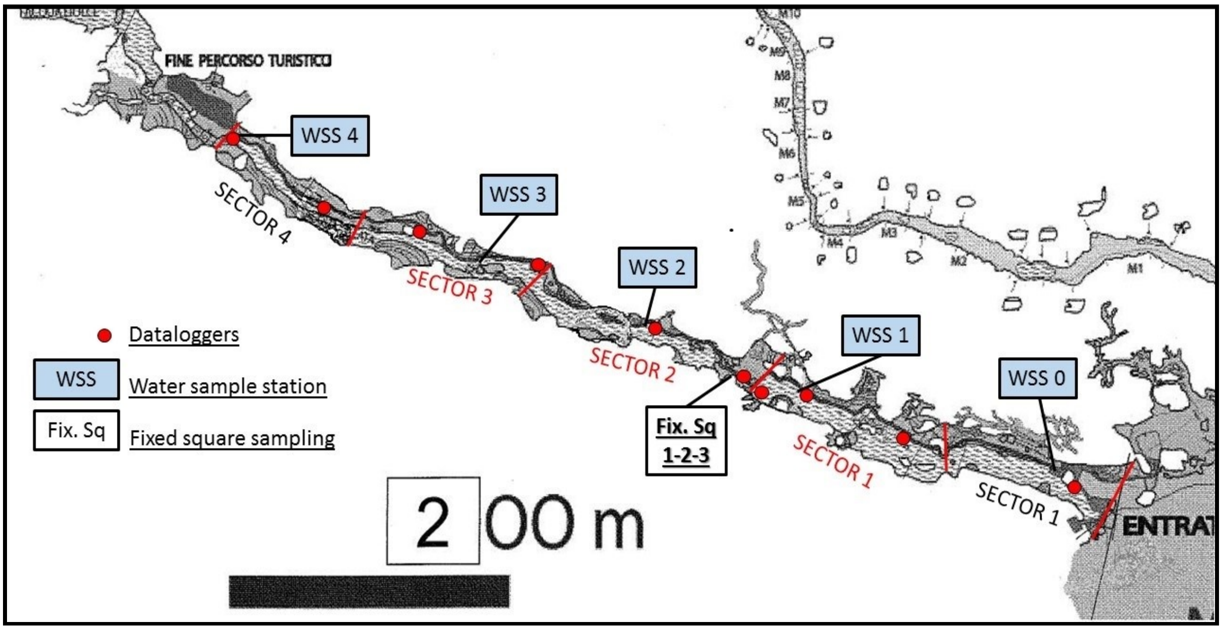
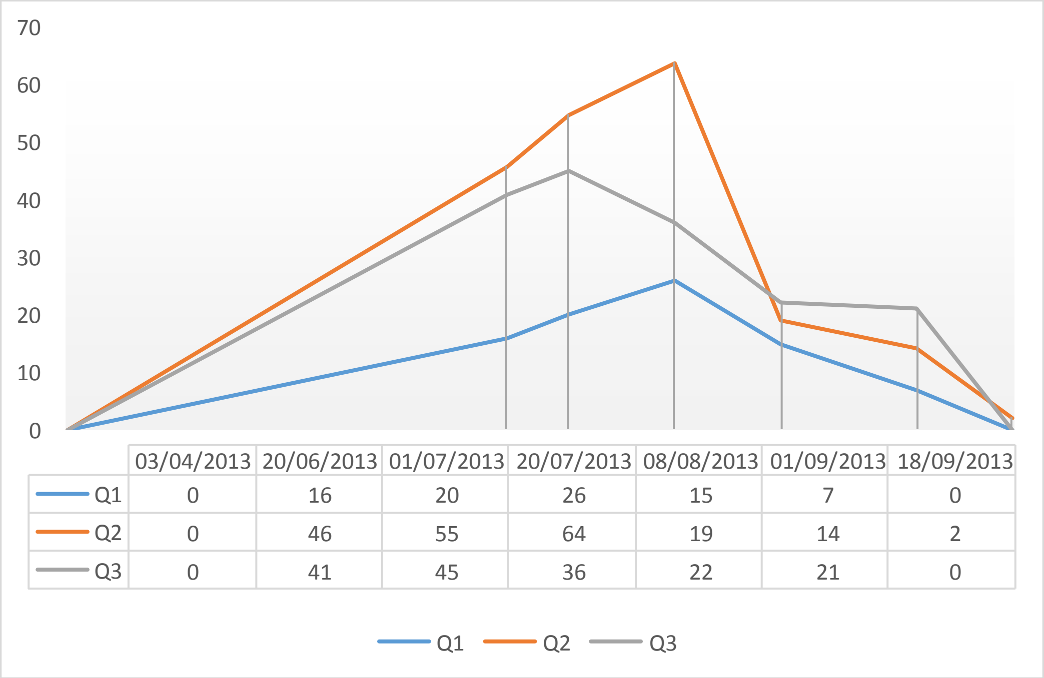
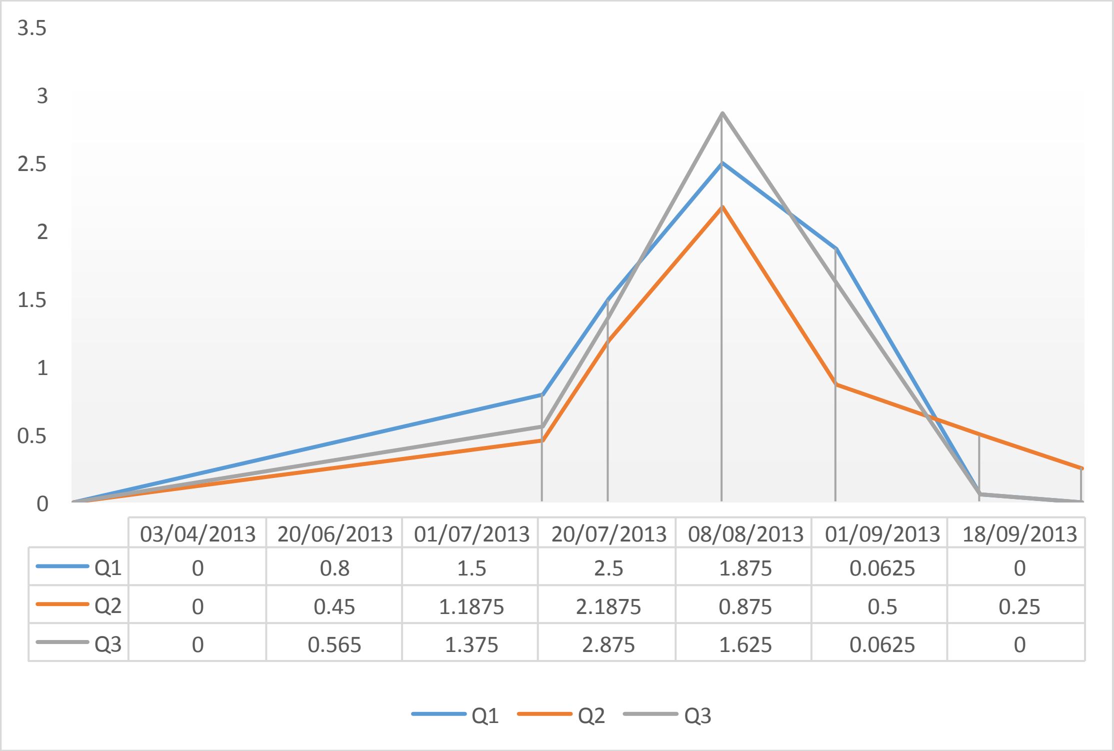


Figure 3. Bue Marino Cave (E-Sardinia). Plan view of southern branch with investigated cave sectors, dataloggers and water sampling stations and location of fixed squares for sponge life cycle monitoring. Station selected for benthos photo-sampling in red.



	03/04/2013	20/06/2013	01/07/2013	20/07/2013	08/08/2013	01/09/2013	18/09/2013
Q1	0	16	20	26	15	7	0
Q2	0	46	55	64	19	14	2
Q3	0	41	45	36	22	21	0

Figure 4. Graphic plot of *Chalinula* sp. density on three fixed squares (Q1, Q2, Q3) during one year.



	03/04/2013	20/06/2013	01/07/2013	20/07/2013	08/08/2013	01/09/2013	18/09/2013
Q1	0	0.8	1.5	2.5	1.875	0.0625	0
Q2	0	0.45	1.1875	2.1875	0.875	0.5	0.25
Q3	0	0.565	1.375	2.875	1.625	0.0625	0

Figure 5. Bue Marino Cave (E-Sardinia). Graphic plot of *Chalinula* sp. area covering on three fixed squares (Q1, Q2, Q3) during one year.

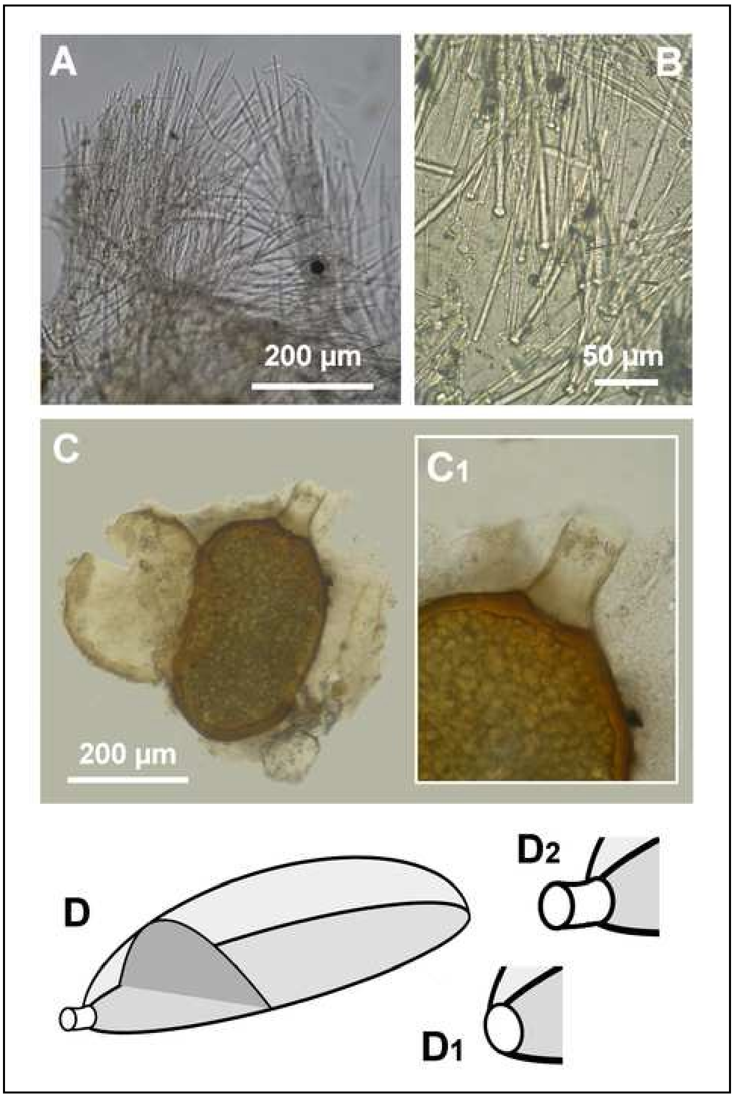


Figure 6. *Protosuberites* sp.. Skeleton, spicules and gemmule-like resting bodies. A. Skeletal architecture with tylostyles arranged arranged in bundles. B. Tylostyles with suboval heads. C. Suboval resting bodies (top view, LM) with flat base and unarmed, protective theca filled by a mass of totipotent cells (right); an empty theca of transparent spongin (left). C1. Theca with sublayered compact spongin and a single foramen bearing a well developed distal collar along the longer axis of the resting body. D. Schematic drawing of a resting body (lateral view) with details of a closed foramen, not evident (apparently lacking) in the dormant phase (D1) and a foraminal collar in the active phase (D2).

Abstract 1

How many sponges live in anchialine caves?

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FABIO DAMIANO LEDDA

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HOW MANY SPONGES LIVE IN ANCHIALINE CAVES?

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To contribute to the assessment of global biodiversity, this paper reviews available information on the cave-dwelling anchialine sponge fauna worldwide.

Key words: biodiversity assessment, Porifera, cave-dwellers, karst caves, lava tunnels, subterranean estuaries.

INTRODUCTION

Faunal diversity in anchialine cave systems is often restricted, by the ecotonal and extreme nature of these biotopes, to a few marine lineages that might represent the first step in the colonization processes of continental aquatic habitats by marine invasions (Riedl, 1966; Sket, 1996). Anchialine caves are centres of endemicity for taxa of marine origin (Riedl, 1966; Sket, 1996; Iliffe, 2000; Humphreys, 2006; Humphreys *et al.*, 2009) although knowledge on the biodiversity of several taxa is extremely poor worldwide.

To contribute to the assessment of global biodiversity, this paper reviews available information on the cave-dwelling anchialine sponge fauna worldwide.

MATERIAL & METHODS

Detailed investigations are in progress to ascertain the existence of published data (including grey literature) on anchialine sponges, and the presence of preserved samples scattered across scientific institutions, or in private collections.

In addition, cooperation is in progress with speleo-teams in Italy, Austria, Croatia, Texas, and Thailand, and new contacts are planned to engage with divers, scientists, and amateurs in the field of biospeleology to acquire more samples and information.

New samples of sponges were analysed that were recently collected from two sites:

– Canary Islands, Lanzarote, Corona lava tube, Tunel de la Atlantida at 722 m from the entrance of the submerged tunnel Montaña de Arena, depth 18 m (courtesy of A. Martinez);

– Sardinia, oriental karst, shallow water in estuarine caves.

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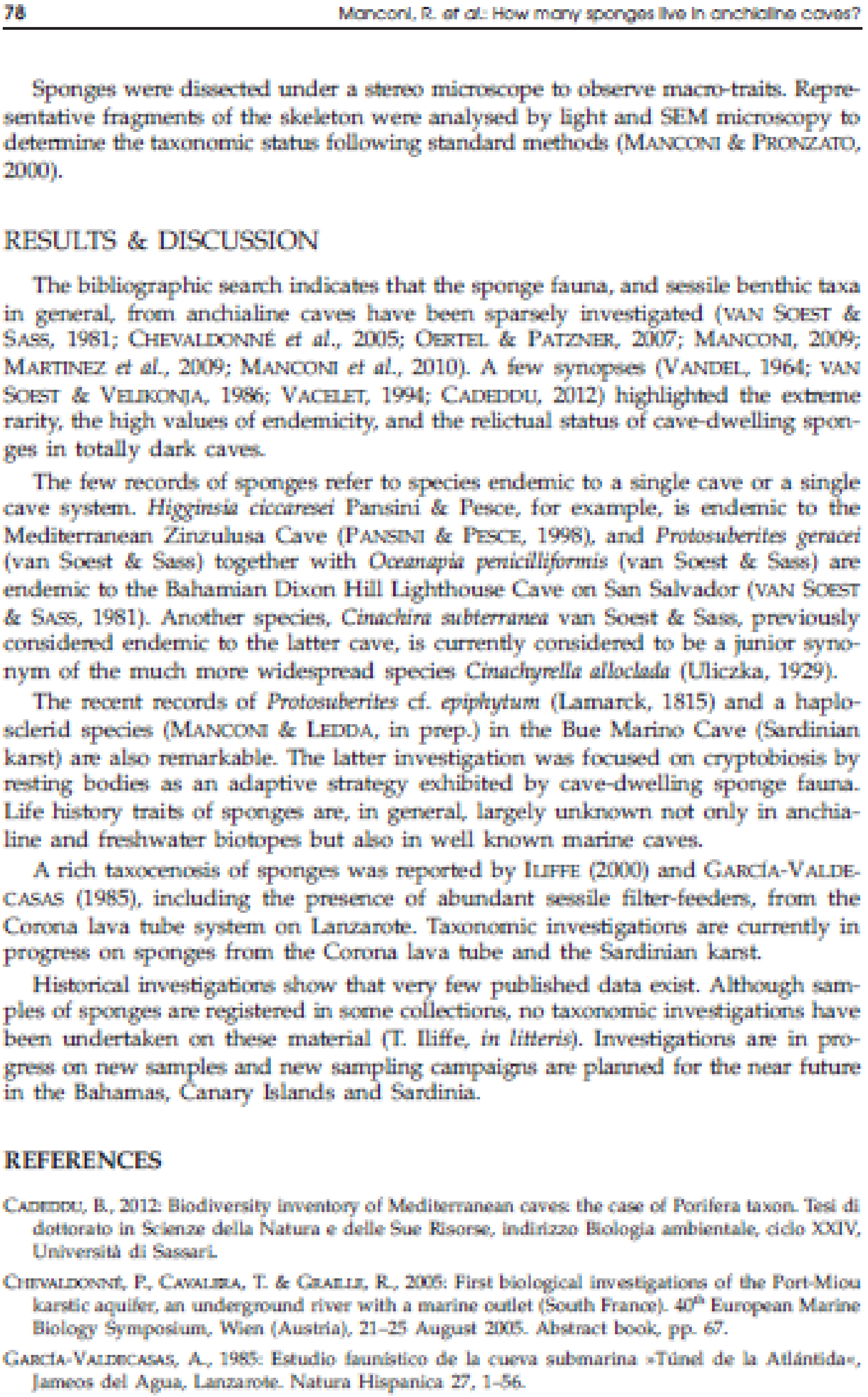

Sponges were dissected under a stereo microscope to observe macro-traits. Representative fragments of the skeleton were analysed by light and SEM microscopy to determine the taxonomic status following standard methods (Manconi & Pronzato, 2000).

RESULTS & DISCUSSION

The bibliographic search indicates that the sponge fauna, and sessile benthic taxa in general, from anchialine caves have been sparsely investigated (van Soest & Sass, 1981; Chevaldonné *et al.*, 2005; Oertel & Patzner, 2007; Manconi, 2009; Martinez *et al.*, 2009; Manconi *et al.*, 2010). A few synopses (Vandel, 1964; van Soest & Velikonja, 1986; Vacelet, 1994; Cadeddu, 2012) highlighted the extreme rarity, the high values of endemicity, and the relictual status of cave-dwelling sponges in totally dark caves.

The few records of sponges refer to species endemic to a single cave or a single cave system. *Higginsia ciccaresei* Pansini & Pesce, for example, is endemic to the Mediterranean Zinzulusa Cave (Pansini & Pesce, 1998), and *Protosuberites geracei* (van Soest & Sass) together with *Oceanapia penicilliformis* (van Soest & Sass) are endemic to the Bahamian Dixon Hill Lighthouse Cave on San Salvador (van Soest & Sass, 1981). Another species, *Cinachira subterranea* van Soest & Sass, previously considered endemic to the latter cave, is currently considered to be a junior synonym of the much more widespread species *Cinachyrella alloclada* (Uliczka, 1929).

The recent records of *Protosuberites* cf. *epiphytum* (Lamarck, 1815) and a haplosclerid species (Manconi & Ledda, in prep.) in the Bue Marino Cave (Sardinian karst) are also remarkable. The latter investigation was focused on cryptobiosis by resting bodies as an adaptive strategy exhibited by cave-dwelling sponge fauna. Life history traits of sponges are, in general, largely unknown not only in anchialine and freshwater biotopes but also in well known marine caves.

A rich taxocenosis of sponges was reported by Iliffe (2000) and García-Valdecasas (1985), including the presence of abundant sessile filter-feeders, from the Corona lava tube system on Lanzarote. Taxonomic investigations are currently in progress on sponges from the Corona lava tube and the Sardinian karst.

Historical investigations show that very few published data exist. Although samples of sponges are registered in some collections, no taxonomic investigations have been undertaken on these material (T. Iliffe, *in litteris*). Investigations are in progress on new samples and new sampling campaigns are planned for the near future in the Bahamas, Canary Islands and Sardinia.

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Abstract 2

Fauna bentonica di una grotta d'estuario: il caso della Grotta del Bue Marino (Golfo di Orosei).

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44° Congresso della Società Italiana di Biologia Marina, Roma, 14-16 maggio 2013. Volume pre-print, pag 99-100.

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FAUNA BENTONICA DI UNA GROTTA D'ESTUARIO: IL CASO DELLA GROTTA DEL BUE MARINO (GOLFO DI OROSEI)

BENTHIC FAUNA OF AN ESTUARINE CAVE: THE CASE OF THE 'BUE MARINO' CAVE (GULF OF OROSEI)

Abstract *- Data on aquatic fauna and water variables from the south branch of the Sardinian 'Bue Marino' Cave are reported. The taxonomic composition and richness suggests that only a few taxa are able to survive the harsh environmental variations due to the unpredictable alternation of floods from the subterranean freshwater system and the marine ingression.*

Key-words: *biodiversity, cave fauna, anchialine habitat.*

Introduzione - Per le grotte d'estuario *sensu* De Waele e Forti (2003), che in parte corrispondono alla definizione di ambiente anchialino, la conoscenza della biodiversità è estremamente scarsa a livello globale (Manconi *et al*., 2010). La vasta area carsica della Sardegna centro-orientale rappresenta un *hot-spot* di biodiversità con alti valori di endemicità per la fauna cavernicola terrestre e d'acqua dolce. La Grotta del Bue Marino (Cala Gonone, Dorgali, Nuoro, 40°14'51"N, 9°37'29"E, 12 SA/NU nel Catasto Speleologico Regionale della Sardegna) è il più vasto di questi sistemi carsici oltre a rappresentare un'importante meta turistica (*ca*. 60.000 visitatori/anno). La grotta, con profilo quasi orizzontale, è divisa in tre rami (nord, centro, sud) e si apre sul Mar Tirreno con un ingresso che rappresenta l'estuario sotterraneo di un grande sistema acquifero (*ca*. 20 km di lunghezza). Scopo del lavoro è quello di presentare i dati relativi all'indagine faunistica e misurazione di alcune variabili ambientali dell'estuario presente nel ramo sud della Grotta del Bue Marino.

Materiali e metodi - L'indagine è stata effettuata lungo un transetto di *ca*. 900 m dall'ingresso alla fine del percorso turistico. La grotta in questo tratto è semisommersa (profondità 0,30-4,5 m) e completamente oscura dopo i primi 100 m. Il censimento faunistico è stato effettuato mediante *visual census* (Fotocamera Canon G10), prelievo mirato di campioni per 'grattaggio' del substrato e trappole fisse o nasse dotate di esca. L'identificazione degli invertebrati è stata effettuata mediante stereomicroscopio e microscopio ottico a trasmissione secondo metodi standard (analisi dei caratteri macroscopici e microscopici, prelievo di piccoli frammenti, preparazione di vetrini). Ad intervalli regolari nel corso delle stagioni (primavera, estate, autunno) sono stati prelevati campioni d'acqua in 5 stazioni lungo il transetto per stimare Salinità, Conducibilità e pH. La temperatura dell'acqua è stata monitorata ogni ora da 10 *datalogger* (HOBO UA-002-64).

Risultati - Il benthos sessile presente lungo il transetto comprende diversi taxa quali foraminiferi (*Planulina* sp.), poriferi (*Protosuberites* cf. *epiphytum*, *Chalinula* sp., *Sycon* sp.), kamptozoi (*Barentsia gracilis* M. Sars, 1835), molluschi bivalvi (*Mytilus galloprovincialis* Lamarck, 1819), anellidi policheti (*Ficopomatus enigmaticus* - Fauvel, 1923; *Sabella* sp.), tunicati (Perophoridae gen. sp.). Tra la fauna vagile sono presenti crostacei decapodi (*Palaemon serratus* - Pennant, 1777) e pesci (*Diplodus vulgaris* Geoffroy Saint-Hilaire, 1817; *Anguilla anguilla*, Linnaeus 1758). La determinazione a livello di specie e genere di alcuni taxa è tuttora in corso in quanto si tratta di possibili specie nuove. I valori di Conducibilità, Salinità, Temperatura e pH mostrano un andamento molto variabile tra le stazioni. Negli anni caratterizzati da forti piogge la grotta è invasa da acqua dolce corrente proveniente dal sistema acquifero sotterraneo. Nelle stagioni di minima portata dell'acquifero è stata evidenziata una marcata e costante stratificazione della colonna d'acqua. Il range di temperatura dell'acqua nell'arco dell'anno va dai 18 °C nel tratto a monte del transetto ai 27 °C dell'ingresso della grotta. In estate l'escursione circadiana media è stata di *ca.* 2 °C in ogni stazione, con un andamento riconducibile alle fasi di marea. La Salinità mostra valori estremamente variabili a seconda del settore e del periodo di campionamento con un *range* compreso tra 22,7‰ e 39,12‰.

Conclusioni - I bassi valori di ricchezza tassonomica della fauna acquatica sono legati alle condizioni ambientali estreme e altamente variabili tipiche della grotta studiata. Analisi sulla correlazione fra i fattori fisico-chimici e la variazione spaziale e temporale della composizione della comunità bentonica lungo il gradiente mare-acqua dolce sono attualmente in corso. La composizione della comunità bentonica, inusuale per una grotta costiera totalmente oscura è da mettere in relazione con il peculiare *status* di fiume-lago-mare presente all'interno del sistema carsico. La produzione di forme di resistenza (gemmule ricche di cellule staminali) nei poriferi indica capacità di adattamento all'ambiente estremo. La presenza di taxa tipicamente fotofili di ambiente salmastro insieme a taxa con strategie morfo-funzionali specializzate per la resistenza conferma che la colonizzazione delle grotte marginali e/o anchialine e/o d'estuario potrebbe essere il primo passo nel processo di colonizzazione delle acque continentali da parte della fauna di origine marina (Riedl e Ozretic,1969; Sket, 1996; Iliffe, 2000; Manconi e Ledda, *in prep*.). L'alto valore paesaggistico-naturalistico del karst della Sardegna centro-orientale e la presenza di diversi e complessi sistemi di grotte marine, anchialine e terrestri sottolinea la necessità di attuare misure di studio, conservazione e valorizzazione della Grotta del Bue Marino e in generale del Golfo di Orosei quale sito turistico ad elevata pressione antropica.

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CHAPTER 2

Paper 2

-Manuscript in preparation-

The hidden diversity of Mediterranean marine caves: A new species of *Protosuberites* (Porifera: Demospongiae: Suberitidae) and a first contribution to the phylogenetic relationships of the genus

PAOLO MELIS, ANNA RIESGO, SERGIO TABOADA, FABIO DAMIANO LEDDA, RENATA MANCONI

The hidden diversity of Mediterranean marine caves: A new species of *Protosuberites* (Porifera: Demospongiae: Suberitidae) and a first contribution to the phylogenetic relationships of the genus

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Abstract

In the present study we used an integrative approach, morphological and genetic, to describe a new species of the genus Protosuberites *from the Bue Marino Cave (Orosei Gulf, Eastern Sardinia) . Micro and macro-morphological traits of the specimens were studied and compared vs. all congeneric species, covering the distribution range of the genus worldwide. From a morphological point of view the new species of* Protosuberites *is similar to P. denhartogi, and* P. epiphytum. Protosuberites mereui *sp. nov. is a thinly encrusting light yellow sponge, devoid of special ectosomal skeleton, with distal portions of spicules or bundles supporting the dermal membrane. The choanosomal skeleton is composed by single tylostyles, with round to suboval heads, irregularly erected on the substratum in thinner areas. Resting bodies (gemmule-like) were found scattered single or in small groups (2-3),* *being suboval, basally plated and firmly adhered to the substratum by a basal spongin plate. For the molecular analysis, three different markers were used (COI, 18S rRNA, and 28S rRNA) and the resulting concatenated alignments were subjected to maximum likelihood analysis and Bayesian inference analyses. Phylogenetic reconstruction recovered a robustly supported sister relationship between* Protosuberites *sp. (from Panama) and the new species (Bue Marino). Genetic distances calculated for the COI gene between the new species and Protosuberites sp. was the lowest (0.29 ± 0.08) within the rest of congeneric species, although it is sufficient to confirm the presence of two distinct species. Both morphological and genetic analysis confirms unanimously* Protosuberites mereui *as a new species. This paper contributes to the biodiversity assessment and to increase the information available on sponge fauna from anchialine caves.*

Key words: *barcoding, integrative taxonomy, anchialine system, Bue Marino Cave*, *Sardinian karstic caves, cryptic species.*

Introduction

In the Mediterranean Sardinia Island, the Bue Marino Cave is an estuarine cave *sensu* De Waele and Forti (2003), matching only in part the definition of anchialine environment (Holthuys, 1973; Sket, 1996) and where the physical chemical parameters such as water salinity varies dramatically during the year depending on both the underground river flow and tide (Manconi et al., 2012; Melis et al., 2013). In Bue Marino,a few sponge species were reported and their identification needs to be defined at genus and species level (Manconi et al., 2012; Melis et al., 2013; see Chapter 1 this thesis). The taxonomy of sponges is sometimes particularly difficult because it mostly relies on spicule morphology (e.g. Lévi, 1973; Solè-Cava et al., 1991; Muricy et al., 1996; Hooper and Van Soest, 2002; Manconi and Pronzato 2002, 2007; Rua et al., 2011) which can vary due to the influence of environmental factors (Xavier et al., 2010). In addition, morphological characters are also subject to personal interpretation (e.g. Klautau et al., 1999), hindering our understanding of sponge diversity. Sponges are often genetically different but morphologically extremely similar, being cryptic speciation very common in many families (e.g., Sarà et al., 1989; Solé-Cava et al., 1990, 1991; Klautau et al., 1994; Muricy et al., 1996; Lazoski et al. 2001; Xavier et al., 2010; Andreakis et al., 2012). In the past, such cryptic speciation has often been considered as "cosmopolitanism", describing species with worldwide distributions. Overall, these problems challenge our ability to define the real diversity of sponge fauna, obtaining estimates for the living species in around 15,000 (Hooper and Van Soest, 2002) against the 7,000 species currently described (Hooper and Van Soest, 2002).

In recent years, however, molecular phylogeny and systematics have been crucial to clarify the taxonomy in complexes of cryptic species or within taxa characterized by a lack or ambiguity of some morphological characters (Blanquer and Uriz, 2007; Erpenbeck et al., 2012; Escobar et al., 2012 ; Xavier et al., 2010).

The genus *Protosuberites* Swartchewsky, 1905 falls well into this latter case, and it has long been considered difficult from a systematic point of view. It currently comprises 24 species (van Soest, 2014) all characterized by a *habitus* thinly to more massively encrusting with a velvety or microhispid surface. The surface skeleton consists of brushes of tylostyles, which are often somewhat smaller than those of the choanosome, and the choanosomal skeleton consists variably of single spicules erected on the substrate (van Soest, 2014). Delineation of species within the genus *Protosuberites* is difficult if based only on the description of the spicules and skeleton. In addition, the boundaries between the species of this genus are often not clear because of the co-occurrence of sympatric species that have overlapping morphologies (van Soest and Kluijver, 2003).

An increasing number of studies demonstrate the potential effectiveness of DNA barcoding in resolving taxonomic and systematic problems in sponges (e.g., Wörheide and Erpenbeck, 2007; Vargas et al., 2012). Although the mitochondrial DNA of the sponges evolves slowly compared to other metazoans (Shearer et al., 2002), DNA barcoding has successfully been used for the identification of species (e.g. Edgar, 2004), and to study the diversification patterns of sponges (Pöppe et al., 2010) and phylogenetic relationships (Cardenas et al., 2009; Erpenbeck et al., 2012). The application of these methodologies on sponges, however, involves a number of inherent difficulties (Vargas et al., 2012) due to microbial contamination (Erpenbeck, 2002; Webster and Taylor, 2011) or presence of bioactive compounds that inhibit the PCR reactions (Chelossi et al., 2004).

Although the knowledge on the biodiversity of anchialine caves is extremely poor worldwide, these caves appear to be hot spots of endemicity for taxa of marine origin (Humphreys, 2006; Humphreys et al., 2009). In particular, the sponge fauna of anchialine caves is very scarcely studied, with a few recorded species in the Mediterranean and Atlantic waters (van Soest and Sass, 1981; Pansini and Pesce, 1998; Manconi et al., 2012).

We report here on a new species belonging to the genus *Protosuberites* from a cave in the eastern Sardinia karst (Bue Marino Cave, Orosei Gulf) focusing on an integrative taxonomic approach combining both morphological and molecular information to characterize its taxonomic status and to better define the phylogeny of the genus.

Materials and Methods

Study area

The Bue Marino Cave is a karstic system with a large opening onto the Tyrrhenian Sea characterised by a developed underground drainage (Cottarelli and Bruno, 1993). The cave, with an almost horizontal profile, is divided in three branches (Northern, Central and Southern), and it is estimated to have a total length of *ca*. 20 Km (Fancello, 2009).

In general, the southern branch is characterized by the presence of a subterranean aquifer intermittently flowing through a series of large shallow freshwater pools. This branch shows impressive columnar speleothems along large subaerial tunnels (10-15 m in diameter) eroded during marine ingressions by the aggressive waters of the mixing zone. In the southern branch, the largest freshwater pool is Lago Lungo (*ca*. 300 m in length) closed downstream by a calcitic dam with a small fall (*ca*. 900 m from the entrance). This calcitic dam represents the end of the “tourist branch”. This region of the cave is characterized by the concomitant presence of freshwater, from the internal aquifer system, and seawater, from the Tyrrhenian Sea. The touristic branch is subjected to flowing freshwater only during high flow phases (rain season) and to marine water intrusion that can occur during low flow phases, storms and tidal fluctuations (0.3 - 0.5 m) through the subaerial cave entrance, and from hypothetical lateral crevices.

Sample processing

Sponge samples were collected at 1 m depth from the Southern branch of Bue Marino Cave (40°14'48.19" N -09°37'22.76" E, Orosei Gulf, Sardinia, Fig. 1). In particular, sampling was carried out in an area of the southern branch (tourist branch). Sponges were collected in the

Lago degli Specchi, a pool of mixoaline water situated in the middle of the touris branch, since June 2008 and particularly from June to August 2013 by snorkeling and SCUBA diving. Sponge samples were detached from the rocks with a knife or/and collected with adhering to substrata. In the lab they were cleaned from epibionts and substrata fragments under a stereomicroscope Samples were finally preserved both dry and in 96 % ethanol and stored at -20ºC for molecular analyses.

Studied materials

Specimens processed for this study include nine different samples of sponges from the Bue Marino Cave (PROTOBM01-09) deposited at the Department of Science for Nature and Environmental Resources (DIPNET), University of Sassari (Italy).

This material was compared morphologically and/or genetically with the following samples: *Protosuberites epiphytum* (UBAPOR01), Cap de Norfeu, Girona, Spain (42.1511 N, 3.1625 E) 3 m of depth, 19.vi.2014, A. Riesgo leg.; *Protosuberites granulosa* (ZMAPOR18657), Chon Buri, Thailand (12.91617 N, 100.7721 E), 6 m depth, 26.ix.2001, Sumaitt Putchakarn leg.; *Protosuberites incrustans* (ZMAPOR20543), Skagerrak, Norway (59.07835 N, 10.73205 E), 112 m depth, 15.iii.2007, R.W.M. van Soest leg.; *Protosuberites denhartogi* (ZMAPOR09801), Nouadhibou, Mauritanie (19.0667 N, -16.4167 E), 12-18 m depth, 11.vi.1988, R.W.M. van Soest and J.J. Vermeulen leg.; *P. denhartogi* (ZMAPOR17181b), Neeltje Jans, Netherlands (51.63 N, 3.72 E), 0.5 m depth, 11.xi.2002, R.W.M. van Soest leg.; *P. denhartogi* (ZMAPOR20358), Outer Hebrides, Mingulay, Scotland, United Kingdom (56.80663N, -7.43173E), 151-159 m depth, 15.vii.2006, R.W.M. van Soest leg.; *P. denhartogi* (MC5141), Ynys Castell, Abercastel, North Pembrokeshire, United Kingdom (51.9629 N, -5.1282 E), 1.iv.2007; *P. denhartogi* (MC5215), Pwlldevi, Albertcastle, United Kingdom (52.00115 N, -5.09425 E), 27.6 m depth, 10.ii.2009; *P. denhartogi* (MC5556), Wendy's Gully, Skomer, Wales, United Kingdom (51.730467 N, -5.09425 E), 16.3 m depth, 10.vii.2009; *P. denhartogi* (MC3715), Glannafeen Cliff, Lough Hyne, Irland (51.49891 N, -9.30054 E), 10 m depth, 10.vii.2009; *P. denhartogi* (MC4908), Keeraunagark rapids, Galway, Ireland (53.2453 N, -9.54766 E), 0 m depth, 10.vii.2009; *P. denhartogi* (MC5288), Aberreidy Quarry, Wales, United Kingdom (51.9378 N, -5.2085 E,), 13.2 m depth, 15.viii.2009.

In addition, samples from the cave of Bue Marino were compared morphologically vs. slides of *Protosuberites epiphytum* (Lamarck, 1815), G. Pulitzer-Finali Collection of slides deposited at the Dipartimento di Scienze della Terra dell'Ambiente e della Vita (DISTAV, Università di Genova), Mediterranean Sea: TRI.274, Porto Tricase, Apulia, conglomerates, 30 m depth, 24.ix.1970; PC.125, Taranto, Mar Piccolo, encrusting on stone, 0.5 m depth, 18.vii.1973; GAR.74, Gargano, Baia di Campi, rocks and mud, 2-10 m depth, 15.ix.1971.

All specimens, fragments, slides and SEM stubs of the new species are deposited in the collection of R. Manconi at the Department of Science of Nature and Territory (DIPNET), University of Sassari (Italy), but holotype and other type material will be formally registered in a Natural History Museum.

Morphological analysis

Morphological macro-traits such as growth form, size and color were evaluated using a Leica Stemi 2000 stereomicroscope. Representative body fragments were dissected to characterize surface morphology and distribution of inhalant and exhalant apertures. Skeleton fragments and spicules were prepared by digestion in sodium hypochlorite at ambient temperature for light microscopy and Scanning Electron Microscopy (SEM). Procedures for SEM followed

Manconi and Pronzato (2000). Spicular measurements were performed in nine specimens using a Leica Leitz DM RB microscope, with a Canon G6 Power Shot photocamera. For each spicule length and thickness were measured using the program TPSDig2 Version 2.16 (Rohlf, 2010). Measurements of spicules are reported as minimum/average/maximum both for length and thickness.

Molecular procedures

A total of 21 individuals of *Protosuberites* belonging to 10 different species, preserved in 96 % ethanol were used for the genetic analysis (Tab. 1; Fig. 2 for details). Total DNA was isolated from a portion of sponge tissue, using both DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) and SPEEDTOOLS tissue DNA Extraction (BioTools, Madrid, Spain), according to the manufacturer's instructions.

Three molecular markers were targeted: the mitochondrial gene *Cytochrome c Oxidase subunit I* (COI) and two nuclear ribosomal genes, the complete *18S rRNA* (18S) and partial *28S rRNA* (28S). Primers used in this study are shown in Table 2. The complete 18S was amplified in three overlapping fragments of about 950, 900 and 850 bp each, using primer pairs 1F–4R, 4F–7R, and 18Sa2.0–9R (Tab. 2). The gene 28S was amplified using the primers pair 28Srd1aF and 28SRd5b (Tab. 2).

For the PCR reaction, the 25-µl mastermix included: 1 µl of total DNA, 18.25 µl of pure distilled water, 2.5 µl of PCR Buffer, 1.25 µl of $MgCl_2$, 0.5 µl of dNTPs, 0.5 µl of each 10 µM primers, and 0.5 µl BioTaq DNA polymerase (BioLine, Madrid, Spain). For the *COI* fragment amplification the PCR program was as follows: denaturation at 94°C for 2 minutes; 30 cycles of denaturation at 94°C for 60 s, annealing at 45°C for 60 s, and extension at 72°C for 60 s; with a final extension step at 72°C for 7 minutes. For the *18S* the amplification program was denaturation at 94°C for 5 minutes; 30 cycles of denaturation at 94°C for 60 s, annealing at 42°C for 30 s, and extension at 72°C for 2,5 minutes; with a final extension step at 72°C for 10 minutes. And finally for the *28S* the following temperature conditions were programmed for PCR: denaturation at 94°C for 3 minutes; 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 20 s, and extension at 72°C for 60 s; with a final extension step at 72°C for 10 minutes.

PCR products of the samples were then purified using MICROCLEAN (MICROZONE, Haywards, UK) and sent to the Serveis Científico-Técnics of the Universitat de Barcelona for sequencing using ABI BigDye Terminator (Applied Biosystems) and an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Chromatograms were visualised and sequences were assembled in CLC Genomics Workbench v.5.1 (QIAGEN, Valencia, CA, USA). These were compared against the GenBank database with the BLAST algorithm (Altschul et al., 1997) to confirm the Porifera assignment.

Phylogenetic analyses

All sequences used in this study, including outgroups (Tab. 2), were aligned using MUSCLE (Edgar, 2004) and the resulting alignments were implemented in jModeltest (Posada, 2008) to select the best evolutionary model. For COI, 28S and 18S the best model was GTR+ Γ +G. Alignments were concatenated and implemented in RAxML 7.2.7 (Stamatakis, 2006) for maximum likelihood (ML) analysis and in MrBayes 3.2 (Ronquist et al., 2012) for Bayesian inference (BI) analysis. Both the BI and the ML analysis were run with partitions set for a unique GTR model of sequence evolution (Tavarè, 1986) with corrections for proportions of invariable and a discrete gamma distribution (GTR+ Γ +G; Yang, 1996), model obtained under the Akaike Information Criterion (Posada and Buckley, 2004). In ML, 100 independent searches were conducted and the nodal support was estimated via the rapid bootstrap algorithm (500 replicates) using the GTR-CAT model (Stamatakis et al., 2008). Bootstrap frequencies were mapped on to the optimal tree obtained from the independent searches. BI analyses were performed with two runs, each with three hot chains and one cold chain with 20 million generations, sampling every 2500th generation, using random starting trees. This analysis was conducted twice, and 25 % of the runs were discarded as a burn-in after checking stationarity with Tracer v.1.4. (Rambaut and Drummond, 2007). The remaining trees were combined to find the maximum a posteriori probability estimate of phylogeny.

The software MEGA (Kumar et al., 2004) was used to calculate mean intra- and inter-clade p-distances for the mitochondrial marker between all the *Protosuberites* species included in the molecular analysis, computed with a bootstrap variance estimation method and 1000 replications, p-distance model and including only transitions and transversions. The gaps were treated with complete deletion and all codon positions were considered.

Results

Systematic accounts

Class Demospongiae Sollas, 1885

Order Hadromerida Topsent, 1894

Family Suberitidae Schmidt, 1870

Genus *Protosuberites* Swartschewsky, 1905

***Protosuberites mereui* Manconi sp. nov.**

(Fig. 3).

Material examined

Paratypes BUEMARCAVE 21, 22, 30, Bue Marino Cave, E-Sardinia, dark cave, Lago degli Specchi pool 0-1 m of depth, in mixohaline water, R. Manconi legit, vi.2008; – PROTOBM01, 02, 03, 04, 05, 06, 07, 08, 09; Bue Marino Cave, E-Sardinia, dark cave, Lago degli Specchi pool 0-2 m of depth in mixohaline water, P. Melis legit, viii.2013.

Comparative material

Details of all material studied from other *Protosuberites* species are given in Table 1. In addition, samples from the Bue Marino Cave were compared morphologically with light-microscope preparations of *Protosuberites epiphytum* (Lamarck, 1815) G. Pulitzer-Finali Collection of slides deposited at the Dipartimento di Scienze della Terra dell'Ambiente e della Vita (DISTAV, Università di Genova), Mediterranean Sea: TRI.274, Porto Tricase, Apulia, conglomerates, 30 m depth, 24.ix.1970; PC.125, Taranto, Mar Piccolo, encrusting on stone, 0.5 m depth, 18.vii.1973; GAR.74, Gargano, Baia di Campi, rocks and mud, 2-10 m depth, 15.ix.1971.

Description

Growth form thinly encrusting (*ca.* 1-2 mm in thickness) in large patches on the rocky substratum (up to 1m² in covered area). Colour light yellow *in vivo*, whitish after preservation in ethanol (Fig. 3a). Consistency firm, slightly jellyish. Sponge surface hispid with Oscules scattered and inconspicuous. Ectosome lacking special skeleton, with distal tips of spicules and tips of specular bundles supporting the dermal membrane. Choanosomal skeleton as single tylostyles irregularly erected on the substratum in thinner areas, and as tylostyles loosely arranged in bundles in the thicker portions of the sponge.

Megascleres tylostyles 100 / 295.3/ 650 µm in length; 3 / 5.03 / 10 µm in thickness; n=150, Tab. 3) straight to slightly bent, entirely smooth, with rounded to suboval heads (4-11 µm in width, Fig. 4). In addition, rare thinner tylostyles (110-232 x 0.5-1 µm) with a sinuous shaft and a typical mushroom-like head were present.

Resting bodies (gemmule-like) scattered single or in small groups (2-3), with a suboval shape, and firmly attached to the substratum by a basal spongin plate. Theca of resting bodies as sublayered compact spongin (*ca.* 12 µm in thickness) with a smooth outer surface not armed by spicules; tylostyles present on the basal spongin plate near the gemmules.

Active and inactive resting bodies present in the same sponge. Active resting bodies (430 µm in total length with foraminal collar) with single foraminal aperture closer to the distal part of the longer axis of the suboval theca, bearing a well-developed (ca. 80 µm in length) thin-walled, transparent collar. Inactive resting bodies (349 x 233 µm in diameter) with no aperture and no collar, but bearing a small area devoid of cells closer to the distal part of the longer axis of the suboval theca. *Habitat and topographic distribution. P. mereui* sp. nov. occurred in typical facies scattered in the dark zone of the cave, on the limestone vertical walls or large boulders in mixohaline pool water at 0-2 m depth. Sponges associated on the same substratum with the serpulid *Ficopomatus enigmaticus,* kamptozoans and sometimes with an unidentified haplosclerid.

Geographic distribution

P. mereui sp. nov. is only known so far from the Bue Marino Cave in the central-eastern Sardinian karst, Thyrrenian Sea.

Etymology

The species is dedicated to the Sardinian speleologist and photographer Luigi Mereu, died prematurely during cave exploration in recognition of his key contribution in the present research conducted in the Bue Marino Cave.

Molecular analyses

The concatenated analysis consisted of 3995 bp (551 bp of COI, 2322 bp of 18S, and 1122 bp of 28S) including 10 terminal taxa, 6 *Protosuberites* OTUs (Operational Taxonomic Units) and 4 outgroups (*Suberites domuncula*, *S. pagurorum*, *Prosuberites laughlini*, *Prosuberites longispinus*). For *P. denhartogi* and *P. mereui* sp. nov. we sequenced the COI of 8 and 7 individuals, respectively, and obtained 5 haplotypes for *P. denhartogi* and 3 haplotypes for *P. mereui* sp. nov. For the 28S gene, 2 distinct haplotypes were found for *P. mereui* sp. nov., and 6 for *P. denhartogi*, while for 18S, there was one single genotype for both *P. mereui* sp. nov.

and *P. denhartogi*. All mitochondrial and nuclear genotypes were deposited in GenBank and accession numbers are listed in Table 1.

The phylogenetic reconstruction of the concatenated set of genes recovered unequivocally a sister relationship between *Protosuberites* sp. (POR14649) and *P. mereui* sp. nov. (Bue Marino) with 0.99 of posterior probability and 98% of bootstrap (Fig. 5). The clade containing both *Protosuberites* sp. and *P. mereui* sp. nov. showed sister relationship with *P. denhartogi* (Fig. 5) with posterior probability and bootstrap values of 1 and 100% respectively. *Protosuberites incrustans* and *P. granulosa* formed a robustly supported clade that was sister to the clade containing *P. mereui* sp. nov., *P.* sp., and *P. denhartogi*. Finally, *P. epiphytum* appeared as the earliest divergent clade within the *Protosuberites* species represented here (Fig. 5).

Genetic distances calculated for the COI gene were always larger than 0.2 % (Tab. 4). The genetic distance calculated between *P. mereui* sp. nov. (BUEMARINO) and *Protosuberites* sp. (POR14649) was the lowest (2.9 ± 0.8, Tab. 4) while the distance between *Protosuberites* sp. and *P. epiphytum* was the highest (6.8 ± 1.1, Tab. 4).

Discussion

Both morphological and genetic analysis confirms unanimously *Protosuberites mereui* sp. nov. as a new species in the Mediterranean waters. The species is assigned to the genus *Protosuberites* Swartschewsky *sensu* van Soest (2002) on the basis of its typical skeletal arrangement, and in particular on the basis of the presence of peripheral tufts of tylostyles. Molecular markers confirm its generic status also. The species occurring in Bue Marino is similar to *P. denhartogi*, *P. epiphytum*, and *Protosuberites prototipus* Swartschewsky, 1905 but it differs in several morphological characters.

Tylostyle measurements of *P. mereui* sp. nov. partially matches those of *P. epiphytum* from the Mediterranean populations (Pulitzer-Finali, 1983) and of *P. denhartogi* from Northern Europe (van Soest, 1977; Van Soest and de Kluijver, 2003). In addition, the peculiar small slender tylostyles with a sinuous shaft and a typical mushroom-like head in *P. mereui* sp. nov. were never been reported for the any *Protosuberites* in the Mediterranean, although this character is shared with *P. prototipus* from the Black Sea (see the original description translation in van Soest, 2002, Fig. 6C, p. 235). Resting bodies here described for the first time for the entire genus *Protosuberites* represent the exclusive diagnostic trait of *P. mereui* sp. nov., a character that has never reported for the cave-dwelling *P. epiphytum* recorded in other western Mediterranean submerged caves (see Pouliquen, 1972; Pulitzer-Finali; 1978; Corriero et al., 2000).

From a morphological point of view, *P. mereui* sp. nov. seems to be close to *P. epiphytum* *sensu* Pulitzer-Finali (1983) and of P. *denhartogi* from Northern Europe (van Soest, 1977; Van Soest & de Kluijver, 2003).

In contrast the phylogenetic analyses presents a different scene. In fact, phylogenetic analyses reveals how the *P. epiphytum* studied in this paper appeared as the earliest divergent clade within the *Protosuberites* speciesconsidered , and the distance between *P. mereui* sp. nov. and *P. epiphytum* was the highest recovered (6.8 ± 1.1). Such results could be indicating a high plasticity of the morphological characters of tylostyles (Chombard, 1998) and the possibility that these might be affected by some environmental factors (McDonald, 2002). These two facts could explain how species so far away from a genetic point of view can present a high similarity of some taxonomic characters due to convergent evolution.

The phylogenetic tree shows that *P. mereui* sp. nov. is sister to *P. denhartogi*, and that *P. mereui* sp. nov. is also sister to *Protosuberites* sp. The still unidentified *Protosuberites* sp. (POR14649) from Panama is characterized by tylostyles in combination with trichodragmas (Nichols, 2005). The genetic distance calculated between *P. mereui* sp. nov. and *Protosuberites* sp. was the lowest found in our study (2.9 ± 0.8). However, this distance is reported as enough to define two distinct species in sponges, where mean *p*-distances using the gene COI in *Cliona. aff. celata* clades ranged from 6.2% to 8.4% (Xavier et al., 2010), 1.6% in *Tedania (Tedania) ignis* (Wulff, 2006), 4.1% in *Astrosclera willeyana* (Wörheide, 2006), and 13-24% in *Scopalina blanensis* (Blanquer and Uriz, 2007).

Besides morphological and phylogenetic features, *P. mereui* sp. nov. also presents distinctive ecological characteristics compared to other congeneric species. In fact, the southern branch of Bue Marino Cave, where *P. mereui* sp. nov was found, is an estuarine anchialine cave where the physico-chemical parameters such as water salinity varies dramatically during the year. This condition is only shared by another congeneric species, *P. geracei* (van Soest and Sass, 1981), the only Suberitidae reported from an anchialine cave so far (Manconi et al., 2012).

In conclusion, given the autoapomorphyc trait ‘presence of gemmules’, the spicule sizes slightly different when compared to related species, and according to the robust results of the analysis of genetic divergence and phylogenetic relationships, we consider the sponge *P. mereui* from the Bue Marino Cave as a new species.

Acknowledgements

The paper is dedicated to the memory of our friend and colleague Luigi Mereu, Sardinian speleologist and photographer, died prematurely in recognition of his contribution in research conducted in Bue Marino Cave and in memory of the experiences we shared with him.

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TABLES

Table 1. *Genus Protosuberites*. Summary of collection details for all studied material in both morphological and molecular analyses.

Taxa	Label	Location	Coordinates	Depth (m)	Collector	Date of collection
Protosuberites mereui **sp. nov.**	BM03,04,05,06,07,08,09	Bue Marino Cave, Goulf of Orosei, Sardinia, Italy	40°14'48.19" N, 09°37'22.76" E	1	R. Manconi and P. Melis	June-August 2008-2013
Protosuberites epiphytum	UBAPOR01	Cap Norfeu, Girona, Spain	42.1511 N, 3.1625 E	15	A. Riesgo	19/06/14
Protosuberites sp. 1	ZMAPOR09801	Nouadhibou, Mauritanie	19.0667 N, -16.4167 E	12 – 18	R.W.M. van Soest and J.J.Vermeulen	19/11/12
Protosuberites sp. 2	MC5141	Ynys Castell, Abercastel, UK	51.9629 N, -5.1282 E	???	???	01/04/07
Protosuberites sp. 3	MC5215	Pwlldevi, Albertcastle, UK	52.00115 N, -5.09425 E	27.6	???	10/02/09
Protosuberites sp. 4	MC5556	Wendy's Gully, Skomer, Wales, UK	51.730467 N, -5.09425 E	16.3	???	10/07/09
	ZMAPOR17181b	Neeltje Jans, Netherlands	51.63 N, 3.72 E	0.5	Excursion Zeeland 2002	11/11/02
	MC3715	Glannafeen Cliff, Lough Hyne, Ireland	51.49891 N, -9.30054 E	10	???	10/07/09
	MC4908	Keeraunagark rapids, Galway, Ireland	53.2453 N, -9.54766 E	10	???	10/07/09
Protosuberites denhartogi	MC5288	Aberreidy Quarry, Wales, UK	51.9378 N, -5.2085 E	13.2	???	15/08/09
Protosuberites incrustans	ZMAPOR20543	Skagerrak, Norway	59.07835 N, 10.73205 E	112	R.W.M. van Soest	15/03/07
Protosuberites granulosa	ZMAPOR18657	Chon Buri, Thailand	12.91617 N, 100.7721 E	6	Sumaitt Putchakarn	26/09/01
Protosuberites sp.	POR14649	East Pacific, Panama	???	???	R.W.M. van Soest	???
Outgroups						
Suberites domuncula	*Suberites domuncula*	- - -	- - -	- - -	- - -	- - -
Suberites pagurorum	*Suberites pagurorum*	- - -	- - -	- - -	- - -	- - -

Table 2. Primer sequences used in this study.

Name	Primer sequence	Citation
ProtoF	5'- GAA TTA CCG CGG CTG CTG G – 3'	**Present communication**
HCOoutout	5' - GTA AAT ATA TGR TGD GCT C - 3'	**Schulmeister, 2003**
jgLCO1490	5' - TIT CIA CIA AYC AYA ARG AYA TTG G - 3'	**Geller et al., 2013**
jgHCO2198	5' - TAI ACY TCI GGR TGI CCR AAR AAY CA - 3'	**Geller et al., 2013**
1F	5'- TAC CTG GTT GAT CCT GCC AGT AG – 3'	**Giribet et al., 1996**
4R	5'- GAA TTA CCG CGG CTG CTG G – 3'	**Giribet et al., 1996**
4F	5'- CCA GCA GCC GCG CTA ATT C – 3'	**Giribet et al., 1996**
7R	5'- GCA TCA CAG ACC TGT TAT TGC – 3'	**Giribet et al., 1996**
18sa2.0	5'- ATG GTT GCA AAG CTG AAA C – 3'	**Whiting et al., 1997**
9R	5'- GAT CCT TCC GCA GGT TCA CCT AC – 3'	**Giribet et al., 1996**
28Srd1aF	5' - CCC SCG TAA YTT AGG CAT AT – 3'	**Giribet et al., 2006**
28SRd5b	5' – CCA CAG CGC CAG TTC TGC TTA C – 3'	**Giribet et al., 2006**

Table 3. Comparative list of morphological traits of spicules for species belonging to the genus *Protosuberites*.

Species	Spicules Type	Length µm	Thickness µm	References	Distribution including the Mediterranean
***P. mereui* sp. nov.**	Tylostyles	100- **295.3**-650	3-**5.03**-10	Present paper	Yes
P. rugosus	Tylostyles	200-1200	8 (style), 12 (head)	Topsent, 1893c	Yes
P. prototipus	Tylostyles	162-417	5-7	Swarthchevsky, 1905	No
P. denhartogi	Tylostyles	110-**258.7**-456 (600 occasionally (Ackers et al., 1992))	4-**6.3**-11	Van Soest and Kluijver, 2003	No
P. epiphytum	Tylostyles	97-**204.97**-320	2.5-**4.71**- 9.5	Present paper	Yes
***P. epiphytum* (sensu Topsent, 1900)**	Tylostyles	100-300	4-7	Topsent, 1900	Yes
P. modestus	Tylostyles	400-1050	8-14	Pulitzer and Finali, 1978	Yes
P. incrustans	Tylostyles	225 – **320.7** - 477	7.38 – **10.02** – 15.11	Present paper	No
P. ferrerhernandezi	Tylostyles	?	?	Boury-Esnault and Lopes, 1985	Yes
P. ectyoninus	Subtlostyles and tylostyles	145 (tylostyles) 350-400 (subtylostyles)	7 (tylostyles) 13 (subtylostyles)	Topsent, 1900	Yes
P. granulosus	Tylostyles	238.3 – **448.1** – 703.3	8,25 – **11.7** - 15	Present paper	No

Table 4. Genetic *p*-distances (in light blue) and standard deviation (light green) computed between the different clades resulting from the phylogenetic analysis. Values of *p*-distance between *Protosuberites mereui* sp. nov. and its closest relative analyzed are shown in red.

	Protosuberites epiphytum	***Protosuberites incrustans***	***Protosuberites granulosa***	***Protosuberites denhartogi***	***Protosuberites* sp. nov. (BUEMARINO)**	***Protosuberites* sp. (POR14649)**
Protosuberites epiphytum		1.1	1.1	0.9	1.1	1.2
Protosuberites incrustans	5.6		0.3	0.9	0.8	1.2
Protosuberites granulosa	5.6	5		0.9	0.9	1
Protosuberites denhartogi	5.5	5.8	5.8		1	1
***Protosuberites mereui* (BUEMARINO)**	5.7	3.4	3.9	6.4		0.8
***Protosuberites* sp. (POR14649)**	6.8	4.4	4.4	6.3	2.9	

FIGURES

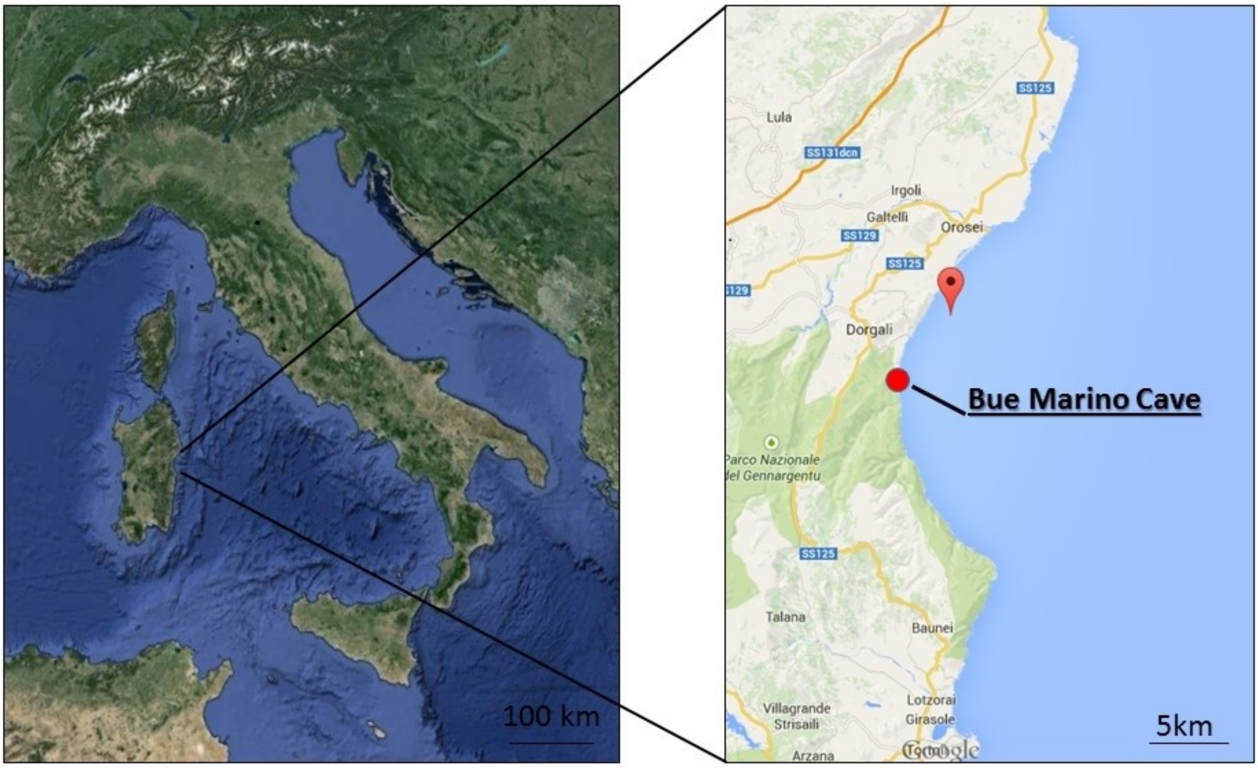

Figure 1. Sardinia island in the Western Mediterranean Sea and study area in the Orosei Gulf karstic region.

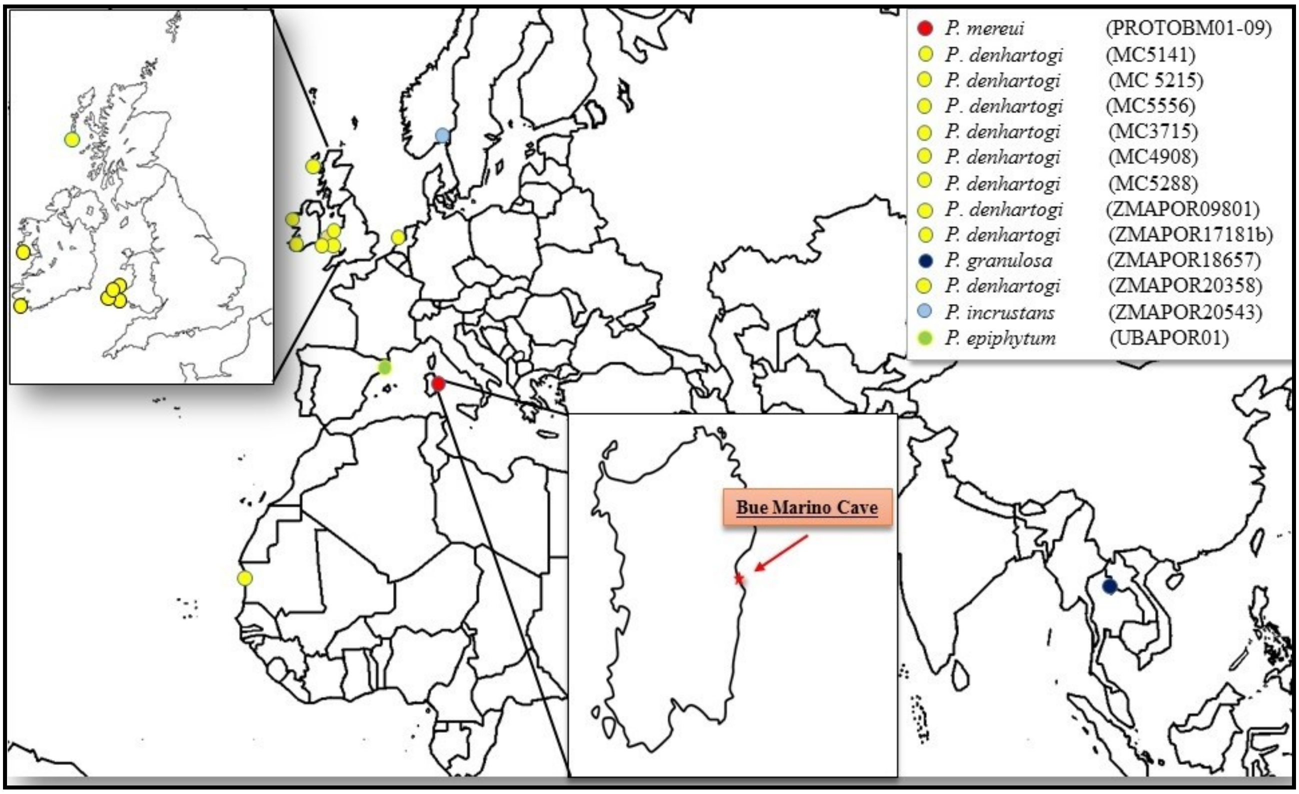

Figure 2- Geographical location of *Protosuberites* samples used in the present study. Inset on the left, showing the approximate localities where *P. denhartogi* were collected in the UK. Inset on the right showing the location of the Bue Marino Cave (Sardinia) where *Protosuberites mereui* sp. nov. was collected.

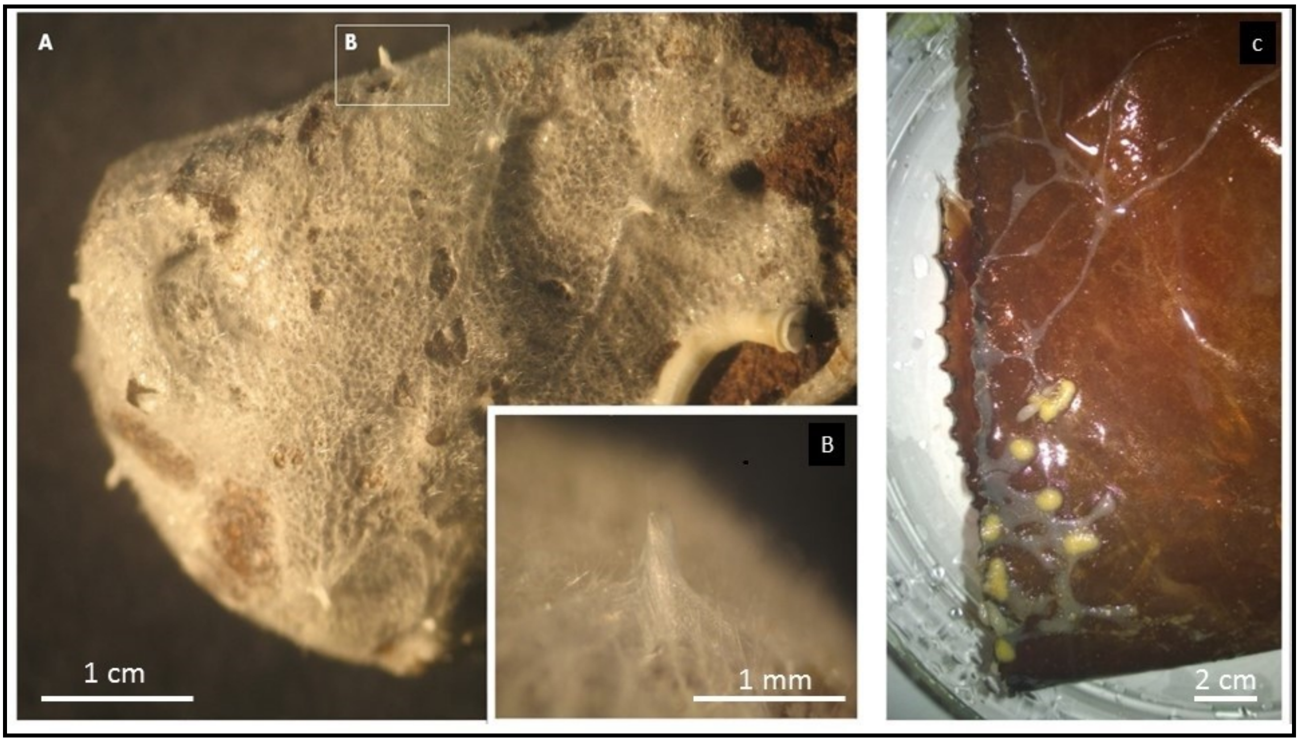

Figure 3. *Protosuberites mereui* sp. nov. A) Specimen (dry) on a rocky fragment . B) Magnification of an osculum. C) Specimen on a plastic fragment (ethanol).

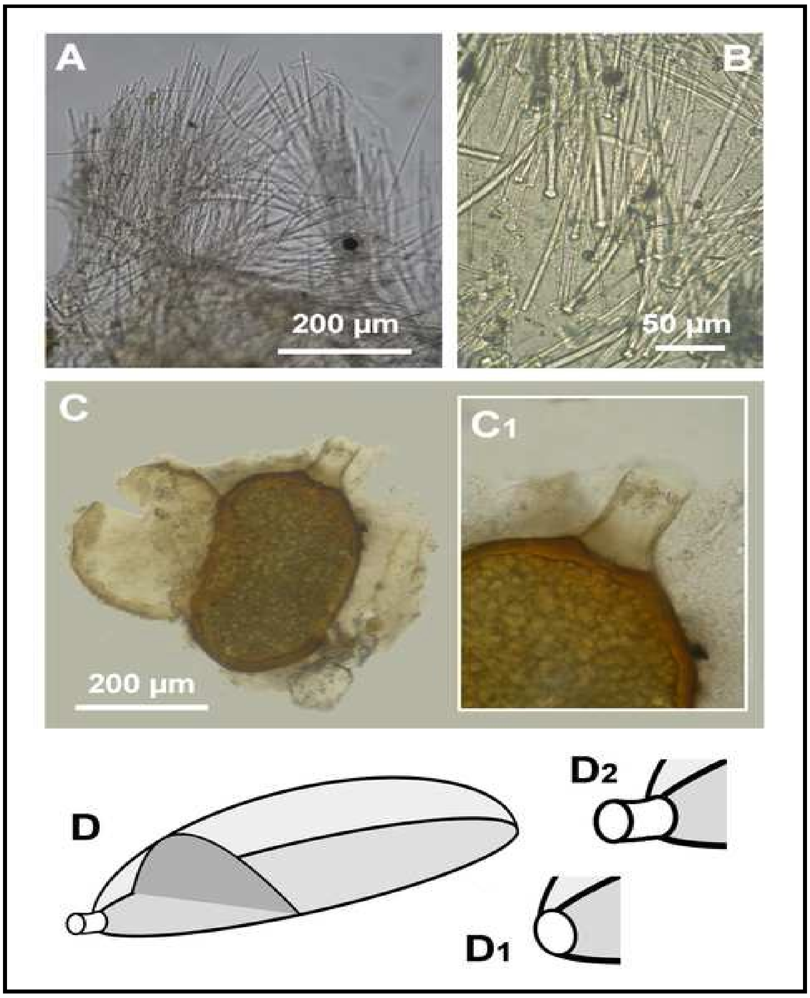

Figure 4. *Protosuberites mereui* sp. nov. Spicules and gemmule-like resting bodies. A. Skeletal architecture with tylostyles arranged in bundles. B. Tylostyles with suboval heads. C. Suboval resting bodies (top view, LM) with flat base and unarmed protective theca filled by a mass of totipotent cells (right); empty theca of transparent spongin (left). C1. Theca with sublayered compact spongin and a single foramen bearing a well developed distal collar along the longer axis of the resting body. D. Schematic drawing of a resting body (lateral view). D1. Details of a closed foramen, not evident (apparently lacking) in the dormant phase; () D2. Foraminal collar of an open foramen in the active phase ().

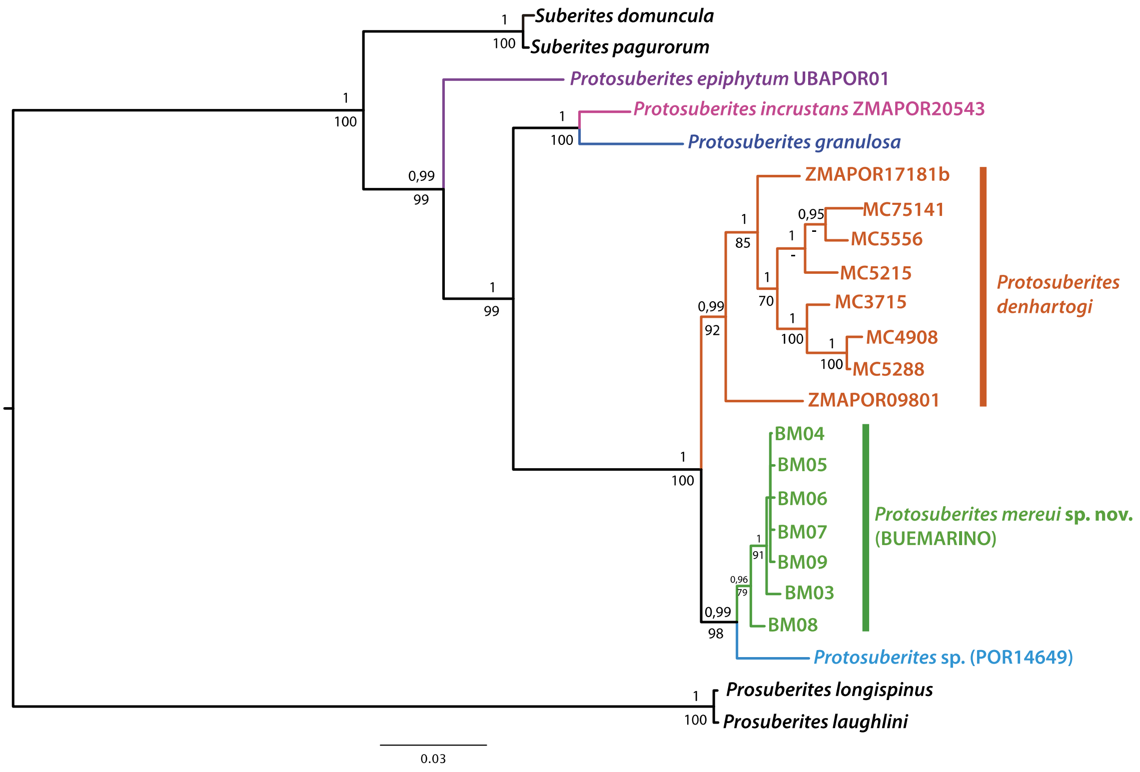

Figure 5. Phylogenetic tree of the genus *Protosuberites* from a concatenated analysis of COI, 18S, and 28S combining Bayesian inference (BA) and maximum likelihood (ML) methods. Numbers on nodes indicate posterior probabilities (BA) and bootstrap values (ML) out of 1 and 100, respectively. Only posterior probabilities over 0.95 and bootstrap values over 70 are indicated.

CHAPTER 3

Paper 3

-Manuscript in preparation-

Spicular morpho-traits of the Mediterranean palaeoendemic genus *Petrobiona* Vacelet & Lévi, 1958 (Porifera: Calcarea) by traditional and geometric morphometric approach

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Spicular morpho-traits of the Mediterranean palaeoendemic genus *Petrobiona* Vacelet & Lévi, 1958 (Porifera: Calcarea) by traditional and geometric morphometric approach

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Abstract

To investigate variation in size and shape of skeletal spicules of two cave-dweller species belonging to the Mediterranean endemic genus Petrobiona (Lithonida: Petrobionidae), we applied the traditional morphometric and geometric morphometric methods. Our aims are i) to clarify the problematic taxonomic status of P. incrustans Sarà, 1963 ii) to investigate in spicule's shape and size variation of P. massiliana Vacelet & Lévi, 1958 distant cave populations.

New records of P. massiliana enlarge the geographic range of this species to the eastern basin. The data indicate significant differences in the spicules shape between P. massiliana and P. incrustans, and more generally between encrusting and massive growth forms.

In addition, we found highly significant differences between sponges of 10 distant and scattered Mediterranean localities, which could indicate a degree of separation between these disjoined populations.

Keywords: *biodiversity, marine caves, cave-dwelling sponges, spicular complement analysis, taxonomy, biogeography.*

Introduction

The Mediterranean genus *Petrobiona* Vacelet & Lévi 1958, and the family Petrobionidae Borojevic, 1979, both monotypic, are problematic taxa apparently related to the Mesozoic fossil order Pharetronida (Vacelet & Lévi 1958; Pickett, 2002). At present, it is believed that the Mediterranean palaeoendemic genus *Petrobiona* is composed of two cave-dweller species. The taxonomic position of *P. incrustans* Sarà, 1963, never recorded after the first description, is problematic because it fluctuated over time between the status of valid species and *junior* synonym of *P. massiliana* Vacelet & Lévi, 1958 (Vacelet, 2002; Manconi et al., 2009). *P. incrustans*, restricted to a semi-submerged cave of the Ionian Sea (Capo di Leuca, Apulian coast) (Sarà, 1963) (Fig. 1) is currently considered, from a formal point of view, a valid species (see Longo e Pronzato, 2011).

Totally, very few records of *P. massiliana* occurred until now for about 50 years (Vacelet & Lévi, 1958; Sarà, 1963; Vacelet, 1964; Rützler, 1966; Pouliquen, 1972; Balduzzi et al., 1982, 1989; Pansini & Pronzato 1982; Bianchi et al., 1986; Bouduresque et al., 1986; Bianchi & Morri 1994; Corriero et al., 1997, 1999; Ben Mustapha et al., 2002; Vacelet et al., 2002; Harmelin et al., 2003; Borg et al., 2004; Hermans et al., 2010; Bianchi et al., 2010; Manconi et al., 2010, 2011; Cadeddu, 2012; Melis et al., 2013; Ricci et al., 2013; Cadeddu et al., 2013, 2014), notwithstanding extensive surveys in the Mediterranean caves (Fig. 1).

At present the geographical range of *P. massiliana* (Fig. 1) includes part of the Eastern Mediterranean (Adriatic Sea, Ionian Sea, Crete, Greece, and Malta Archipelago) and part of the western basin (Gulf of Lions, Sardinia Sea, Bonifacio Strait, Tyrrhenian Sea, and Tunisia).

The absence of this species despite focused surveys, from the westernmost area of the Mediterranean basin as the Balearic Islands, the Iberian coast, Algeria, and Morocco is peculiar (Vacelet et al., 2002; Manconi et al., 2009).

The disjunct distribution of cave-dwelling populations of *P. massiliana* in scattered Mediterranean caves, and its life history together with the status of protected species, focus on *P. massiliana* as a potential umbrella species for protection of cave-dwelling biocenosis and in general of the Mediterranean marine caves biotope (Manconi et al., 2009). *P. massiliana* was included in IUCN Red List since 1979, and more recently in the SPA/BIO Protocol of the Barcelona Convention (Manconi et al., 2009; Relini e Tunesi, 2009).

The morphological comparative analysis versus previous data from the entire geographic range highlighted that spicular traits seem to be highly conservative in contrast with a notable plasticity of growth form (Manconi et al., 2009). *P. massiliana* is characterized by a variability of growth forms ranging from massive to encrusting while *P. incrustans* displays only the encrusting form (Vacelet, 1964; Manconi et al., 2009; Longo & Pronzato, 2011).

In addition, *P. massiliana* presents a distinct category of diactines viz. macrodiactines (Vacelet, 1964; Cadeddu, 2012) and a distinct category of tetractines with short basal actins that are not present in *P. incrustans* (Sarà, 1963) (Fig. 2). Conversely, *P. incrustans*, according to the original description, presents larger triactines, smaller tetractines (pugioles), and a spiny surface of diapasons (Sarà, 1963) (Fig. 3).

The spot-like distribution pattern of *P. massiliana* and the life cycle characterized by short larval phases and low power of dispersal of lecitotrophic larvae (Gallissian & Vacelet, 1992; Manconi et al., 2009) suggest a high isolation between the populations.

The genetic differentiation in marine invertebrates is strongly influenced by their ability to dispersal (Lévin, 2006) and, consequently, their mode of reproduction (Palumbi, 1995). When species have a discontinuous patchy distribution, a high potential dispersal can allow even a moderate exchange of individuals between local subpopulations, while for marine organisms characterized by a low potential of dispersion, will create a spatial network of genetically differentiated populations (Dahdouh-Guebas et al., 2002) as in the case of *Petrobiona.* It has been shown that the level of genetic structure might be reflected in phenotypic differences (Palumbi, 1995; Madeira et al., 2012).

Some studies show that in some marine invertebrates the local presence of some morphological variants be explained by the ecophenotypic plasticity (Miner et al., 2005; Silva & Paula, 2008), particularly in sponges that are characterized by a high plasticity of some taxonomic characters (e.g. Carballo et al., 2006; Pronzato et al., 2003; López-Legentil et al., 2010). The relative contribution by genetic and environmental factors to the phenotypic expression is evident. Among sponges the key environmental factors are seasonal climate (Manconi & Pronzato, 1991), water physical-chemical characteristics, food availability and quality, predation pressures (Hill & Hill, 2002), and parasitism (Pawlik, 1983) all acting on the ecophenotypic plasticity.

The geometric morphometrics (Bookstein, 1991; Rohlf & Marcus, 1993) takes into account the spatial relationships between Landmarks, collected in the form of Cartesian coordinates in two or three dimensions, allowing the quantification and statistical analysis of the differences in shape (Bookstein, 1989, 1991; Rohlf & Marcus, 1993). With this new algebraic-geometric approach is possible to quantify the shape differences between two or more objects and decompose the component "shape" from the component "size", during the analysis of changes. The two components are then analysed both separately and jointly. In summary, we applied traditional and geometric morphometric methods to verify the presence of significant differences in shape and size of spicules at different levels: *i)* two species (*P. massiliana* vs. *P. incrustans*), *ii)* two growth forms (massive vs. encrusting of two species), and *iii)* 10 populations of two species (*P. massiliana* and *P. incrustans* from different localities).

All published and unpublished data on geographic distribution were also considered to define in detail the biogeographic pattern of the genus *Petrobiona*.

In this paper, we use an integrative approach to study the spicular complement of nine distinct karstic cave populations of *P. massiliana* from scattered areas of the Mediterranean Sea (Fig. 1) and the holotype of *P. incrustans*, either by a traditional morphometric analysis and a geometric morphometric method.

Materials and methods

Geographic distribution

All records of *P. massiliana* and *P. incrustans* were considered from the literature and unpublished dataset to obtain a detailed map.

Studied material for morphological analysis

Several specimens (n=25) of *P. massiliana* and *P. incrustans* were studied from 10 different locations in the Mediterranean Sea (Fig. 1). All the records refer to caves and all specimens are at present registered in the private collection of R. Manconi at the Department of Science of Nature and Territory (DIPNET), University of Sassari (Italy).

The following samples of *P. massiliana* are from shallow water caves (visual census SCUBA), preserved in alcohol 70-100 % or dry: RMNEREO02, Nereo Cave, Capo Caccia, Alghero (40°33'40"N, 8°9'41"E), 25 m depth, 6.viii.2009, R. Manconi leg., massive growth form; RMMAZZERE04, RMMAZZERE05, RMMAZZERE07, Mazzere Cave, Plemmirio, Siracusa, Sicily (37°00'18"N, 15°18’35”E), 10 m depth, 15.viii.2009, R. Manconi leg. massive growth form; RMGAMBERI02, RMGAMBERI03, RMGAMBERI05, Gamberi Cave, Plemmirio, Siracusa, Sicily (37°0'13.40"N, 15°18'47.36"E), 10 m depth, 15.viii.2009, R. Manconi leg., massive growth form.

The following samples of *P. massiliana* were kindly provided by Prof. Jean Vacelet: RMCALANQUE01, RMCALANQUE02, Endoume Cave, Marseille (43°16'47.24"N, 5°21'0.40"E), 7 m depth, 17.vi.1992, J. Vacelet leg., encrusting growth form; RMLEPLAN01, RMLEPLAN02, Plan Cave, Plane Island, Marseille (43°11'12.71"N, 5°23'18.52"E), 15 m depth, 9.ii.1982, J. Vacelet leg., encrusting growth form; RMBONIFACIO01, Cave du Phare, Bonifacio, Corsica (41°23'14.16"N, 9° 8'38.49"E), 8 m depth, 6.viii.1961, J. Vacelet leg., massive growth form; RMCROATIA01, RMCROATIA02, Milna Islan , Croatia (43° 9'N, 16°29' E), 38 m depth, 19.vi.2000, J. Vacelet leg., massive growth form; RMCRETE01, RMCRETE02, Crete Island, unknown location, 10 m depth, v.2008, J. Vacelet leg., massive growth form.

The following samples of *P. massiliana* were kindly donated by Dr. Sandra Ricci: PROVINON5, PROVINOT9, CERTA113, CERTB182-a, CERTB182-b, CERTA151, CERTA152-a, CERTA152-b, Azzurra Cave, Isola di Capri, Naples Gulf (40°33'38.63"N, 14°12'21.89"E), 2 m depth, 15.viii.2012, S. Ricci leg., massive growth form.

The previous samples were compared vs. the only available material: three slides of the holotype (specimen not available) of *P. incrustans* kindly lent by the Dipartimento di Scienze della Terra dell'Ambiente e della Vita, University of Genova, Genova, Italy: SLIDEINCRUSTANS01, SLIDEINCRUSTANS02, SLIDEINCRUSTANS03, Teatrino Cave, Apulia (39°47'29"N, 18°20' 38"E), 10 m depth, 1963, M. Sarà leg.

Morphological macro-traits such as growth form, size, and color were evaluated for all specimens by the stereomicroscope Zeiss Stemi 2000, and representative body fragments were dissected to characterize surface morphology and distribution of inhalant and exhalant apertures. Calcareous skeleton fragments and spicules were prepared by digestion in sodium hypochlorite at ambient temperature for a morphometric analysis of all spicular types by Leica Leitz DM RB microscope.

Traditional morphometric analysis

All 25 cave-dwelling specimens from 10 different localities (Fig. 1), which we consider as distinct populations, were subjected to traditional morphometric analysis.

The approach now referred to a traditional morphometrics is only a few decades old and it is characterized by the application of multivariate statistical methods to sets of variables that usually correspond to various measured distance of morpho-traits (Rohlf & Marcus, 1993).

In this case, we used spicular micrometries as main descriptors of the shape. For this purpose, for each of the 25 specimens included in the study, have been taken into consideration four distinct spicular categories of *P. massiliana* and *P. incrustans*: diactine, diapason, tetractine and triactine according to Vacelet & Lévi (1958), and Sarà (1963).

For each of the 10 populations 50 digital images were taken for each of the four mentioned spicular categories (200 images for each population). The images were taken at the LM Leica Leitz DM RB with a digital camera Canon Power Shot G6 with zoom 35 mm using a lens with a standard magnification (40x). On each of the 2,000 images obtained was placed a ladder. The spicular micrometries were detected with the program TpsDig2 (Rohlf, 2006).

The micrometries detected for different spicular types (Fig. 2) are:

- diactine: length (D1) and width (D2)
- triactine: actins length (D1, D2, D3), the distance between the actins tips (D4, D5, D6), thickness actin basal (D7)
- tetractine: length actin baseline (D1), lateral length Actin (D2), length of the two arms of actin baseline (D3, D4), length of the two arms of actin side (D5, D6) and thickness of basal actin (D7) and lateral (D8).
- diapason: basal actin length (D1), lateral actins length (D2, D3), distance between lateral actins (D4), central spine length (D5) and thickness of basal and lateral actins (D6, D7).

We obtained a dataset of 12,000 distances (Excel 2013), that have been ordered and subjected to logarithmic transformation $x = ln (x + 1)$ to reduce the variance (Rising et al., 1989).

This dataset was analyzed with the free package for statistical analysis R v.3.1.1, to perform exploratory statistical measures of central tendency, variability, dispersion and graphs such as trend lines, box plots, size-frequency histograms in order to identify trends and potential differences in the size classes. The degree of correlation between distances was also verified and expressed graphically with pair-plot graphs.

Some distances/spicular micrometries according to their degree of correlation were selected for multiparametric techniques of analysis as Principal Component Analysis (PCA). This type of analysis is the central core of traditional morphometric method, based on statistical study of distances (or lengths), used as the main descriptors of a shape (Mitteroecker & Gunz, 2009).

Geometric morphometric analysis

The morphology of the spicules was characterized in two dimensions for 25 specimens from 10 different sites.

This method yields detailed information about variation in the shape of objects at the same time as retaining a visual representation of them throughout the analysis (Mitteroecker & Gunz, 2009). Landmarks, which are coordinates of points, were recorded in each spicule, from high-resolution digital images, taken with a Canon Power Shot G6 digital camera with a 35-mm micro lens and the light microscope Leica Leitz DM RB, using consistent capture conditions for all specimens.

For each of the 10 populations 200 photos were collected, 50 for each considered spicular category.

The following landmarks were considered (Fig. 4): triactine: six landmarks; tetractine: eight landmarks; diapason: eight landmarks; diactine: four landmarks.

Totally 26 homologous landmarks were digitized on the acquired spicules images (2.000 photos) using TPSDIG version 2.16 (Rohlf, 2010a). The criteria used to choose the landmarks were their easy identification across samples and the ability of landmarks to capture the general shape of the spicule. In addition, we followed the homology criteria for landmark location according to Bookstein (1997).

Landmarks of each specimen were optimally aligned by MorphoJ v.1.05f (Klingenberg, 2008) and subjected to generalized Procrustes analysis (Rohlf & Slice, 1990) to obtain superimposed configurations of specimens *via* translation, scaling, and rotation, using the minimal bending energy method (Mitteroecker & Gunz, 2009).

Centroid size, considered as scaling factor during the superimposition process, was subsequently used as a measure of size for each specimen.

The term ‘shape’ used in the present study is thus defined as a geometric representation of an object after removing all non-shape variation as a result of measurement-associated errors (Claverie et al., 2011).

After alignment and standardization of landmarks configurations, Coovariance Matrix values were calculated. In particular, three Coovariance Matrix values were calculated for each of the four spicular categories, taking into account different categories of dataset such as membership of a population, species and growth form for each specimen.

A Relative Warp analysis (Bookstein, 1991) was performed on each Coovariance Matrix, which is the analogue of a Principal Components Analysis (Zelditch et al., 2004). This analysis reduces the dimensionality of multivariate data by transforming a set of many correlated variables into a small number of significant uncorrelated variables (Claverie et al., 2011) called RWs. These new sets of shape variables are then used for statistical comparisons of shape variation within and among groups.

We also used the thin-plate spline approach, by the software TPSRELW version 1.49 (Rohlf, 2010b), which allowed the visualization of shape change as deformation grids. This type of analysis has produced as graphical output a PC shape changes (diagram showing the shape changes associated with the PCs (eigenvectors), a Eigenvalues (diagram showing the percentages of total variance for which the PCs account) and a PC scores (scatter plot of PC scores).

In the PC score graphs, to graphically highlight the main groups of data in graphs plot has been used a confidence ellipse means clustering, with probability 0.9.

For each spicular dataset three different Canonical Variate Analysis (CVA) were conducted, taking into account different categories of dataset such as membership of a locality/population, species and growth form for each specimen. Canonical Variate Analysis (CVA) is a method used to find the shape features that best distinguish among multiple groups of specimens. Group membership is assumed to be known *a priori* (Klingenberg & Monteiro, 2005).

The graphical output of this analysis consists of two separate graphs. One of them shows the shape changes associated with the Canonical Variates (CVs). This change is the same as can be obtained by regression of shape onto the scores for the respective CV (Rohlf et al., 1996).

The second graph is a scatter plot of the CV scores.

In the PC score graphs, to graphically highlight the main groups of data in graphs plot, a confidence ellipse means clustering, with probability 0.9 were used.

With the CVA analysis matrices of pairwise Procrustes distances (Rohlf & Slice, 1990) and Mahalanobis distances (Penny, 1996) were calculated among all possible pairs of groups (populations, species and *habitus*). The Mahalanobis distances for all pairs are computed using the pooled within-group covariance matrix for all the groups jointly. This usage reflects the assumption of CVA that the within-group covariance matrices are identical.

For this reason, Discriminant Function analysis was used when were compared only two groups as in the case of *P. massiliana* vs. *P. incrustans* or massive vs. encrusting growth forms.

A permutation test (e.g. Klingenberg & Monteiro, 2005) was also performed, for Mahalanobis and Procrustes distances. The test was set up on a basis of 10,000 permutations and the P-values for all tests in pairs were presented separately for Procrustes and Mahalanobis distances.

In this way, we determined the degree and significance of shape and size variation between populations/geographical areas, between encrusting and massive growth forms, and between species. Finally, we have performed a cluster analysis for each spicular dataset of Mahalanobis distances between populations (Fig. 5) with the software Primer 6 (Primer-E Ltd, Plymouth, UK).

Results

Geographic distribution

At present the geographical range of *P. massiliana* (Fig. 1) includes part of the Eastern and western Mediterranean basins. Records from the Gulf of Lions refer to several cave as follows Endoume Cave, Figuier Cave, Trèmies Cave, Niolon Cave, Réseau de Veyron, Mont Rose Cave, Triperie Cave, Grand Congloué Cave, Oule Cave, La Vesse Cave, and Bagaud Island at Port-Cros. The Ligurian Sea harbour this species in a few caves: Santa Margherita Island near Cannes, Punta Falconara Cave at the Gallinara Island, and Bergeggi Cave. In the Sardinia Sea and Bonifacio Strait *P. massiliana* was recorded from: Fantasmi Cave, Terrazze Cave, Galatea Cave, Falco Cave, Bisbe Cave, Nereo Cave. Records from the Tyrrhenian Sea are from Stanze di Aladino Cave at Tavolara Island, Mitigliano Cave and Tuffo Tuffo Cave in the Gulf of Naples, together with Azzurra Cave at Capri, Cattedrale Cave at Capo Palinuro, and Verde Cave at the Ustica Island,. From North Africa only one record is known from Zembra Cave in northern Tunisia. The geographic range includes also part of the eastern Mediterranean basin with records in the Ionian Sea from Mazzere Cave, Gamberi Cave, and Gymnasium Cave at Siracusa, and Three Paléokastriza caves, and Navarin Cave from Greece. Only two records are known from the Sicily Channel: Gozo (Malta Archipelago), and Taccio Vecchio Cave (Lampedusa Island); Aegean Sea: Kalithea Bay (Rodi).

In this paper we report a new record from the Adriatic Sea that represent the first recordv from Croatia on two specimens of *P. massiliana*, with massive growth form, from an unnamed karstic cave of the Milna Island, 38 m depth, 19 June 2000, preserved in ethanol.

Three specimens of *P. massiliana* with massive growth form are recorded for the first time from Crete Island, unknown locality m 10 m depth, May 2008, preserved in ethanol.

Traditional morphometric analysis

The traditional morphometric analysis based on D1 distance of diactine spicular category of all investigated populations, revealed the presence of conspicuous number of macrodiactines (Tab. 1).

The analysis of the distance D1 as diactine length (Fig. 2) on which the traditional morphometric analysis was based, was conducted on a basis of 500 spicule belonging to 10 different populations (50 diactines for each population).

Macrodiactines are presents in populations of the Azzurra Cave (Capri), Gamberi cave and Mazzere cave (Siracusa). Data show that the range of macrodiactine measurements variation from Capri populations is of 82.5-120 x 5.35-6.8 μm, from Gamberi Cave population of 97-135 x 4.5-6.4 μm and from Mazzere cave population of 75-135 x 5.4-7 μm.

The analysis by LM of the holotype of *P. incrustans* revealed the absence of spines at the surface of diapason in contrast with Sarà (1963) considering this trait as one of the main apomorphies of *P. incrustans.*

For each spicular category, an analysis of the correlation between distances of spicular micrometries was performed with the R program. The results expressed in a scatterplot graph (Fig. 6) show a moderate positive correlation between the length and the thickness of diactines.

As for diapason the detected distances are all strongly positively correlated one with each other, except the distance D5 that describes the length of the central spine, not always present.

However, an unexpected correlation is present on diapasons between the thickness of the lateral and basal actins and the distance between the apices of the lateral actins.

As for tetractines the less correlated distances are those related to the thickness of the lateral and basal actins and the length of basal actin.

The higher levels of positive correlation are obtained for spicular micrometries detected in triactines, but the thickness of actins baseline is not correlated with any other distance, and length of actins between them is on average correlated.

A PCA analysis performed on each spicular category.

This analysis reveals that, for distances dataset for diactines, the PC1 explains 80% of the variation and PC2 7.6%.

In plot (PC1-PC2) (Fig. 7-a), the diactines of *P. incrustans* are better grouped than those of all other populations with negative values on PC1 component, whereas the diactines of Mazzere and Gamberi caves display the most positive values.

In the PCA analysis performed on diapason distances dataset, the PC1 explains 42% of the variation and PC2 18.4% (Fig. 7-b).

In the PCA analysis performed on tetractine distances dataset, PC1 explains 65% of the variation and PC2 16.4%.

In plot (PC1-PC2) (Fig. 7-c), two main groups are present and attributable to two main morphotypes.

In the PCA analysis performed on triactine distances dataset, the PC1 explains 86% of the variation and PC2 05.4% (Fig. 7-d).

Morphometric geometric analysis

A Relative Warp (RW) analysis performed with the program TPSRELW, version 1.49 (Rohlf, 2010b), conducted on a basis of 500 configurations of landmarks digitized on tetractines (50 for each of the 10 populations included in the study), has detected the presence of two distinct main morphotypes: tetractine A (TA) and tetractine B (TB) (Fig. 8).

The morphotype TA is the most frequent among tetractines and characterized by a larger central actin compared to lateral actins (Fig. 8). There are two variants of this morphotype in which the actin baseline can be more or less than the upper central (Fig. 8). In contrast the morphotype TB is characterized by a short basal actin and particularly long lateral actins (Fig. 8). An RW analysis exclusively performed on tetractines of *P. incrustans* from the type locality highlighted the presence of both morphotypes TA and TB (Fig. 9).

A comparative analysis of the triactines and tetractines size (1,000 spicules in total) of different populations was carried out by using the Centroid Size (CS) as a measure of spicules size.

For the triactines CS analysis, a boxplot (Fig. 10) shows that the *P. massiliana* populations have an average higer value from Capri vs. a smaller value from the Grotta di Nereo (Sardinia). In the same analysis *P. incrustans* shows intermediate values of triactine CS.

Most variability for triactine CS is associated with the population of Crete and the lower with Nereo Cave, while populations of Sicily and Capri show the absolute highest dimensions of triactines, and Nereo the lowest.

With regard to the comparative analysis of tetractines, a boxplot (Fig. 11) shows how the median values are highest for the encrusting forms of Plan Cave (Marseilles) which present even more outliers, while the average values are lower for the Crete and Gamberi Cave specimens.

The higher variability of tetractine CS was observed in the encrusting morphs of the Plan Cave, and the lower of the Nereo Cave. *P. incrustans*, compared with others populations shows intermediate values also in tetractine size.

CVA analysis conducted on all spicular dataset, taking into account the shape differences between *P. massiliana* and *P. incrustans* showed significant values only in the diapason shape with Procustes distances value of 0.0905 and P-value from permutation tests (10,000 permutation rounds) of 0.0011 (Tab. 2).

Another CVA analysis was performed on all spicular datasets, taking into account the growth form differences of all the specimens subjected to the study. It was found that there are highly significant differences (P-values <.0001) in the diapason and tetractine shape of encrusting vs. massive growth forms, with PD values of 0.0872 and 0.0668, respectively (Tab. 2).

The latter CVA analysis was performed on all spicular datasets, taking into account the population of origin of the studied specimens.

Most of the Mahalanobis Distances among populations (Tab. 3) are highly significant for all spicular categories.

The pairwise test reveals that 18 comparisons are not significant between diactines of different locations, 3 for the diapasons, 6 for tetractines and 15 for triactines.

In fact, CVA analysis between populations show the most obvious results if conducted on diapason and triactine dataset.

The plot of the CVA conducted on diapason dataset (Fig. 12) shows a greater separation for *P. incrustans* and Croatia population.

The diapason of *P. incrustans* shows a tendency towards central values in CV1, which gives us information about changes in the opening angle and thickness of the lateral actins, and negative values in CV2, which indicates changes in the basal actin length and central plug.

Croatia population shows diapason positive values in the CV1 and slightly in CV2, which indicates a larger angle of aperture of lateral actins, thicker lateral actins and presence of a central spine.

The plot of the CVA conducted on tetractine dataset (Fig. 13) shows a greater separation for Crete, Plan and Nereo Cave populations.

Plan and Nereo take positive values in CV2 indicating a lowering of lateral actins joined to a reduction of baseline actin dimensions, while the tetractines from Crete have a more marked shortening baseline actin (Fig. 13).

All three latter populations (Plan, Nereo, and Crete) have tetractines with predominant morphotype 2 (Fig. 13).

The population with the highest number of significant comparisons Mahalanobis Distance based is Croatia while the least number of comparisons is assigned to the holotype of *P. incrustans* from Teatrino Cave (Tab. 4).

The cluster analysis (Fig. 5) performed on the same "between populations distance matrix distance" shows different results depending on the used marker (spicular category). In the diactine based analysis the more similar populations are those from Nereo and Endoume cave, while the most diverging one is from Siracusa (Mazzere Cave).

The diapason based analysis shows that more similar populations are the encrusting form from Plan and Endoume Caves, while Nereo is the most divergent.

In the tetractine based analysis, the more similar populations are Nereo and Mazzere and the more divergent is that of Croatia.

In the triactine based analysis most similar population are Nereo and Plan, and the farthest is from Crete.

Major variations (Mahalanobis Distance values) are observed for the diapasons and the tetractines (Fig. 5).

Discussion

Geographic distribution

Until now, the species was reported in a few scattered sites in the Gulf of Lions, Ligurian Sea, Sardinian Sea, Strait of Bonifacio, Tyrrhenian Sea, Ionian Sea, Adriatic Sea, Strait of Sicily, Tunisia and Aegean Sea (Fig. 1) (Vacelet & Lévi, 1958; Sarà, 1963; Vacelet, 1964; Rützler, 1966; Pouliquen, 1972; Balduzzi et al., 1982, 1989; Pansini & Pronzato 1982; Bianchi et al., 1986; Bouduresque et al., 1986; Bianchi & Morri 1994; Corriero et al., 1997, 1999; Ben Mustapha et al., 2002; Vacelet et al., 2002; Harmelin et al., 2003; Borg et al., 2004; Hermans et al., 2010; Bianchi et al., 2010; Manconi et al., 2010, 2011; Cadeddu, 2012; Melis et al., 2013; Ricci et al., 2013; Cadeddu et al., 2013, 2014).

The synthesis of all records contribute to better define the biogeographic pattern of the species in the Western and Eastern Mediterranean basin (Fig. 1). The new records of *P. massiliana* enlarge its distribution range towards the Eastern Mediterranean Basin i.e. eastern Adriatic Sea and Aegean Sea.

P. massiliana was not found in the westernmost portion of the Mediterranean basin as the Balearic Islands and the Iberian coast, despite the proximity of these areas with a some site of records i.e. Sardinia Sea, Gulf of Lions. This finding may be explained by the fact that these are rare subject to a low sampling effort (Manconi et al., 2009). Then it is necessary to broaden the base sample, investigating under sampled areas of the Mediterranean (e.g. Eastern Basin, North Africa) to piece together the entire distribution range of the genus.

Our data supports the hypothesis by Manconi *et al.* (2009) that *P. massiliana* is not so rare in the Mediterranean basin but it is represented by several cryptic populations highly fragmented in space mostly restricted to the Cave biotope.

Growth form

According to the literature, the growth form of *P. massiliana* is highly variable (Vacélet 1964; Manconi et al 2009).

This variability is also confirmed by the specimens subjected to this study that varies from thin encrusting morphs (Fig. 14) (Plan, Endoume and Teatrino Caves), to erected finger-like morphs (Fig. 14) (Nereo Cave) to erected globular morphs (Fig. 14) (Cave du Phare, Azzurra Cave, Croatia, Crete, Mazzere Cave, and Gamberi Cave).

The phenotypic plasticity within *P. massiliana* can be explained by action of hydrological and biotic factors or phenomena of spatial competition with other benthic organisms (mainly sponges, bryozoans, serpulids and madreporarians) or dependent to predation pressure (Manconi et al., 2009). The species has never been found in the same microhabitat colonized by lithistids (Manconi et al., 2006), as previously suggested by Pouliquen (1972).

In any case, the growth form variability supports the opinion of Vacelet et al. (2002) on the state of *P. incrustans* as a junior synonym of *P. massiliana*, but this thesis was formulated without any study of the type material (see Longo & Pronzato, 2011).

Macrodiactines

Some authors (e.g. Manconi et al., 2009; Cadeddu, 2012; Melis et al., 2013) argue that in contrast to the variability in growth form, the spicular complements characters seem to be highly conservative.

However, it was never carried out a statistical analysis on spicular micrometries, and only Manconi et al., 2009 report in literature number of samples and spicules analyzed. We created a database of spicular micrometries obtained on a broad-based of spicules and specimens, on which has been applied traditional morphometrics method.

In addition to for the first time has been applied the geometric morphometric method to statistically study the shape variability of sponge's spicular complement.

Macrodiactines belong to a spicular type ranging from absent to rare in most of the dataset published until now by Vacelet (1964) for a few populations of some Marseilles caves Vacelet (1964) and cave-dwelling populations Sardinian (Falco Cave) and Sicilian (Mazzere, Gamberi and Gymnasium Cave from Plemmirio MPA and Taccio Vecchio Cave from Lampedusa) (Cadeddu, 2012; Melis et al., 2013). The morphometries of macrodiactines reported by Vacelet (1964) match the range 270-480 x 11-20 µm. The morphometries of macrodiactines reported by Cadeddu (2012) match the range: Mazzere Cave (87.5-235 x 2.5-7), Gamberi Cave (97.5-3).

The presence of macrodiactines in presently considered populations of Capri (n=1) and Siracusa (n=2) confirms the importance of this spicular category as a diagnostic spicular trait.

These three populations located in the central region of the Mediterranean basin, geographically closer than the other populations we studied, showing the macrodiactines markedly smaller than those reported by Vacelet (1964), but significantly larger than the diactines.

Analysis of P. incrustans

The application of both morphological methods to check the validity of *P. incrustans* as species, has given conflicting results.

In contrast with what is reported in the original description (Sarà, 1963) *P. incrustans* present both morphotypes of tetractines, while we did not find spines on diapason.

No significant differences was found in triactine and tetractine size of *P. incrustans*, compared with populations of *P. massiliana*.

Conversely the absence of macrodiactine in the analysed slides of *P. incrustans* represents the first evidence to support the validity of this species according to original description. It is necessary to consider that in accordance with Vacelet (1964), the location of macrodiactine, when present, is exclusive of sponge basal portion, the one in contact with the skeleton.

The absence of macrodiactine in the *P. incrustans* holotype of could therefore not be distinctive character, as influenced by the way in which the holotype was studied.

Also a significant difference is highlighted in shape of diapason of *P. incrustans* compared to those of *P. massiliana*. In particular, the former species would have lateral actines more spread apart and thicker, with higher probability of presenting the central spines.

However the number of significant comparisons of *P. incrustans* based on pairwise tests on Mahalanobis distance on all spicular traits is globally the lowest. This result is reinforced by the comparison between massive and encrusting forms.

Comparison between massive and encrusting forms

Highly significant differences in shape of diapason and tetractine among encrusting and massive growth forms of *Petrobiona* were found.

The spicular difference in shape recorded in the holotype of *P. incrustans*, at the level of diapason, and in general the difference among encrusting and massive growth form at diapason and tetractine level, could be suggestive of some kind of disturbance produced, for example, by the action of physico-chemical factors as water turbulence.

Several authors argue that *P. massiliana* prefer low water movement conditions (Pouliquen 1969 ; Corriero et al. 2000; Vacelet 1976; Pansini et al. 1977). It was observed that water movement seems to strongly affect the topographic distribution of this sponge in Sardinian caves (Manconi et al. 2009), where few scattered encrusting specimens or isolated specimens occur in high-water movement conditions, while large facies of massive specimens are prevailing when water movement is lower. Indeed Manconi et al. (2009) reported a facies of the most developed specimens (i.e. erected fan-shaped growth forms) restricted to the inner totally dark and confined zone of the Fantasmi Cave in western Sardinia.

A further potential environmental constraint for this species could be represented by freshwater infiltrations into marine caves Manconi et al. (2009). It is possible that in the long term, the degree of freshwater input and the related salinity variations may play a key role in the control of settlement and persistence of *P. massiliana* populations in submerged caves, as well as changes in the morphology of spicules in response to these changes Manconi et al. (2009).

The differences of some spicular traits observed in *P. incrustans* and in general between encrusting and massive forms may be connected to the mechanical function to which absolve these spicules.

Differences between populations

It is evident the contribution that a reduced panmissis, within the distribution range of *Petrobiona*, brings in explaining the differences among populations studied in this paper. In fact significant differences in all populations subjected to comparison were found.

Some populations result to be more "different" than others, such as those from Crete and Croatia and other less, like Teatrino Cave population and encrustings forms of Endoume Cave.

The only environmental factors alone can not explain these differences, because it would mean that each cave has exclusive and unique environmental and biotic condition, which is only partially true.

The observed differences suggest a genetic isolation between populations, dependent on the low power dispersal of larvae.

The presence of *P. massiliana* in distant karstic areas matches a biogeographic model of disjunct distribution with populations isolated in cryptic habitats as suggested by Manconi et al. (2009).

Conclusions

The use of a multidisciplinary approach to the study of morphological characters of the genus *Petrobiona* has given good results.

Was emerged a substantial differentiation degree in diapasons shape of *P. incrustans* vs. *P. massiliana* however they are not yet sufficient evidence to consider *P. incrustans* valid species and will need to investigate this issue with further studies expanding basis sample.

The high degree of differentiation between the analysed populations especially in diapasons and tetractine shape suggests i) the use of these two markers as reliable descriptors of the differences in shape and ii) the use of a molecular approach to confirm hypotheses of a reduced gene flow between geographically distant populations.

Further investigations are in progress to verify the degree of genetic diversity between these populations and the correlation between morphological and genetic diversity, and between these and some factors such as geographical distance between populations.

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TABLES

Table 1. *P. massiliana* and *P. incrustans*. Sample summary: species, number of specimens, growth form, and presence/absence of macrodiactines from each Mediterranean locality included in the present study.

Cave/Locality	**Species**	**Number of specimens**	**Growth form**	**Macrodiactines µm**
Teatrino Cave, S-Apulia	*P. incrustans*	1 (holotype)	Encrusting	absent
Cave du Phare , S-Corsica	*P. massiliana*	1	Massive	absent
Endoume Cave, Marseille	*P. massiliana*	3	Encrusting	absent
Azzurra Cave, Capri	*P. massiliana*	8	Massive	82.5–120 x 5.35–6.8
Crete	*P. massiliana*	3	Massive	absent
Milna Island, Croatia	*P. massiliana*	2	Massive	absent
Gamberi Cave, NE-Sicily	*P. massiliana*	1	Massive	97– 135 x 4.5– 6.4 µm
Plan Cave, Marseille	*P. massiliana*	2	Encrusting	absent
Mazzere Cave, NE-Sicily	*P. massiliana*	3	Massive	75–135 x 5.4–7 µm
Nereo Cave, NW-Sardinia	*P. massiliana*	3	Massive	absent

Table 2. *P. massiliana* and *P. incrustans*. Procustes Distances (left) between species and between growth forms. Shape changes in diactines (line 1), diapason (line 2), tetractines (line 3), and triactines (line 4) are indicated in each box. P-values (right) from permutation tests (10,000 permutation rounds) for Procustes Distances between species and growth form. Red = highly significant, orange=significant, and black= not significant.

	P. massiliana	***P. incrustans***	**Massive growth form**	**Encrusting growth form**
P. massiliana		0.7310 0.0011 0.7855 0.5373		
P. incrustans	0.0085 0.0905 0.0167 0.0177			
Massive growth form				0.5835 <.0001 <.0001 0.3025
Encrusting growth form			0.0066 0.0872 0.0668 0.0145	

Table 3. *P. massiliana* and *P. incrustans*. Mahalanobis Distances among populations (left). Shape changes in diactines (line 1), diapason (line 2), tetractines (line 3), and triactines (line 4) are indicated in each box. P-values (right) from permutation tests (10,000 permutation rounds) for Mahalanobis Distances among populations. Red = highly significant, orange=significant, and black= not significant.

	Bonifacio	**Endoume**	**Capri**	**Crete**	**Croatia**	**Plan**	**Mazzere**	**Nereo**	**Teatrino**	**Gamberi**
Bonifacio		0.0347 0.1617 <.0001 0.0574	0.1910 0.0041 <.0001 0.0023	0.0055 0.0001 <.0001 <.0001	0.0051 <.0001 0.0004 0.0001	0.0806 <.0001 <.0001 0.7757	<.0001 0.0126 <.0001 0.0003	<.0001 0.0211 <.0001 <.0001	0.0038 <.0001 0.0041 0.1940	0.0008 0.0006 0.0041 0.0053
Endoume	0.6402 0.8174 1.3050 0.7672		0.9208 0.0516 <.0001 0.0023	0.0156 0.0002 0.3837 0.0001	0.1707 <.0001 0.0115 0.0002	0.0787 0.0312 0.0011 0.1164	0.0021 0.2125 0.0372 0.0056	0.0591 0.0143 <.0001 <.0001	0.5960 0.0018 0.0760 0.1024	0.0992 <.0001 0.1309 0.0752
Capri	0.5205 0.9151 1.3101 0.8283	0.1791 0.7996 1.3726 0.7717		0.0912 <.0001 <.0001 0.0302	0.0610 <.0001 0.0061 0.0056	0.2357 <.0001 <.0001 0.0011	0.0015 <.0001 <.0001 0.1532	0.0151 0.0001 <.0001 0.0026	0.3951 <.0001 <.0001 0.0103	0.0501 0.0010 <.0001 0.0217
Crete	0.8117 1.2352 1.4779 1.2336	0.6581 1.2755 0.7433 1.1373	0.5575 1.2755 1.5708 0.7024		<.0001 0.0013 0.0001 0.0030	0.0001 <.0001 <.0001 0.0005	0.0004 0.0016 0.0055 0.0602	<.0001 0.0001 <.0001 0.0892	0.0051 0.0048 0.0324 0.0066	<.0001 0.0416 0.0052 0.0061
Croazia	0.7709 1.6274 1.1200 1.1952	0.4431 1.6771 0.9468 1.0473	0.5672 1.3448 0.8661 0.7754	1.0398 1.2036 1.3565 1.0358		0.0182 <.0001 0.0007 0.0001	0.0041 <.0001 0.0015 0.0010	0.0328 <.0001 <.0001 <.0001	0.2629 <.0001 0.0044 0.0002	0.1268 0.0054 0.0248 0.0010
Plan	0.5623 1.2437 1.1678 0.4396	0.4956 0.9234 1.1272 0.6910	0.4376 1.1053 1.7926 0.8372	0.9188 1.6284 1.6197 1.0839	0.6047 1.7037 1.1791 1.1205		<.0001 0.0018 <.0001 0.0010	0.0003 0.0003 <.0001 <.0001	0.1003 0.0021 0.0004 0.7230	0.1132 <.0001 0.1066 0.0095
Mazzere	1.2861 0.9822 1.2507 1.1308	0.7673 0.8111 0.8960 0.9286	0.9069 1.0180 1.2707 0.5766	0.9998 1.1565 1.1666 0.8278	0.7962 1.3645 1.0988 1.0809	1.1066 1.0556 1.3849 1.0796		0.2186 0.0044 0.0007 0.6964	0.0906 0.0038 0.4718 0.0736	0.0011 0.0048 0.2271 0.0788
Nereo	1.2062 1.0298 1.5889 1.4419	0.5830 1.1342 1.5137 1.2088	0.7393 1.1443 1.3403 0.8389	1.0149 1.3885 1.9838 0.8002	0.6362 1.5581 1.4326 1.1648	0.9184 1.2305 1.6365 1.3325	0.5110 1.1629 1.2268 0.4987		0.2785 0.0091 0.0175 0.0005	0.1672 0.0119 0.0021 0.0769
Teatrino	0.8155 1.5276 1.1048 0.6584	0.3142 1.3536 0.9153 0.6998	0.4055 1.3622 1.2447 0.7069	0.7727 1.2917 1.1045 0.9315	0.4369 1.8027 1.1319 1.1927	0.5222 1.2293 1.3361 0.4458	0.5934 1.2314 0.7344 0.7566	0.4655 1.3183 1.0835 1.0459		0.2901 0.0070 0.5556 0.0279
Gamberi	1.0317 1.1605 0.9434 0.9703	0.5691 1.3382 0.8369 0.7702	0.6719 1.0274 1.2996 0.7126	1.1528 0.9757 1.2068 1.0085	0.5534 1.0951 0.9852 1.0687	0.5618 1.3624 0.9008 0.9270	0.9321 1.0749 0.8125 0.7990	0.5623 1.0969 1.1905 1.0459	0.4806 1.2375 0.7292 0.8427	

Table 4. *P. massiliana* and *P. incrustans*. Number of comparisons statistically significant for each population based on the Mahalanobis distances between populations. The lowest and the highest values are in bold.

	N° of significant P - values
Bonifacio	29
Endoume	21
Capri	28
Crete	31
Croatia	**32**
Plan	27
Mazzere	25
Nereo	28
Teatrino	**20**
Gamberi	21

FIGURES

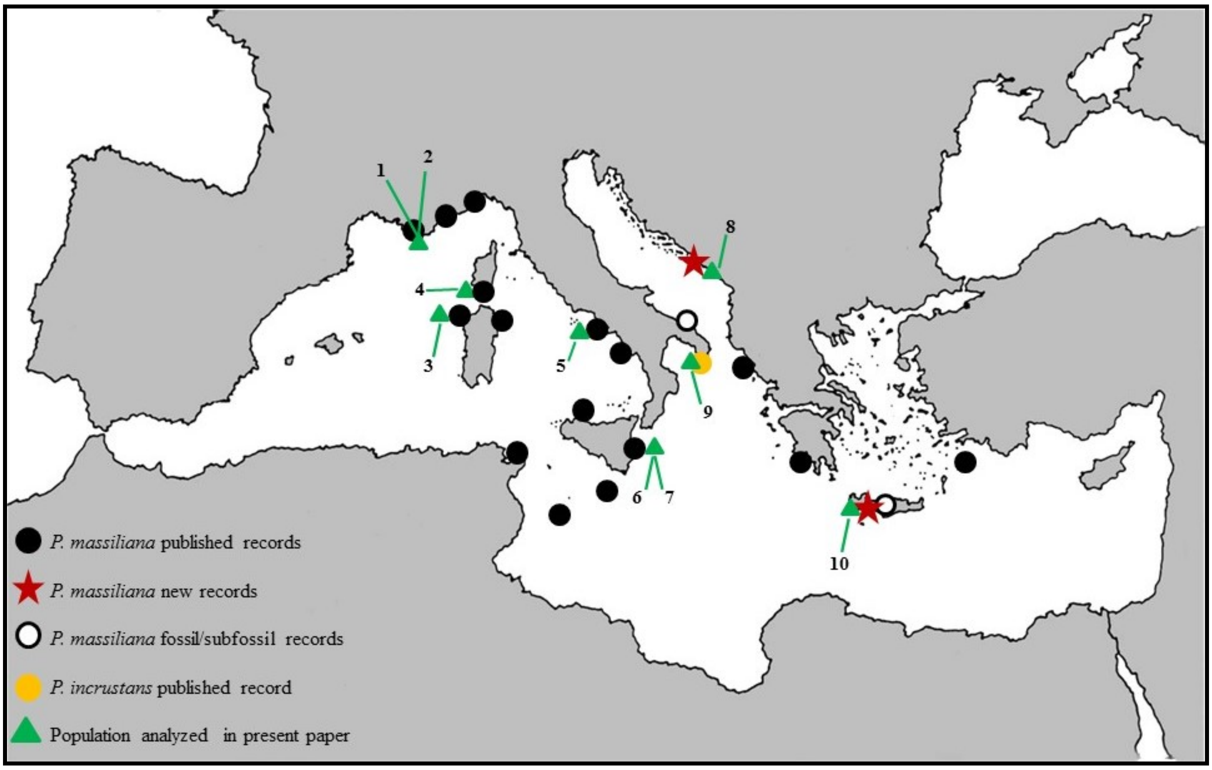

Figure 1. Biogeographic pattern of the genus *Petrobiona* endemic to the Mediterranean Sea. *P. massiliana* and *P. incrustans*. In black geographical localisation of published record of *P. massiliana.* geographical localisation of the populations (green triangle) of *P. massiliana* and *P. incrustans* studied in this paper as follows: Endoume Cave, Marseille (1); Plan Cave, Marseille (2); Nereo Cave, Alghero (3); Cave du Phare. Bonifacio, Corsica (4); Azzurra Cave, Capri (5); Gamberi Cave, Siracusa (6); Mazzere Cave, Siracusa (7); Ile Miljet, Croatia (8); Teatrino Cave, Puglia (9); Crete Island, unknown location (10).

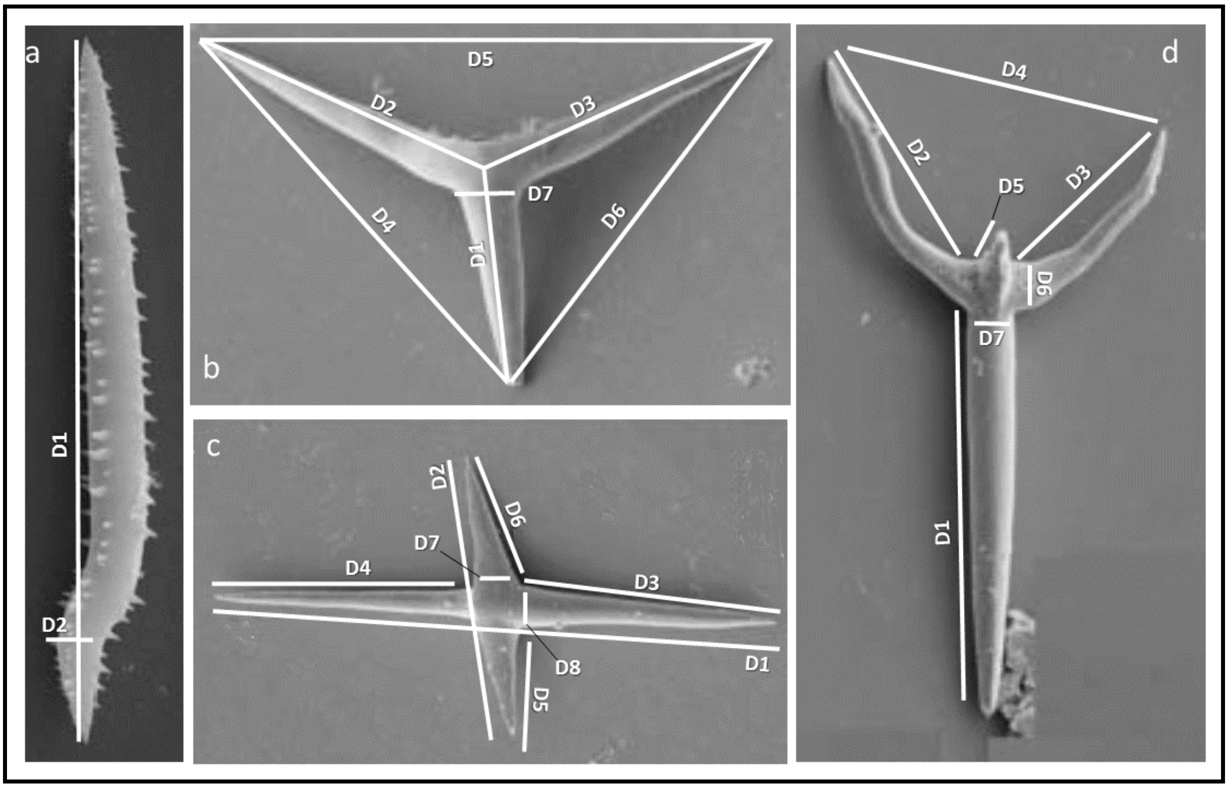

Figure 2. *Petrobiona massiliana*. Spicular complement by SEM. Graphic representation of the 24 distances or spicular micrometries taken into account in traditional morphometric analysis, divided into spicular categories: diactine (a), triactine (b), tetractine (c), diapason (d). (Modified from Manconi et al., 2009).

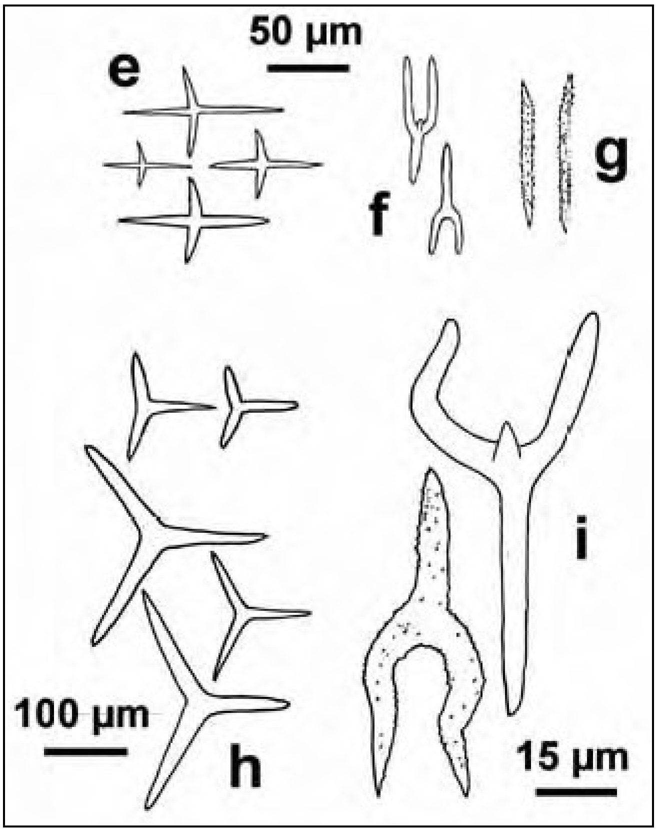

Figure 3. *Petrobiona incrustans*. Spicular complement of tetractine (e), diapason (f), diactine (g), triactine (h), Tuning- fork with rough surface and diapason whit central plug (i). (Modified from Sarà, 1963).

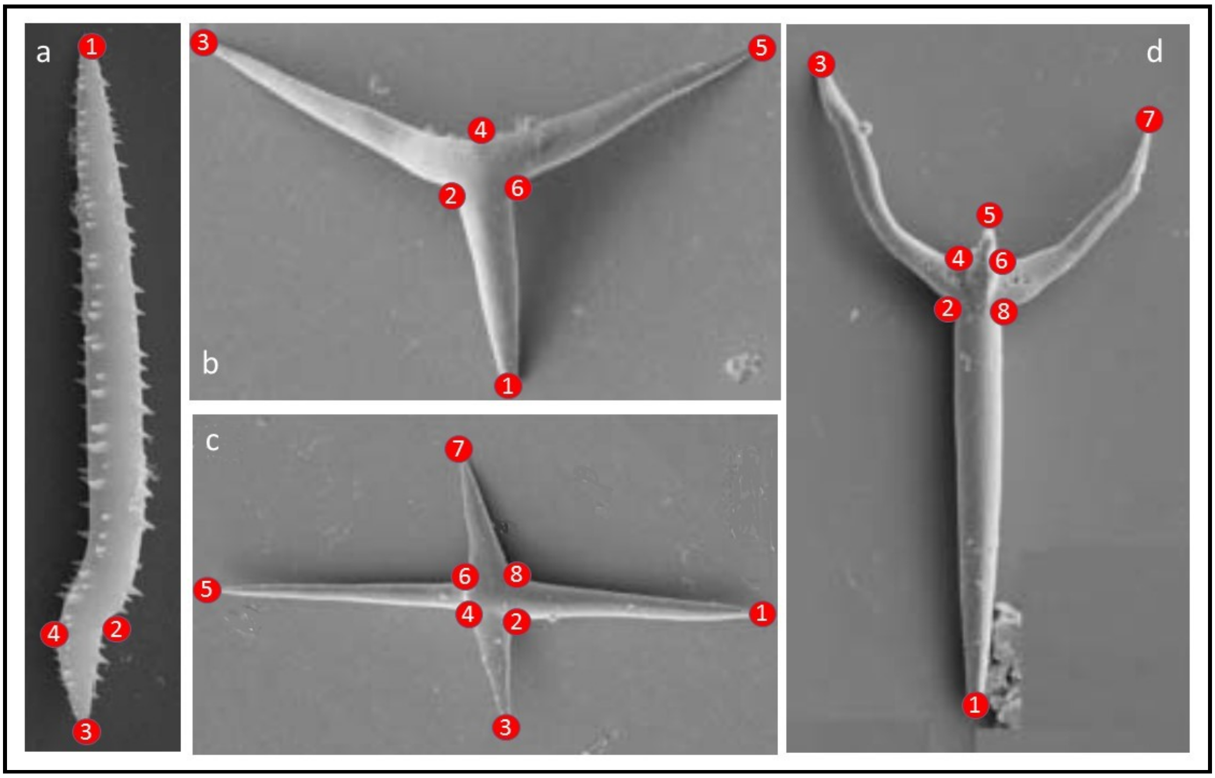

Figure 4. *Petrobiona massiliana*. Spicular complement by SEM. Graphic representation of the 26 Landmarks on which the morphometric geometric analysis is based, divided into spicular categories: diactine (a), triactine (b), tetractine (c), diapason (d). (Modified from Manconi et al., 2009).

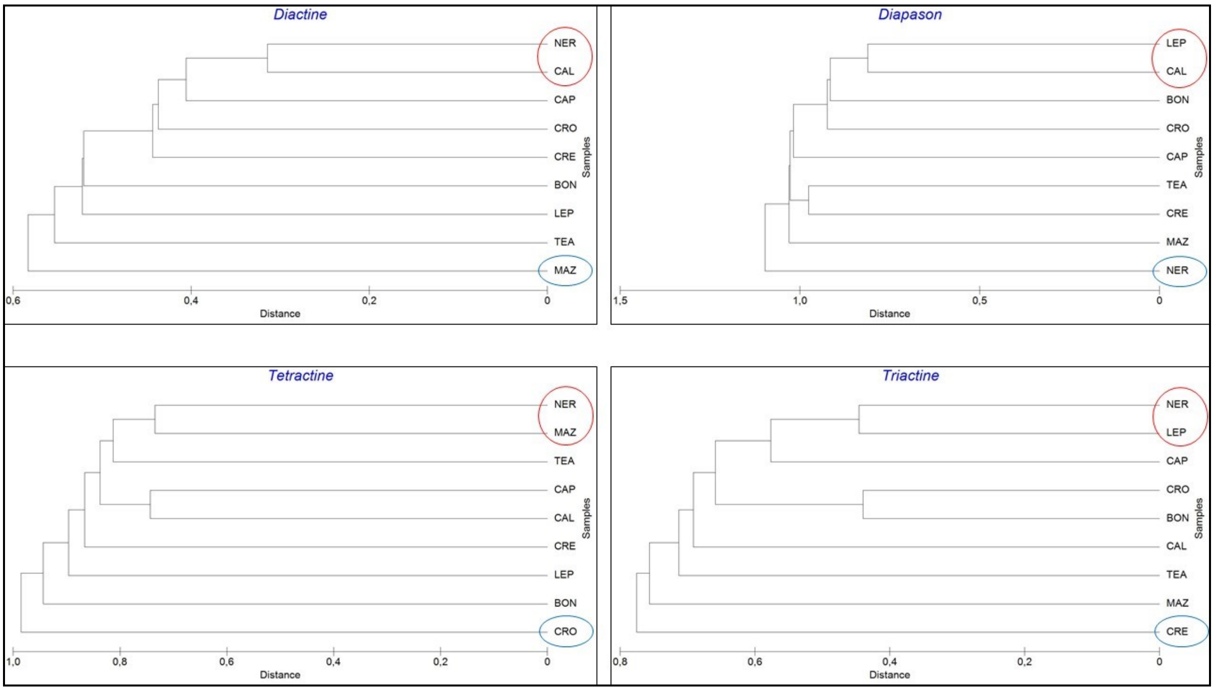

Figure 5. *P. massiliana* and *P. incrustans*. Cluster analysis for each of the four considered spicular dataset (xx spicules for each population) using Mahalanobis distances between populations. The red circle indicates the nearest populations and the blue one the most distant.

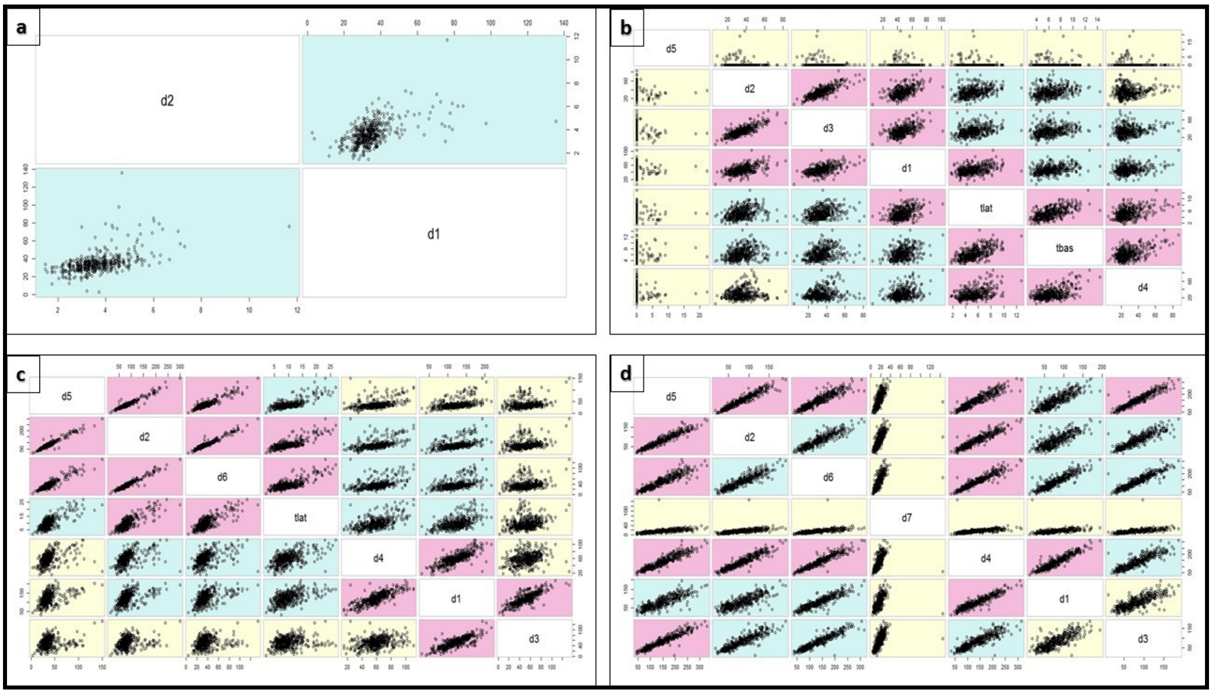

Figure 6. *P. massiliana* and *P. incrustans*. Scatterplot of the transformed distances. a) diactine; b) diapason; c) tetractine; d) triactine. Yellow indicates low values of correlation, blue medium values, and purple higher values.

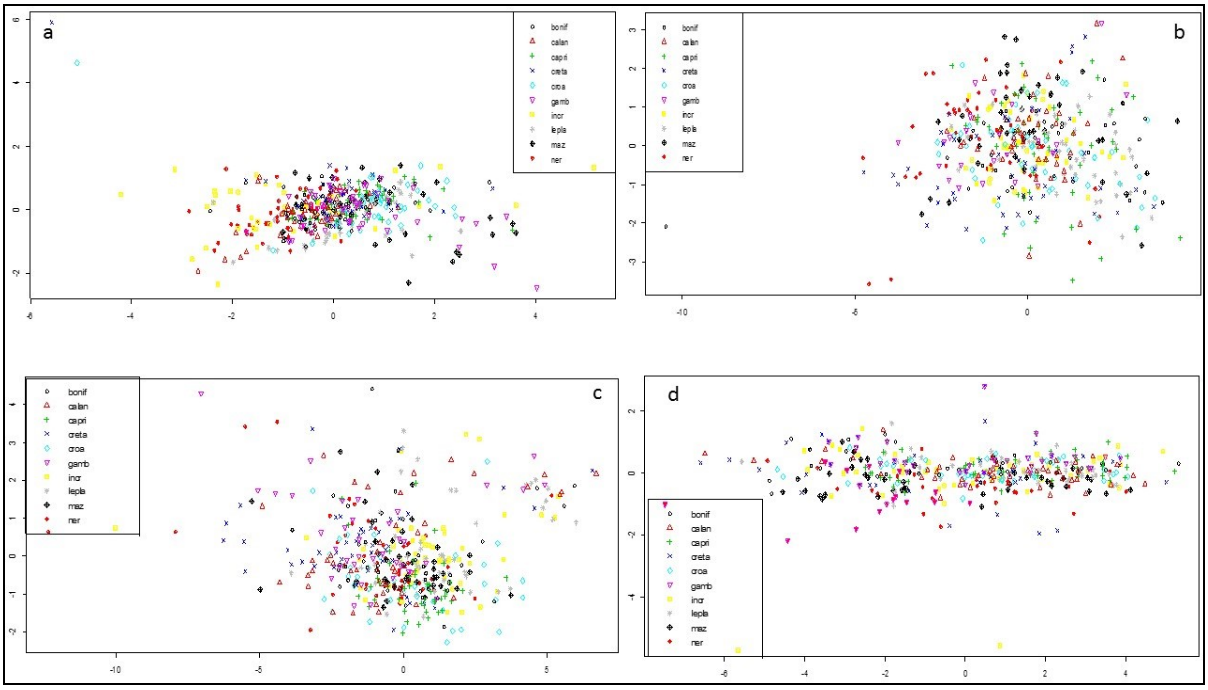

Figure 7. *P. massiliana* and *P. incrustans*. Plot of PCA analysis performed on the distance dataset of diactine (a), diapason (b), tetractine (c), triactine (d). In the x-axis PC1 component and y-axis PC2.

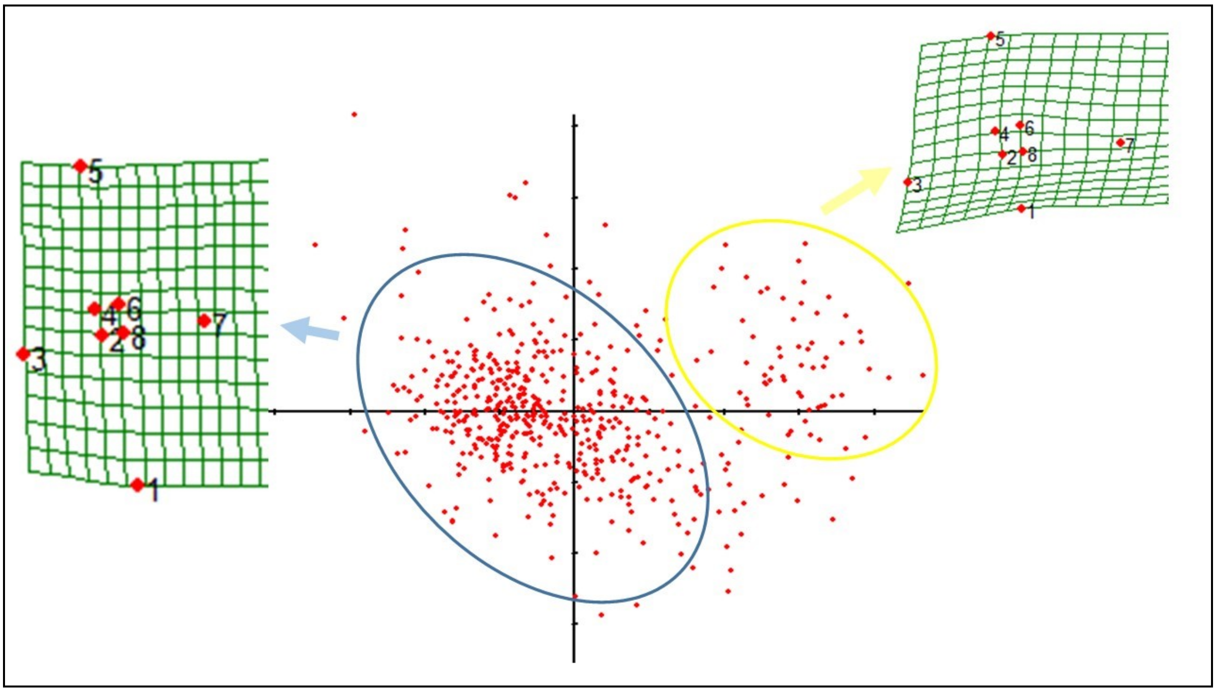

Figure 8. *P. massiliana* and *P. incrustans* (10 populations). Plot of Relative Warp analysis performed on Landmarks configuration of 500 tetractines shows two clusters. The morphotype 1 (blue circle) characterizes specimens with tetractines bearing central actine larger than the lateral one. The morphotype 2 (yellow circle) characterize specimens with tetractine bearing a short basal actin and particularly developed lateral actins.

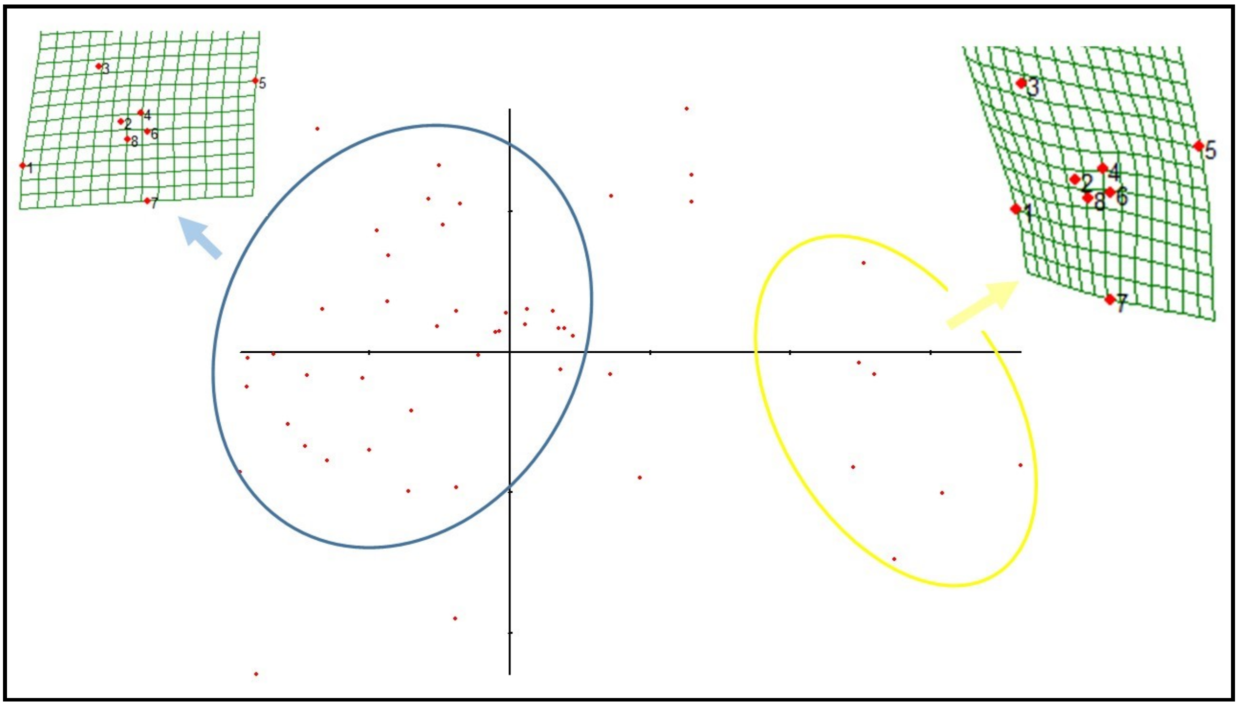

Figure 9. *Petrobiona incrustans*. Plot of Relative Warp analysis performed on Landmarks configuration of 50 tetractines shows two clusters. The morphotype 1 (blue circle) characterize specimens with tetractines bearing central actine larger than the lateral one. The morphotype 2 (yellow circle) characterize specimens with tetractine bearing a short basal actin and particularly developed lateral actins .

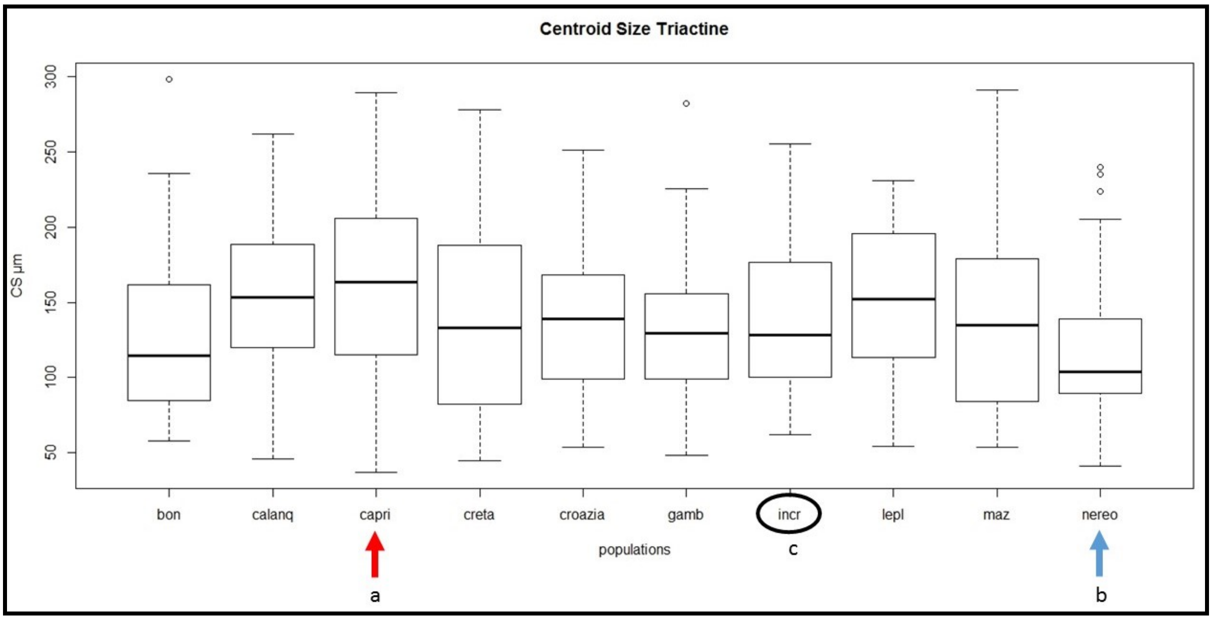

Figure 10. *P. massiliana* and *P. incrustans*. Box plots showing the size of the Centroid of triactines of all populations included in the study. (a) higher mean values (Capri population), (b) lower mean values (Nereo population).

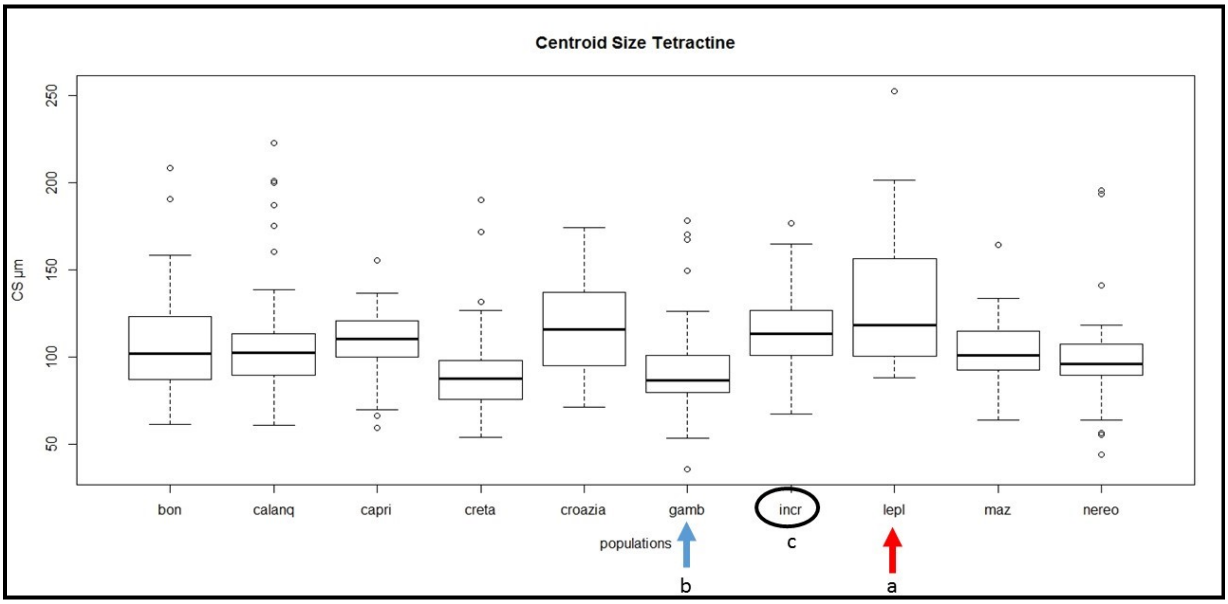

Figure 11. *P. massiliana* and *P. incrustans*. Box plots showing the size of the Centroid of tetractines of all populations included in the study. (a) higher mean values, (b) lower mean values.

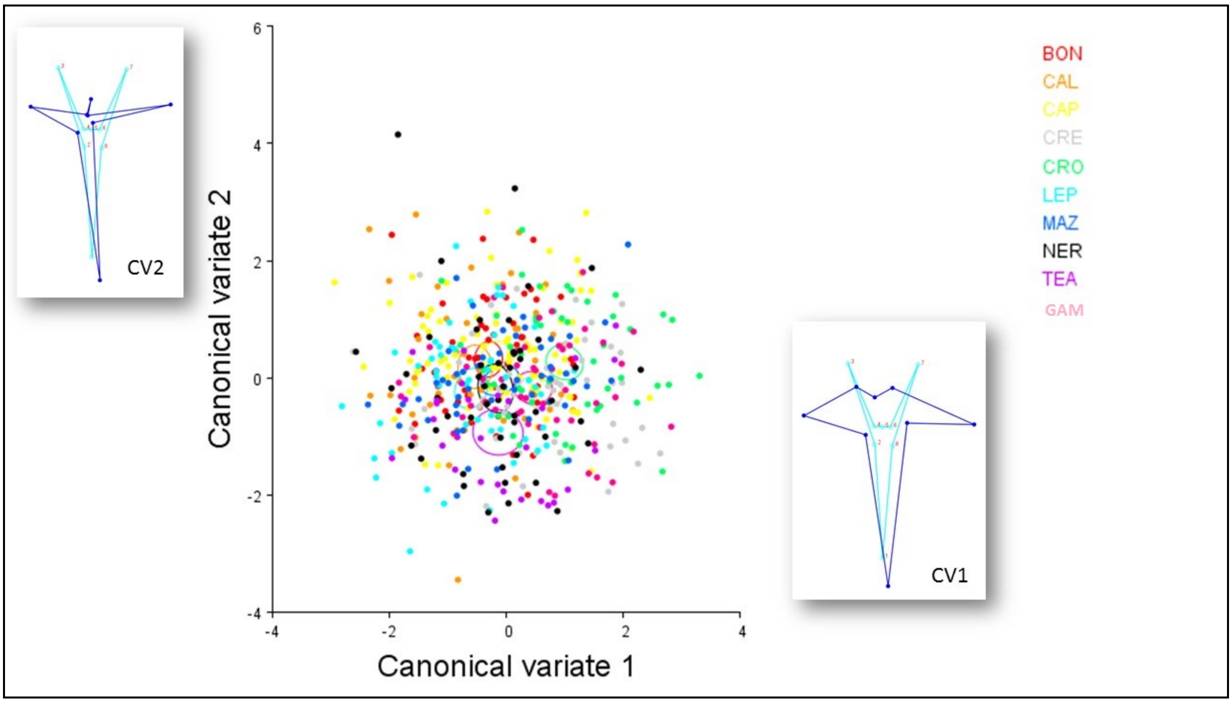

Figure 12. *P. massiliana* and *P. incrustans*. CVA analysis among population on diapason dataset. The left pane shows the changes in shape associated with the y-axis (CV2), where light blue represented an average morph and dark blue the maximum. The right pane shows the shape changes associated with the x-axis (CV1).

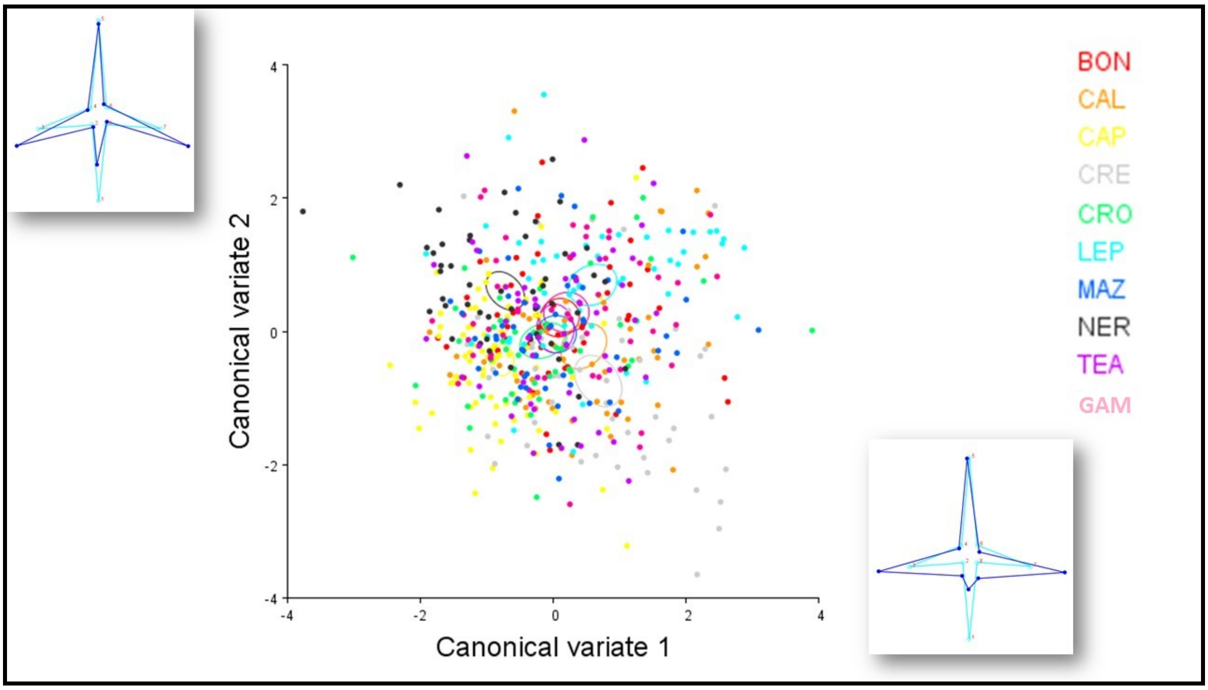

Figure 13. *P. massiliana* and *P. incrustans*. CVA analysis among population on tetractine dataset. The left pane shows the changes in shape associated with the y-axis (CV2), where light blue represented an average form and dark blue the maximum. The right pane shows the shape changes associated with the x-axis (CV1).

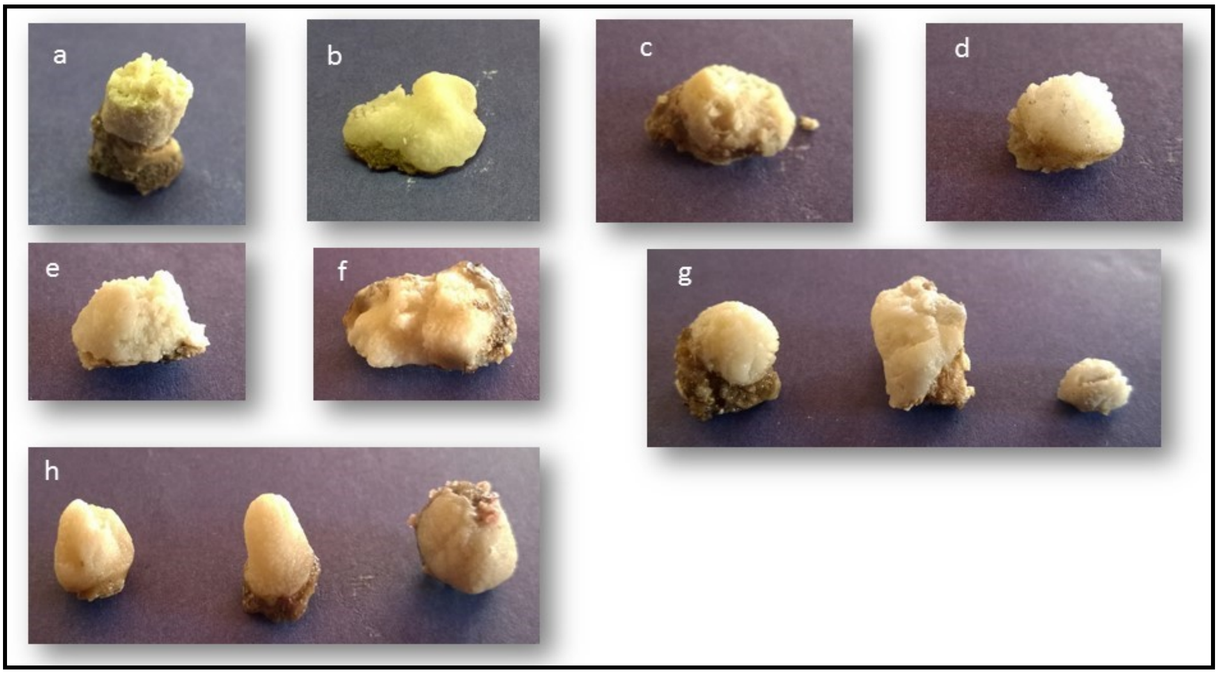

Figure 14. (a-i) Specimens of *Petrobiona massiliana* from some Mediterranean karstic caves preserved in ethanol, included in the study. **a,** Cave du Phare, Bonifacio, Corsica; **b**, one of three encrusting morphs from the Endoume cave, Marseille. **c**, one of three morphs from Crete. **d**, one of two specimens of Milna Island, Croatia. **e**, specimen of Gamberi Cave, Siracusa. **f**, one of two encrusting morphs from the Plan Cave, Marseille. **g**, the three specimens from Mazzere, Siracusa. **g**, the three specimens from Nereo Cave **h**, Capo Caccia, Alghero. **i**, one of the eight specimens from Azzurra Cave, Capri.

Abstract 1

Petrobiona massiliana (Porifera, Calcarea):distribuzione e analisi morfometrica di alcune popolazioni del Mediterraneo

PAOLO MELIS, BARBARA CADEDDU, FABIO DAMIANO LEDDA, SANDRA RICCI, RENATA MANCONI

Boll. Mus. Ist. Biol. Univ. Genova, 75: 87-88

PETROBIONA MASSILIANA (PORIFERA, CALCAREA): DISTRIBUZIONE E ANALISI MORFOMETRICA DI ALCUNE POPOLAZIONI DEL MAR MEDITERRANEO

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Petrobiona massiliana Vacelet & Lévi, 1959 (Lithonida) è un paleoendemita del Mar Mediterraneo con distribuzione limitata ad habitat criptici semi-oscuri o oscuri. Lo *status* di endemita riguarda non solo la specie ma anche il genere *Petrobiona* Vacelet & Lévi, 1958 e la famiglia Petrobionidae Borojevic, 1979, entrambi monotipici.

In questo lavoro viene riportato un nuovo ritrovamento dalla Grotta Azzurra di Capri nel Mar Tirreno centrale. L'areale di *P. massiliana* comprende parte del bacino orientale del Mar Mediterraneo (Mar Adriatico, Mar Ionio, Canale di Sicilia, Mar Egeo, Rodi) e parte del bacino occidentale (Golfo del Leone, Mar Ligure, Mar di Sardegna, Mar di Corsica, Mar Tirreno).

L'analisi morfometrica è stata condotta su 325 spicole della popolazione della Grotta Azzurra di Capri (Tab. 1) in comparazione con popolazioni delle grotte di Provenza (località tipo), Sardegna e Penisola Sorrentina (Vacelet & Lévi, 1958; Rützler, 1966; Vacelet, 1964; Manconi et al., 2009).

Nonostante la distribuzione disgiunta delle popolazioni considerate, l'analisi sulla morfometria spicolare non ha evidenziato divergenze nei caratteri diagnostici. Nella popolazione di Capri come in altre popolazioni in fase di studio si riscontra tuttavia la presenza di un tipo spicolare peculiare (macrodiactina) riportata soltanto da Vacelet (1964) per alcune popolazioni cavernicole di Marsiglia e considerata finora rara o assente in altre popolazioni.

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Località/ Grotta	**Triactine (µm)**	**Diapason (µm)**	**Tetractine (µm)**	**Microdiactine (µm)**	**Macrodiactine (µm)**	
Marsiglia Olotipo	50-200 x 20-40	70-100	300 ax x 80 lat x 8-27 thick	30-40 x 2	assenti	Vacelet & Levi 1958
Marsiglia/ Varie località	25-200 x 6-40	30-70 x 5-8.5 bas 20-50 x 4-7 lat	40-130 x 22-28 lat 8-100 x 10-28 Ax	30-60 x 2-3	270-480 x 11-20	Vacelet 1964
Penisola Sorrentina/ GrottaTuffo-Tuffo	45-300 x 15-48	70-130 x 6-8 total	130-150 ax x 80-135 lat x7,5-18 thick	30-65 x 2-4	assenti	Rützler 1966
Sardegna Punta Giglio/ Grotta Terrazze	45-170 x 10-25	35-60 x 3-8 bas 23-48 x 3-5 lat	100-165 ax x 60-105 lat x 5-10 thick	25-40 x 3	assenti	Manconi et al. 2009
Sardegna Punta Giglio/ Grotta Galatea	55-175 x 10-25	45-63 x 5-8 bas 20-55 x 4-5 lat	80-155 ax x 50-95 lat x 5-19 thick	25-38 x 2-3	assenti	Manconi et al. 2009
Sardegna Punta Giglio/ Grotta Fantasmi	50-200 x 10-30	35-63 x 3-8 bas 20-55 x 3-8 lat	70-190 ax x 45-100 lat x 5-15 thick	28-55 x 2-4	assenti	Manconi et al. 2009
Sardegna Capo Caccia/ Grotta Nereo	35-200 x 5-30	40-73 x 5-10 bas 20-50 x 4-8 lat	50-180 ax x 35-170 lat x 4-20 thick	18-43 x 3	assenti	Manconi et al. 2009
Capri/ Grotta Azzurra	42,7-183 x 7-31,5	22-76 x 5-8 bas 30-75 x 2-8 lat	68,5-192 ax x 30-75 lat 3,5-11 thick	38,5-83 x 2,5-6	92,5-124 x 5,5-6,5	Presente lavoro

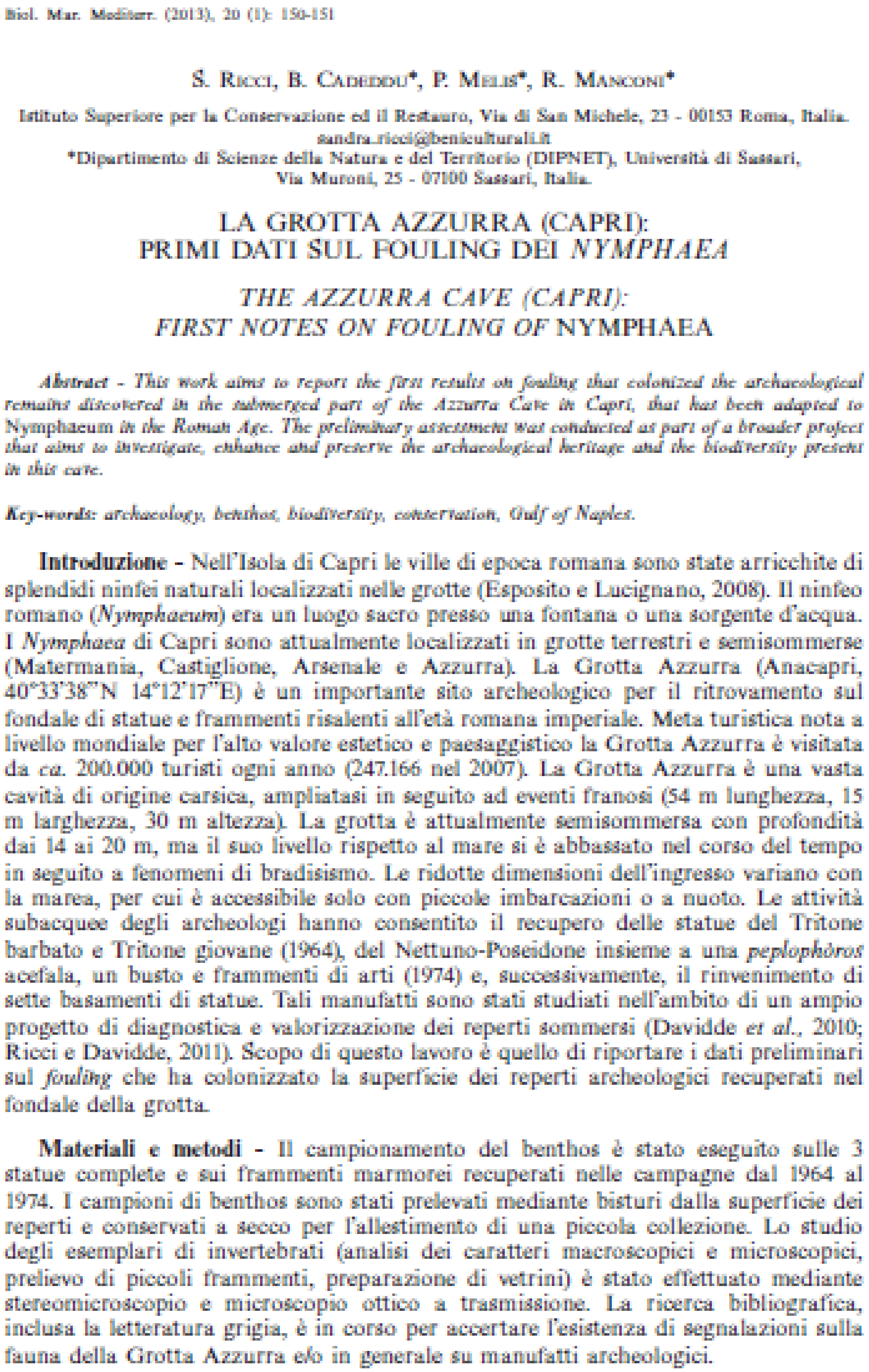
Tabella 1. *Petrobiona massiliana*. Principali tratti spicolari della popolazione di Capri e di altre popolazioni cavernicole del Mediterraneo.

Abstract 2

La Grotta Azzurra (Capri): Primi dati sul fouling dei Nymphaea

SANDRA RICCI, BARBARA CADEDDU, PAOLO MELIS, RENATA MANCONI

Biol. Mar. Mediterr. (2013), 20 (1): 150-151



Biol. Mar. Mediterr. (2013), 20 (1): 150-151

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LA GROTTA AZZURRA (CAPRI): PRIMI DATI SUL FOULING DEI *NYMPHAEA*

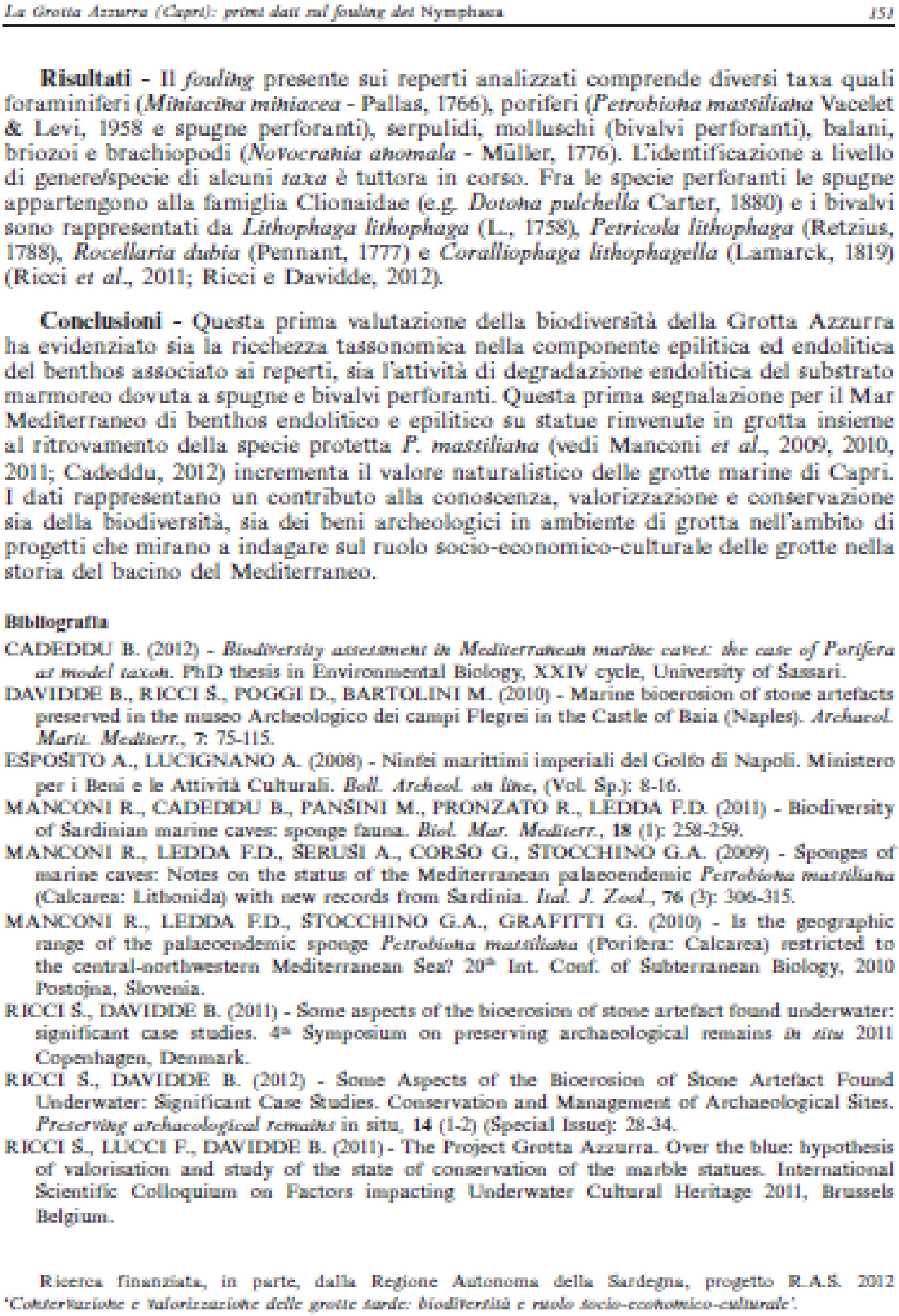
THE AZZURRA CAVE (CAPRI): FIRST NOTES ON FOULING OF NYMPHAEA

Abstract *- This work aims to report the first results on fouling that colonized the archaeological remains discovered in the submerged part of the Azzurra Cave in Capri, that has been adapted to* Nymphaeum *in the Roman Age. The preliminary assessment was conducted as part of a broader project that aims to investigate, enhance and preserve the archaeological heritage and the biodiversity present in this cave.*

Key-words: *archaeology, benthos, biodiversity, conservation, Gulf of Naples.*

Introduzione - Nell'Isola di Capri le ville di epoca romana sono state arricchite di splendidi ninfei naturali localizzati nelle grotte (Esposito e Lucignano, 2008). Il ninfeo romano (*Nymphaeum*) era un luogo sacro presso una fontana o una sorgente d'acqua. I *Nymphaea* di Capri sono attualmente localizzati in grotte terrestri e semisommerse (Matermania, Castiglione, Arsenale e Azzurra). La Grotta Azzurra (Anacapri, 40°33'38"N 14°12'17"E) è un importante sito archeologico per il ritrovamento sul fondale di statue e frammenti risalenti all'età romana imperiale. Meta turistica nota a livello mondiale per l'alto valore estetico e paesaggistico la Grotta Azzurra è visitata da *ca*. 200.000 turisti ogni anno (247.166 nel 2007). La Grotta Azzurra è una vasta cavità di origine carsica, ampliatasi in seguito ad eventi franosi (54 m lunghezza, 15 m larghezza, 30 m altezza). La grotta è attualmente semisommersa con profondità dai 14 ai 20 m, ma il suo livello rispetto al mare si è abbassato nel corso del tempo in seguito a fenomeni di bradisismo. Le ridotte dimensioni dell'ingresso variano con la marea, per cui è accessibile solo con piccole imbarcazioni o a nuoto. Le attività subacquee degli archeologi hanno consentito il recupero delle statue del Tritone barbato e Tritone giovane (1964), del Nettuno-Poseidone insieme a una *peplophòros* acefala, un busto e frammenti di arti (1974) e, successivamente, il rinvenimento di sette basamenti di statue. Tali manufatti sono stati studiati nell'ambito di un ampio progetto di diagnostica e valorizzazione dei reperti sommersi (Davidde *et al*., 2010; Ricci e Davidde, 2011). Scopo di questo lavoro è quello di riportare i dati preliminari sul *fouling* che ha colonizzato la superficie dei reperti archeologici recuperati nel fondale della grotta.

Materiali e metodi - Il campionamento del benthos è stato eseguito sulle 3 statue complete e sui frammenti marmorei recuperati nelle campagne dal 1964 al 1974. I campioni di benthos sono stati prelevati mediante bisturi dalla superficie dei reperti e conservati a secco per l'allestimento di una piccola collezione. Lo studio degli esemplari di invertebrati (analisi dei caratteri macroscopici e microscopici, prelievo di piccoli frammenti, preparazione di vetrini) è stato effettuato mediante stereomicroscopio e microscopio ottico a trasmissione. La ricerca bibliografica, inclusa la letteratura grigia, è in corso per accertare l'esistenza di segnalazioni sulla fauna della Grotta Azzurra e/o in generale su manufatti archeologici.



Risultati - Il *fouling* presente sui reperti analizzati comprende diversi taxa quali foraminiferi (*Miniacina miniacea* - Pallas, 1766), poriferi (*Petrobiona massiliana* Vacelet & Levi, 1958 e spugne perforanti), serpulidi, molluschi (bivalvi perforanti), balani, briozoi e brachiopodi (*Novocrania anomala* - Müller, 1776). L'identificazione a livello di genere/specie di alcuni *taxa* è tuttora in corso. Fra le specie perforanti le spugne appartengono alla famiglia Clionaidae (e.g. *Dotona pulchella* Carter, 1880) e i bivalvi sono rappresentati da *Lithophaga lithophaga* (L., 1758), *Petricola lithophaga* (Retzius, 1788), *Rocellaria dubia* (Pennant, 1777) e *Coralliophaga lithophagella* (Lamarck, 1819) (Ricci *et al.*, 2011; Ricci e Davidde, 2012).

Conclusioni - Questa prima valutazione della biodiversità della Grotta Azzurra ha evidenziato sia la ricchezza tassonomica nella componente epilitica ed endolitica del benthos associato ai reperti, sia l'attività di degradazione endolitica del substrato marmoreo dovuta a spugne e bivalvi perforanti. Questa prima segnalazione per il Mar Mediterraneo di benthos endolitico e epilitico su statue rinvenute in grotta insieme al ritrovamento della specie protetta *P. massiliana* (vedi Manconi *et al.*, 2009, 2010, 2011; Cadeddu, 2012) incrementa il valore naturalistico delle grotte marine di Capri. I dati rappresentano un contributo alla conoscenza, valorizzazione e conservazione sia della biodiversità, sia dei beni archeologici in ambiente di grotta nell'ambito di progetti che mirano a indagare sul ruolo socio-economico-culturale delle grotte nella storia del bacino del Mediterraneo.

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Abstract 3

Checklist of cave-dwelling Porifera from the Ligurian Sea.

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Boll. Mus. Ist. Biol. Univ. Genova, 75: 78-80

CHECKLIST OF CAVE-DWELLING PORIFERA FROM THE LIGURIAN SEA

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The checklist of the cave-dwelling sponge fauna from the Ligurian Sea is reported from literature (Cadeddu, 2012; Manconi et al., 2013). Sponges are recorded from 12 caves: 1. Punta Falconara caves (44°01'32"N 8°13'33"E); 2. Punta Sciusciaù caves (44°01'32"N 8°13'33"E); 3. Bergeggi Cave (44°14'38"N 8°26'37"E); 4. San Fruttuoso Cave (44°18'58"N 9°10'32"E); 5. Punta Carega Cave (44°19'02"N 9°10'38"E); 6. Paraggi Cave (44°18'43"N 9°12'36"E); 7. W-Zoagli Cave (44°20'N 9°16'E); 8. Galleria Zoagli-Chiavari Cave (44°19'N 9°17'E); 9. Piccola Zoagli-Chiavari Cave (44°19'N 9°17'E); 10. Corridoio Punta Manara (44°15'N 9°24'E); 11. Punta Manara Cave (44°15'N 9°24'E); 12. W-Bonassola Cave (44°11'N 9°35'E); 13. Tinetto Cave (44°01'24"N 9°51'03"E); 14. Lerici Cave (44°04'33"N 9°55'00"E). Taxonomic richness of cave-dwelling Porifera resulted in a total value of 60 species, belonging to 42 genera, 31 families, 15 orders, and 3 classes. The chorological categories are dominated by Cosmopolitan (C, n=23; 38%) and Atlanto-Mediterranean (AM, n=22; 37%) species, whereas lower values refer to Mediterranean endemics (E, n=12; 20%), Amphiatlantic (AA, n=2; 3%), and Indo-Mediterranean (IM, n=1; 2%) species.

The following species were recorded:

Clathrina clathrus (Schmidt, 1864) (E; 3, 10; Sarà, 1964; Bianchi et al., 1986; Bianchi & Morri, 1994); *C. coriacea* (Montagu, 1818) (C; 7, 8, 9, 10, 11; Sarà, 1964); *C. rubra* Sarà, 1958 (AM; 8; Sarà, 1964); *Leucetta solida* (Schmidt, 1862) (IM; 8; Sarà, 1964); *Sycon elegans* (Bowerbank, 1845) (C; 8; Sarà, 1964); *S. raphanus* Schmidt, 1862 (C; 5, 8; Sarà, 1964); *Leucandra aspera* (Schmidt, 1862) (C; 8; Sarà, 1964); *Petrobiona massiliana* Vacelet & Lévi, 1958 (E; 3; Bianchi et al., 1986; Bianchi & Morri, 1994); *Oscarella lobularis* (Schmidt, 1862) (AA; 13; Balduzzi et al., 1994; Cocito et al., 2002); *Plakortis simplex* Schulze, 1880 (C; 12; Sarà, 1964); *Stelletta grubii* Schmidt, 1862 (AA; 5; Sarà, 1964); *Penares euastrum* (Schmidt, 1868) (AM; 13; Cocito et al., 2002); *P. helleri* (Schmidt, 1864) (AM; 13; Cocito et al., 2002); *Erylus discophorus* (Schmidt, 1862) (AM; 3; 11; 12; Sarà, 1964; Bianchi et al., 1986); *Delectona madreporica* Bavestrello et al., 1997 (E; 6; Bavestrello et al., 1997); *Cliona celata* Grant, 1826 (C; 8; Sarà, 1964); *C. viridis* (Schmidt, 1862) (C; 3, 8; Sarà, 1964; Bianchi et al., 1986); *Diplastrella bistellata* (Schmidt, 1862) (AM; 3; Bianchi et al., 1986); *Spirastrella cunctatrix* Schmidt, 1868 (AM; 3; Bianchi et al., 1986); *Aaptos aaptos* (Schmidt, 1864) (C; 3, 5; Sarà, 1964; Bianchi et al., 1986; Bianchi & Morri, 1994); *Suberites carnosus* (Johnston, 1842) (C; 9, 12; Sarà, 1964); *Terpios fugax* Duchassaing & Michelotti, 1864 (C; 9; Sarà, 1964); *Chondrosia reniformis* Nardo, 1847 (C; 1, 2, 3, 7, 9, 11, 13; Sarà, 1964; Bianchi et al., 1986; Balduzzi et al., 1994; Bianchi & Morri, 1994; Cocito et al., 2002); *Chondrilla nucula* Schmidt, 1862 (C; 6; Arillo et al., 1993); *Clathria* (*Clathria*) *toxivaria* (Sarà, 1959) (E; 8; Sarà, 1964); *Clathria* (*Microciona*) *toxitenuis* Topsent, 1925 (AM; 11; Sarà, 1964); *Antho* (*Antho*) *involvens* (Schmidt, 1864) (C; 11; Sarà, 1964); *Rhabderemia topsenti* van Soest & Hooper, 1993 (AM; 12; Sarà, 1964); *Crambe crambe* (Schmidt, 1862) (AM; 5, 7, 8, 9, 10, 11, 12; Sarà, 1964); *Hemimycale columella* (Bowerbank, 1874) (AM; 8; Sarà, 1964); *Hymedesmia* (*Hymedesmia*) *castanea* Sarà, 1964 (E; 11; Sarà, 1964); *H. paupertas* (Bowerbank, 1866) (AM; 8, 9; Sarà, 1964); *Phorbas fictitius* (Bowerbank, 1866) (AM; 11, 12; Sarà, 1964); *P. tenacior* (Topsent, 1925) (AM; 1, 2, 3, 11; Sarà, 1964; Balduzzi et al., 1994; Bianchi & Morri, 1994); *Mycale* (*Aegogropila*) *tunicata* (Schmidt, 1862) (AM; 12; Sarà, 1964); *Axinella cannabina* (Esper, 1794) (E; 3; Bianchi & Morri, 1994); *A. damicornis* (Esper, 1794) (AM; 13; Cocito et al., 2002); *A. verrucosa* (Esper, 1794) (AM; 3, 4; Pansini, 1984 Bianchi & Morri, 1994); *Acanthella acuta* Schmidt, 1862 (AM; 3; Bianchi & Morri, 1994); *Agelas oroides* (Schmidt, 1864) (E; 3; Bianchi

& Morri, 1994); *Haliclona* (*Haliclona*) *varia* (Sarà, 1958) (E; 11, 12; Sarà, 1964); *Haliclona* (*Reniera*) *cinerea* (Grant, 1826) (C; 7, 8, 12; Sarà, 1964); *Haliclona* (*Soestella*) *mucosa* (Griessinger, 1971) (AM; 4; Pulitzer-Finali, 1983); *Petrosia* (*Petrosia*) *ficiformis* (Poiret, 1789) (AM; 1, 2, 3, 6, 13; Bianchi et al., 1986; Arillo et al., 1993; Balduzzi et al., 1994; Bianchi & Morri, 1994; Cocito et al., 2002); *Aplysilla rosea* (Barrois, 1876) (C; 7, 13, 14; Sarà, 1964; Pulitzer-Finali & Pronzato, 1980); *Dysidea avara* (Schmidt, 1862) (C; 3; Bianchi & Morri, 1994); *D. fragilis* (Montagu, 1818) (C; 7, 8, 9, 12; Sarà, 1964); *D. incrustans* (Schmidt, 1862) (AM; 14; Pulitzer-Finali & Pronzato, 1980); *Ircinia oros* (Schmidt, 1864) (E; 7; Sarà, 1964); *I. variabilis* (Schmidt, 1862) (C; 11; 12; 13; Sarà, 1964; Cocito et al., 2002); *Sarcotragus fasciculatus* (Pallas, 1766) (AM; 8, 11; Sarà, 1964); *S. spinosulus* Schmidt, 1862 (C; 13; Cocito et al., 2002); *Spongia lamella* (Schulze, 1879) (E; 3; Bianchi & Morri, 1994); *S. officinalis* Linnaeus, 1759 (C; 3, 5, 8, 12; Sarà, 1964; Bianchi et al., 1986); *S. virgultosa* (Schmidt, 1868) (AM; 5, 9, 11, 12; Sarà, 1964); *Cacospongia scalaris* Schmidt, 1862 (E; 9, 12; Sarà, 1964); *Hyrtios collectrix* (Schulze, 1880) (AM; 12; Sarà, 1964); *Halisarca dujardini* Johnston, 1842 (C; 7; Bianchi et al., 1986); *Aplysina cavernicola* (Vacelet, 1959) (AM; 3; Bianchi & Morri, 1994).

Chorological categories (letters), recorded caves (numbers) and references (records) are reported in brackets for each species. For cited records see Cadeddu (2012) and Manconi et al. (2013) and references therein. The status of taxa was checked from Van Soest et al. (2013).

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OTHER ABSTRACT

Abstract 1

Maricoltura di *Crambe crambe* nel Mar di Sardegna.

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MARICOLTURA DI *CRAMBE CRAMBE* NEL MAR DI SARDEGNA

MARICULTURE OF CRAMBE CRAMBE *IN THE SARDINIA SEA*

Abstract *- This study aims to test suitable substrates to farm the endemic Mediterranean sponge* Crambe crambe*. Growth and survival values are reported for both tested methodologies in an experimental sponge culture plant in the Sardinia Sea.*

Key-words: *macrobenthos, Porifera, farming methods, W-Mediterranean Sea.*

Introduzione - La spongicoltura è una valida soluzione per la produzione sostenibile di poriferi di interesse commerciale per vari scopi (Pronzato *et al.*, 2012; Pronzato e Manconi, 2008; Ledda *et al.*, 2008, 2013; Murray *et al.*, 2013; Manconi *et al.*, 2005). I metodi di allevamento *in situ* delle spugne si basano su tecnologie semplici ed economiche. La maricoltura finalizzata alla produzione di spugne mira alla gestione sostenibile di questa biorisorsa mediterranea. Molti autori hanno sperimentato la potenzialità di allevare *C. crambe* (Schmidt, 1862) con diverse metodologie *in situ* ed *ex situ*. Questo lavoro, basato sul metodo U.S.A.M.A.®, ha lo scopo di sperimentare tipologie e strutture di impianto modulari per la valutazione di substrati idonei all'allevamento *in situ* di *C. crambe* nel Mar di Sardegna.

Materiali e metodi - I campioni per gli espianti, prelevati in immersione nella Baia di Porto Conte in aprile 2012 a profondità di 2-5 m, sono stati immediatamente trasportati, in contenitori areati e refrigerati, nel sito dell'impianto localizzato nella stessa baia. I 10 campioni sono stati frammentati ottenendo 28 espianti con dimensioni di 7-9 cm^2. Sono stati allestiti 2 moduli in PVC con 14 espianti ciascuno secondo 2 metodologie: a) retine in nylon, e b) piastrelle in travertino. Ogni campione è stato identificato a livello di specie sulla base del complemento spicolare al microscopio ottico. La sopravvivenza è stata valutata in base allo stato di salute dell'esemplare, quali colore e consistenza. L'accrescimento di ogni espianto è stato valutato sulla base delle aree di ricoprimento del substrato, da aprile 2012 a gennaio 2014.

Risultati - La popolazione di *C. crambe* di Porto Conte è caratterizzata da un complemento spicolare costituito esclusivamente da due categorie di stili. Alla fine degli esperimenti (gennaio 2014) il valore di sopravvivenza era pari a 86% degli espianti allevati in retina, e del 93% in quelli allevati su piastrella. Gli esperimenti hanno evidenziato un accrescimento nettamente superiore per la metodologia di allevamento su piastrelle di travertino con un'area di ricoprimento medio di 15β cm^2 rispetto a 62 cm^2 registrata nelle retine nel corso di 22 mesi (Fig. 1) con un incremento medio pari a 1800% per le piastrelle e ca. 700% per le retine

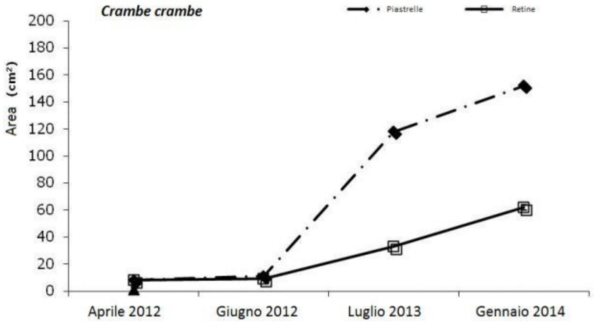

Fig. 1 - Accrescimento medio di *C. crambe* allevati su piastrella *vs*. retina nel Mar di Sardegna.
AVG *growth of* C. crambe *farmed on tile* vs. *mesh in the Sardinia Sea.*

Conclusioni - Gli alti valori di sopravvivenza e di crescita di *C. crambe* indicano che la semina è avvenuta in un periodo favorevole per il ciclo vitale della specie e che le condizioni ambientali del sito e le caratteristiche dell'impianto sono ottimali per questa specie. Il substrato più idoneo per l'allevamento di *C. crambe* sembra essere la piastrella di travertino. Queste spugne caratterizzate da habitus incrostante aderiscono saldamente alla superficie della piastrella in breve tempo e si accrescono in assenza di competitori sul substrato calcareo vergine.

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