



Coping with brackish water: A new species of cave-dwelling *Protosuberites* (Porifera: Demospongiae: Suberitidae) from the Western Mediterranean and a first contribution to the phylogenetic relationships within the genus

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Abstract

We used both morphological and genetic approaches to investigate and to describe a new Mediterranean sponge species of the genus *Protosuberites* from the estuarine-anchialine Bue Marino Cave of Sardinia (Tyrrhenian Sea). The morpho-traits of the specimens were compared versus congeneric species with the strongest affinities, covering the genus geographic range worldwide. *Protosuberites mereui* **sp. nov.** is light yellow, thinly encrusting, devoid of any special ectosomal skeleton, with spicular complement of tylostyles of three size classes, single or arranged in bundles/tufts, with round to suboval heads. The new species is characterized by an exclusive diagnostic trait recorded for any cave-dwelling *Protosuberites* i.e. suboval and basally plated resting bodies with a foraminal aperture ornate by a collar. Resting bodies were found in the basal spongin plate firmly adhering to the substratum singly or in small groups. Also the rare, small tylostyles with a sinuous shaft and a typical mushroom-like head were never recorded in the Western Mediterranean and Atlantic species of the genus. The phylogenetic reconstruction using maximum likelihood (ML) and Bayesian Inference (BI) analyses (*COI*, *18S* rRNA, and *28S* rRNA) recovered a robustly supported sister relationship between the Mediterranean *P. mereui* **sp. nov.** and *Protosuberites* sp. ‘Panama’ from the Eastern Pacific Ocean. The genetic distances based on *COI* sequences between all compared *Protosuberites* species were always higher than 2%, a value sufficient to confirm that *P. mereui* **sp. nov.** is a distinct species within the genus. Morphological and genetic analyses confirm unanimously *P. mereui* **sp. nov.** as a new species. Our results contribute to the assessment of biodiversity in anchialine/estuarine caves and increase data on sponge adaptive strategies in these extreme ecosystems.

Key words: sponges, integrative taxonomy, Sardinia, Bue Marino Cave, estuarine/anchialine karstic caves

Introduction

The biodiversity of estuarine caves *sensu* De Waele & Forti (2003) that match in part the definition of anchialine environments (Holthuys 1973; Sket 1996) is poorly known worldwide, but they appear to be hot spots of endemism 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 records in the Mediterranean and Atlantic water (van Soest & Sass 1981; Pansini & Pesce 1998; Manconi *et al.* 2012; Melis *et al.* 2013) i.e. the estuarine/anchialine Bue Marino Cave of Sardinia (Western Mediterranean Sea) where the physical-chemical parameters, e.g. water salinity and water movement, vary dramatically around the year. Among these sponges, a species of the genus *Protosuberites* Swartschewsky, 1905 (order Suberitida) preliminarily identified as *Protosuberites* cf. *epiphytum* (Manconi *et al.* 2012; Melis *et al.* 2013, Melis 2015) is particularly interesting, since only a single cave-dwelling species of the genus i.e. *Protosuberites geracei* (van Soest & Sass 1981) is known to date from the Caribbean Sea.

The taxonomic status of the genus *Protosuberites* is problematic. It currently comprises 23 valid species (van Soest *et al.* 2016) defined as “Suberitidae with encrusting habit and a skeleton differentiated in a choanosomal arrangement of single tylostyles or bundles of tylostyles, and ectosomal brushes of tylostyles” (van Soest 2002, p. 235). Delineation of species within the genus *Protosuberites* is difficult because they only possess tylostyles of

variable size and a few other distinctive morphotraits. In addition, the boundaries between the species within this genus are often unclear because of the co-occurrence of sympatric species with overlapping morphologies (van Soest & de Kluijver 2003).

It is well known that sponge taxonomy is particularly difficult because it mostly relies on spicule morphology constrained by phenotypical plasticity (e.g. Lévi 1973; Solè-Cava *et al.* 1991; Muricy *et al.* 1996; Klautau *et al.* 1999; Hooper & van Soest 2002; Manconi & Pronzato 2002, 2007; Xavier *et al.* 2010; Rua *et al.* 2011). In such cases, molecular analyses have been crucial to confirm and/or clarify the phylogeny and systematics in problematic taxa (Blanquer & Uriz 2007; Erpenbeck *et al.* 2012; Escobar *et al.* 2012; Xavier *et al.* 2010). Although the mitochondrial DNA of Porifera evolves slowly compared to other metazoans (Shearer *et al.* 2002). An increasing number of studies demonstrate the potential effectiveness of DNA barcoding (e.g. Wörheide & Erpenbeck 2007; Vargas *et al.* 2012) for species identification (e.g. Edgar 2004), for the elucidation of diversification patterns (Pöppe *et al.* 2010) and to resolve phylogenetic relationships (Cárdenas *et al.* 2009; Erpenbeck *et al.* 2012; Morrow & Cárdenas 2015). 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 & Taylor 2011) and/or the presence of bioactive compounds that may inhibit the PCR reactions (Chelossi *et al.* 2004).

We report here on a new species belonging to the genus *Protosuberites* from the Sardinian Bue Marino Cave (Orosei Gulf, western Tyrrhenian Sea) using an integrative taxonomic approach combining both morphological and molecular information to characterize its taxonomic status and to place the new Mediterranean species within the genus *Protosuberites* phylogenetic context.

Materials and methods

Study area. The Bue Marino Cave belongs to a karstic system characterized by a developed underground drainage with an estimated length of *ca.* 20 Km and a large opening onto the western Tyrrhenian Sea (Cottarelli & Bruno 1993; Fancello 2009). The cave, with an almost horizontal profile, is divided in three branches (northern, central, and southern). The semi-submerged southern branch shows large columnar speleothems along large subaerial tunnels (10–15 m in diameter) eroded during marine ingressions by the aggressive waters of the mixing zone (e.g. De Waele & Forti 2003). The upper zone of this branch is indeed characterized by a subterranean aquifer intermittently flowing through a series of large shallow freshwater pools showing downstream a small calcitic dam (*ca.* 900 m from the entrance). Downstream of the dam, in the terminal mixohaline pool Lago degli Specchi, a concomitant presence of freshwater and seawater, with long term stratification during summer, occurs from the subterranean aquifer system and from the Tyrrhenian Sea, respectively. The pool is sporadically subjected to both harsh freshwater flooding (high flow phases in the rain season) and to marine water intrusions (low freshwater flow phases) by storms and tidal fluctuations (0.3–0.5 m) through the subaerial cave entrance, and from hypothetical lateral crevices.

Sample processing. Sponges were collected in June 2008 and June–August 2013 from the southern branch of the Bue Marino Cave (40°14'48.19" N, 09°37'22.76" E; Orosei Gulf, E-Sardinia, western Tyrrhenian Sea; Fig. 1, Table 1). Samplings were carried out in mixohaline water (from 24‰ up to 39.6‰ of salinity at *ca.* 0.30–1 m of depth, depending on the day and on the point of sampling) in the Lago degli Specchi by snorkelling and SCUBA diving. Sponge samples were detached from the rocks with a knife and/or collected together with their substrate. In the lab, samples were cleaned from epibionts and substrate fragments under a stereomicroscope. Samples were dried or preserved in 96% ethanol, and stored at -20 °C for molecular analyses.

Morphological analysis. Morphotraits i.e. growth form, colour, inhalant/exhalant apertures, surface, skeletal architecture, and resting bodies were evaluated by Light Microscopy (LM, Leica Stemi 2000 stereomicroscope) and Scanning Electron Microscopy (SEM, Vega3 TESCAN type LMU). To characterize spicule microtraits by LM, sponge fragments were prepared by digestion in sodium hypochlorite at ambient temperature and mounted in slides with DPX. Spicules preparation for SEM follows Manconi & Pronzato (2000). Spicule measurements (length x thickness; n=200) of samples (Table 1) were done by LM (Leica Leitz DM RB with a Canon G6 Power Shot photcamera) using the program TPSDig2 Version 2.16 (Rohlf 2010) and reported as minimum (average) maximum. Morphometries of other comparative samples were obtained from the literature.

TABLE 1. Genus *Protosuberites*. Summary of type materials and voucher collection details for all studied species in both morphological and molecular analyses.

Species	Voucher number	Location Depth	Collector Date	Accession number (COI/18S/28S)
<i>P. mereui</i> sp. nov.	PROTOBM01	Bue Marino Cave	P. Melis	NO
	PROTOBM02	Gulf of Orosei	June-August	NO
	PROTOBM03	Sardinia, Italy	2012-2014	KX601197/ KX601222/ KX601212
	PROTOBM04	40.2467 N, 9.6229 E		KX601198/-/-
	PROTOBM05	1 m		KX601199/-/-
	PROTOBM06			KX601200/-/-
	PROTOBM07			KX601201/-/-
	PROTOBM08			KX601202/-/-
	PROTOBM09			KX601203/-/-
<i>P. ectyoninus</i>	UBAPOR01	Cap Norfeu Girona, Spain 42.1511 N, 3.1625 E 15 m	A. Riesgo 6/19/14	KX601188/ KX601214/ KX601205
<i>P. denhartogi</i>	ZMAPOR09801	Nouadhibou Mauritanie 19.0667 N, 16.4167 E 12–18 m	R.W.M. van Soest J.J. Vermeulen 11/19/12	KX601196/ KX601221/
	MC5141	Ynys Castell Abercastel, UK 51.9629 N, 5.1282 E unknown depth	B. Picton 4/1/07	KX601190/
	MC5215	Pwlldevi Albertcastle, UK 52.00115 N, 5.09425 E 27.6 m	B. Picton 2/10/09	KX601194/ KX601219/ KX601210
	MC5556	Wendy's Gully Skomer, Wales, UK 51.730467 N, 5.09425 E 16.3 m	B. Picton 7/10/09	KX601193/ KX601218/ KX601209
	ZMAPOR17181b	NeeltjeJans Netherlands 51.63 N, 3.72 E 0.5 m	R.W.M. van Soest 3/23/12	NO
	MC3715	Glannafeen Cliff Lough Hyne, Ireland 51.49891 N, 9.30054 E 10 m	B. Picton 7/10/09	KX601191/ KX601216/ KX601207
	MC4908	Keeraunagark rapids Galway, Ireland 53.2453 N, 9.54766 E 10 m	B. Picton 7/10/09	KX601192/ KX601217/ KX601208
	MC5288	Aberreidy Quarry Wales, UK 51.9378 N, 5.2085 E 13.2 m	B. Picton 8/15/09	KX601195/ KX601220/ KX601211

.....continued on the next page

TABLE 1. (Continued)

Species	Voucher number	Location Depth	Collector Date	Accession number (<i>COI/18S/28S</i>)
<i>P. incrustans</i>	ZMAPOR20543	Skagerrak, Norway 59.07835 N, 10.73205 E 112 m	R.W.M. van Soest 3/21/12	KX601189/ KX601215/ KX601206
<i>P. sp.</i> 'Thailand'	ZMAPOR18657	Chon Buri, Thailand 12.91617 N, 100.7721 E 6 m	S. Putchakarn 11/26/01	KX601204/-/-
<i>P. sp.</i> 'Panama'	POR14649	Panama, East Pacific unknown depth	S. Nichols ---	AY561915/--/ AY561979

Acronyms. MSNG, Museo civico di Storia Naturale “Giacomo Doria” di Genova, Italy; BMNH, The Natural History Museum of London, United Kingdom; DIPNET, Department of Science for Nature and Environmental Resources, University of Sassari, Sassari, Italy; DISTAV, Dipartimento di Scienze della Terra dell'Ambiente e della Vita, Università di Genova, Italy.

Molecular procedures. A total of 19 specimens of *Protosuberites* belonging to 5 species from different geographic areas were preserved in 96% ethanol and used for the molecular analyses (Table 1, Fig. 2). 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), following the manufacturer's instructions.

Three molecular markers were targeted i.e. 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. For *COI*, 551 base pairs were amplified using either the specific forward primer ProtoF and the reverse primer HCOoutout (Schulmeister 2003) or the pair jgLCO1490-jgHCO2198 (Geller *et al.* 2013; Table 2). The complete 18S (1690 base pairs) was amplified in three overlapping fragments of about 950, 900 and 850 base pairs each, using primer pairs 1F–4R, 4F–7R, and 18Sa2.0–9R (Table 2). Also, 1104 base pairs of the gene *28S* were amplified using the primers pair 28Srd1aF and 28SRd5b (Table 2).

TABLE 2. Primer sequences used in this study.

Name	Primer sequence	References
ProtoF	5'- GAA TTA CCG CGG CTG CTG G – 3'	Present paper
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 <i>et al.</i> 2013
gHCO2198	5' - TAI ACY TCI GGR TGI CCR AAR AAY CA - 3'	Geller <i>et al.</i> 2013
1F	5'- TAC CTG GTT GAT CCT GCC AGT AG – 3'	Giribet <i>et al.</i> 1996
4R	5'- GAA TTA CCG CGG CTG CTG G – 3'	Giribet <i>et al.</i> 1996
4F	5'- CCA GCA GCC GCG CTA ATT C – 3'	Giribet <i>et al.</i> 1996
7R	5'- GCA TCA CAG ACC TGT TAT TGC – 3'	Giribet <i>et al.</i> 1996
18sa2.0	5'- ATG GTT GCA AAG CTG AAA C – 3'	Whiting <i>et al.</i> 1997
9R	5'- GAT CCT TCC GCA GGT TCA CCT AC – 3'	Giribet <i>et al.</i> 1996
28Srd1aF	5' - CCC SCG TAA YTT AGG CAT AT – 3'	Giribet <i>et al.</i> 2006
28SRd5b	5' – CCA CAG CGC CAG TTC TGC TTA C – 3'	Giribet <i>et al.</i> 2006

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 MgCl₂, 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 min; 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 min. For the *18S* the amplification program

was denaturation at 94 °C for 5 min; 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 min; with a final extension step at 72 °C for 10 min. And finally for the 28S the following temperature conditions were programmed for PCR: denaturation at 94 °C for 3 min; 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 min. PCR products of the samples were then purified using MICROCLEAN (MICROZONE, Haywards, UK) and sent to the Serveis Científico-Tècnics 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.

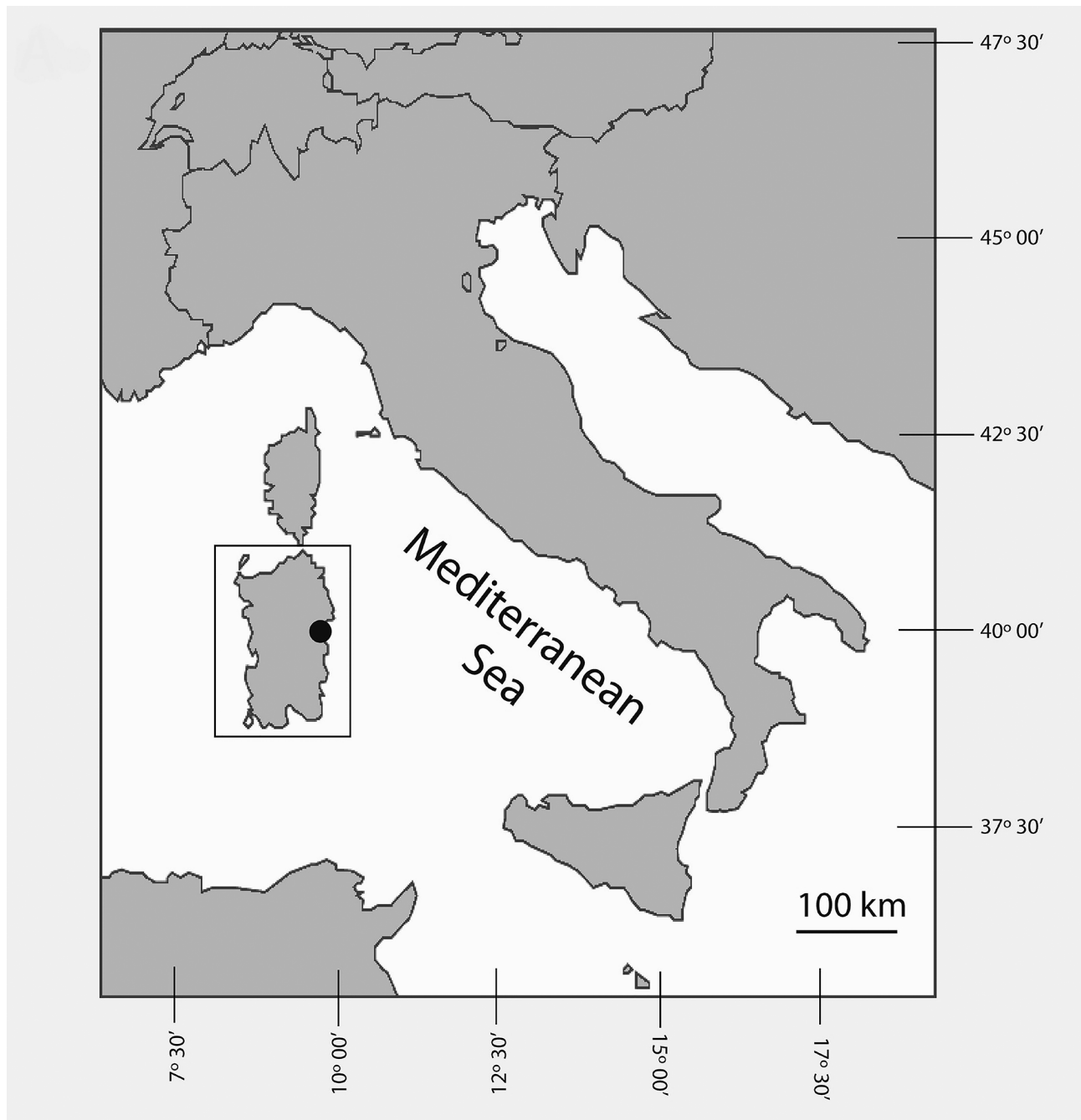


FIGURE 1. *Protosuberites mereui* sp. nov. from the western Mediterranean Sea. Map with type locality (black dot) in the Bue Marino Cave, 40°14'48.19"N, 9°37'22.76"E, Tyrrhenian Sea, Eastern Sardinia, Orosei Gulf karstic area.

Phylogenetic analyses. Additional sequences for 4 outgroups of Suberitidae, *Prosuberites laughlini* (Díaz Alvarez & van Soest 1987) (*COI*: AY561960, *18S*: EF654529, *28S*: AY561927), *Prosuberites longispinus* Topsent, 1893 (*18S*: KC902182, *28S*: HQ379245), *Suberites domuncula* (Olivi 1792) (*COI*: JX999078; *18S*: AJ620112, *28S*: AJ620113), and *Suberites pagurorum* Solé-Cava & Thorpe 1986 (*COI*: KC869422, *28S*: HQ379248) were obtained from the ncbi Genbank database. All sequences were then 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 then concatenated (3399 base pairs) and implemented in RAxML 7.4.2 (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 analyses were run with partitions set for a unique GTR model of sequence evolution (Tavaré 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 & Buckley 2004). In ML, 100 independent searches were conducted and the nodal support was estimated via the rapid bootstrap algorithm with 500 replicates (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 2500 generations, 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 & Drummond 2007). The remaining trees were combined to find the maximum *a posteriori* probability estimate of phylogeny.

The software MEGA (Kumar *et al.* 2008) was used to calculate mean intra- and inter-clade *p*-distances for *COI* between all the *Prosuberites* 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 Suberitida Chombard & Boury-Esnault, 1999

Family Suberitidae Schmidt, 1870

Genus *Prosuberites* Swartschewsky, 1905

Prosuberites mereui Manconi sp. nov.

Figs 1–4; Tables 1, 3, 4

Material examined. Holotype: MSNG 59362, BUEMARCAVE 21, Bue Marino Cave, 40°14'48.19" N, 9°37'22.76"E, southern branch, mixohaline pool 'Lago degli Specchi', Eastern Sardinia, western Tyrrhenian Sea, ca. 0.30–1 m of depth, R. Manconi legit, vi.2008.

Paratypes: MSNG 59363, BUEMARCAVE 22, *ibid.*; MSNG 59364, BUEMARCAVE 30, *ibid.*; BMNH 2016.10.28.1, PROTOBM 03, *ibid.*; BMNH 2016.10.28.2, PROTOBM 04, *ibid.*; BMNH 2016.10.28.3, PROTOBM 05, *ibid.*; BMNH 2016.10.28.4, PROTOBM 06, *ibid.*; BMNH 2016.10.28.5, PROTOBM 07, *ibid.*; BMNH 2016.10.28.6, PROTOBM 08, *ibid.*, P. Melis legit, viii.2013.

Other material: PROTOBM01, 02, 09, *ibid.*, P. Melis legit; BUEMARCAVE, several specimens, slides and stubs, *ibid.*, Daniela Demurtas legit, R. Manconi's collection at DIPNET.

Comparative materials. Details of all material studied from other comparable *Prosuberites* species and from the literature are given in Table 1. In addition, *Prosuberites* cf. *epiphytum* (Lamarck, 1815) was studied from the Mediterranean Sea G. Pulitzer-Finali Collection at DISTAV, slides: TRI.274, Porto Tricase, Apulia, conglomerates, 30 m depth, 24.ix.1970; PC.125, Taranto, Mar Piccolo, Apulia, encrusting on stone, 0.5 m depth, 18.vii.1973; GAR.74, Gargano, Baia di Campi, Apulia, rocks and mud, 2–10 m depth, 15.ix.1971.

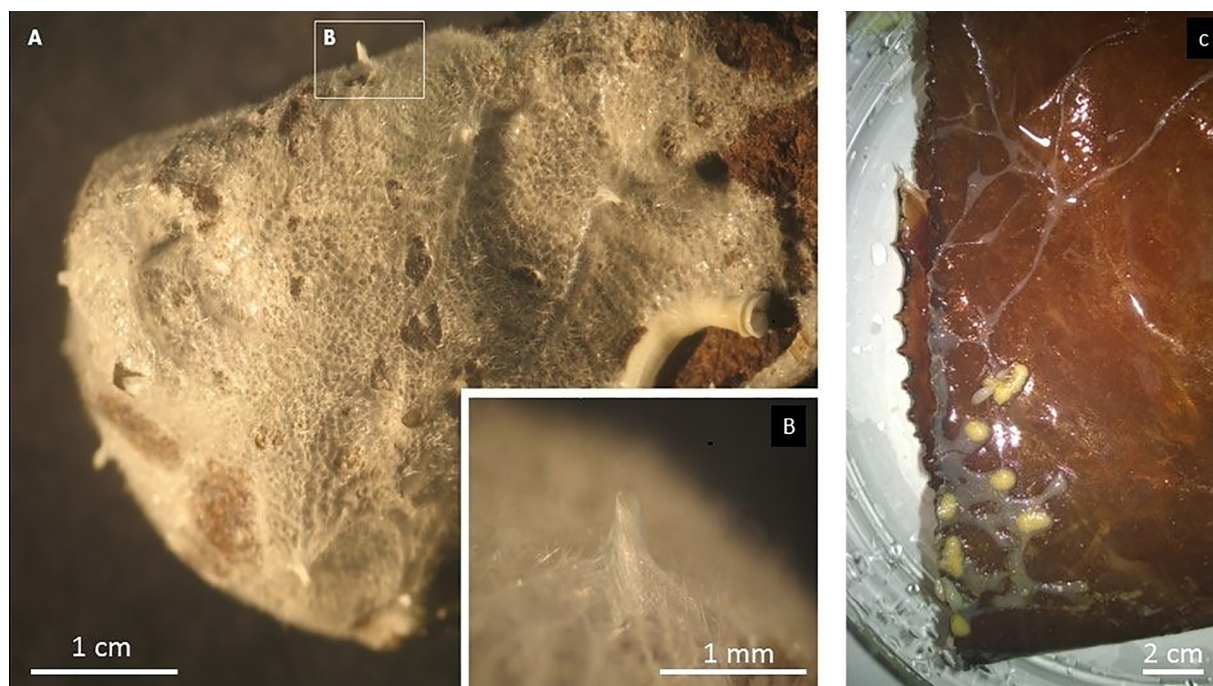


FIGURE 2. *Protosuberites mereui* **sp. nov.** A. Encrusting specimen (dry) on a rocky fragment, with branched subdermal canals leading to small, scattered oscules. The serpulid *Ficopomatus enigmaticus* is evident on the right. B. Magnification of an osculum. C. Specimen on a plastic fragment (ethanol).

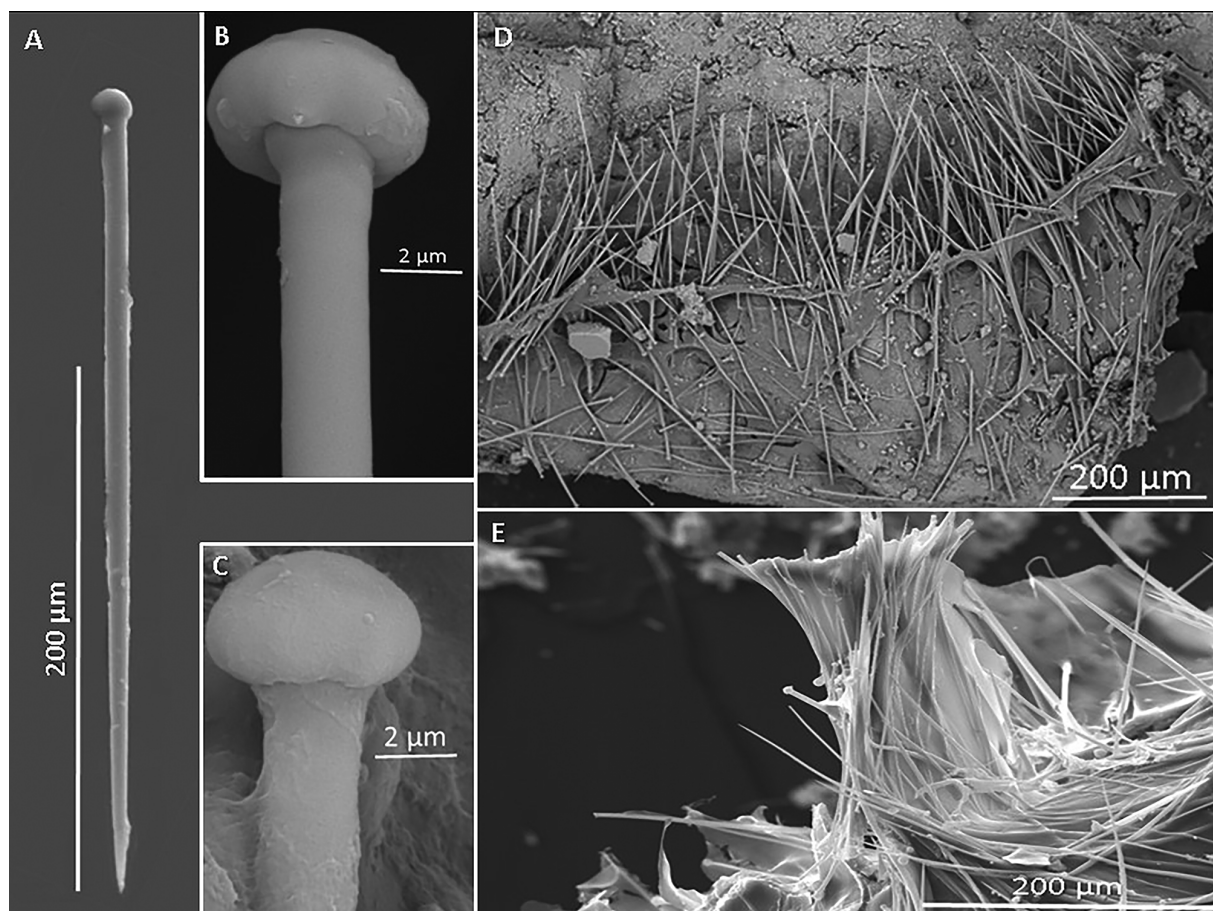


FIGURE 3. *Protosuberites mereui* **sp. nov.** Spicules and skeleton (SEM). A. Smooth tylostyle with suboval head. B-C. Head of tylostyles. D. Skeletal architecture with hispidation due to erect tylostyles. E. Detail of the osculum with tylostyles loosely arranged in bundles in the thicker portions of the thinly encrusting sponge.

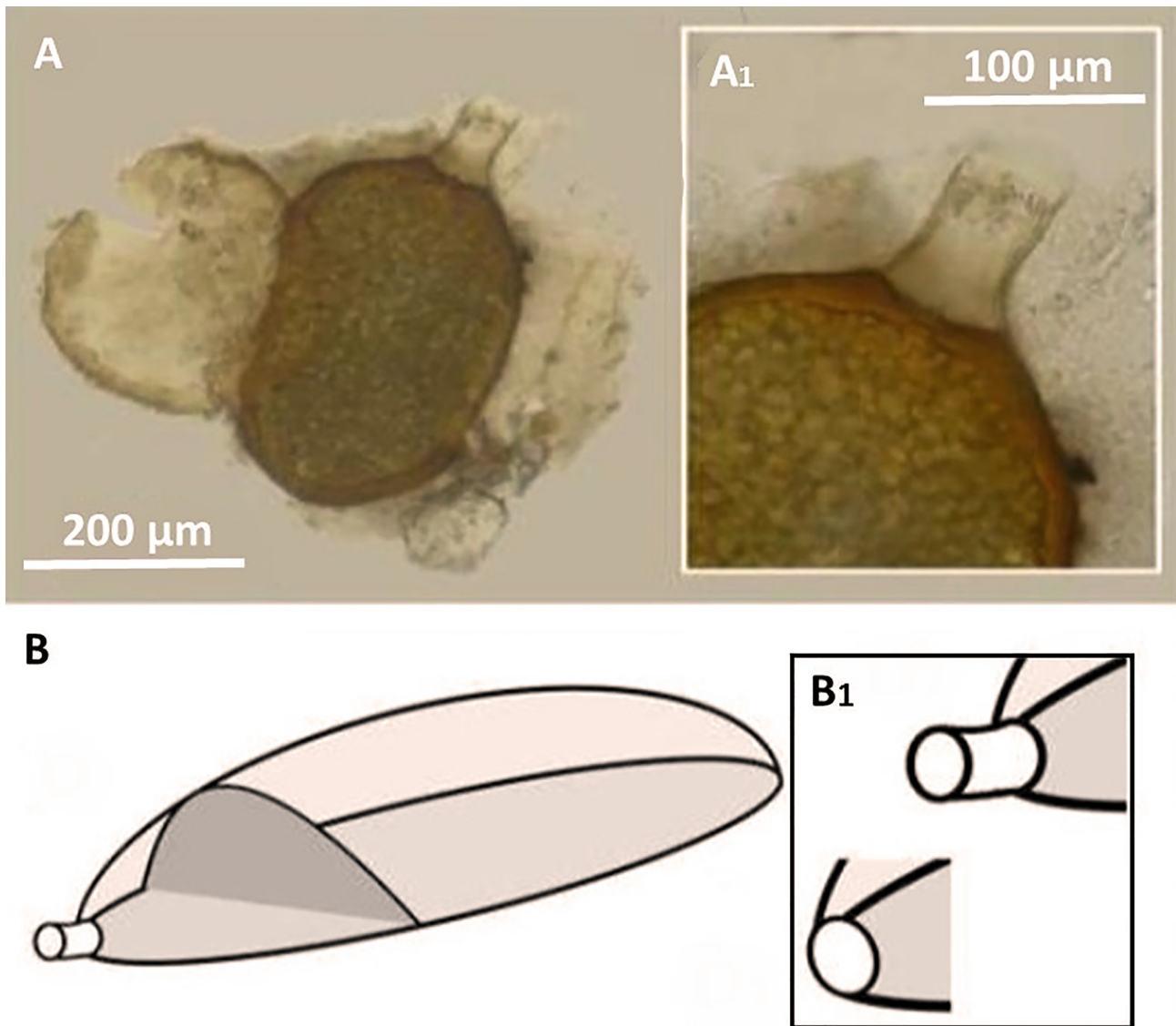


FIGURE 4. *Protosuberites mereui* sp. nov. Resting bodies (gemmule-like). A. Suboval resting body (LM) with a flat base and unarmed protective theca filled by a mass of totipotent cells (centre); empty theca of transparent spongin (left). A1. Theca of sublayered compact spongin and a single, distal, evident foramen bearing a well-developed collar (LM). B. Schematic drawing of a resting body (lateral view). B1. Foramen detail with protruding collar (top), and closed foramen lacking collar (bottom). B-B1 by courtesy of R. Pronzato.

Description. **Growth form** encrusting, very thin (*ca.* 1–2 mm in thickness) in patches (from a few cm² up to 1 m²) on rocky substrata. **Colour** light yellow *in vivo*, whitish after preservation in ethanol (Fig. 2). **Consistency** firm. **Surface** hispid due to tips of spicules, with subdermal canals and scattered oscules. **Ectosomal skeleton** lacking special architecture, with distal tips of spicules supporting the dermal membrane. **Choanosomal skeleton** of erected brushes of tylostyles from single in thinner portions to loosely arranged as tylostyles bundles/tufts in the thicker portions (Fig. 3d). **Basal spongin plate** well developed in the surroundings of resting stages and in general armed by tylostyles heads. **Megascleres** tylostyles 100(295.3)650 x 3(5.03)10 µm (n=200; Table 3) belonging to three length size classes 130–180 µm; 280–330 µm; 380–430 µm, straight to slightly bent, frequently sinuous, entirely smooth, with heads from rounded to suboval (4–11 µm in width, Fig. 3a, b, c). Very rare small tylostyles with mushroom-like head, sinuous thin and short shaft (110–232 x 0.5–1 µm) also present. Very rare subtylostyles also present (Table 3). **Resting bodies** (gemmule-like) suboval, flat, firmly adhering to the substratum and scattered singly or in small groups (2–3) (Fig. 4). **Theca** of resting bodies as sublayered compact spongin (*ca.* 12 µm in thickness) with a smooth outer surface not armed by spicules (Fig. 4a1). **Active resting bodies** with evident

TABLE 3. Genus *Protosuberites*. Comparative list of spicular complement morphotraits for species with the strongest affinities with *P. mereui* sp. nov. Minimum, average (in bold), and maximum length x thickness of spicules are given when available.

Species	Spicule type	Length x Thickness μm	Resting bodies	Geographic range	Presence in brackish water	References
<i>P. mereui</i> sp. nov.	Tylostyles (very rare subtylostyles)	(100– 295.3 –650) x (3– 5.03 –10)	YES	W-Mediterranean Sea	YES	Present Paper
<i>P. rugosus</i> (Topsent, 1893)	Tylostyles	(200–1200) x 8 (shaft) x 12 (head)	NO	Mediterranean Sea	NO	Topsent, 1893
<i>P. prototipus</i> Swartschewsky, 1905	Tylostyles	(162–417) x (5–7)	NO	Black Sea	NO	Swartschewsky, 1905
<i>P. denhartogi</i> Soest & Kluijver, 2003	Tylostyles	(110– 258.7 –456) x (4– 6.3 –11) (rarely 600)	NO	N-Atlantic Ocean	NO	Soest & Kluijver, 2003
<i>P. ectyoninus</i> (Topsent, 1900)	Subtylostyles Tylostyles	(97– 204.97 –320) x (2.5– 4.71 –9.5) (145–400) x (7–13)	NO	N-Atlantic Ocean Mediterranean Sea	NO	Ackers <i>et al.</i> , 1992 Present paper
<i>P.</i> cf. <i>epiphytum</i> (<i>sensu</i> Topsent, 1900) now <i>P. Denhartogi</i>	Tylostyles	(100–300) x (4–7)	NO	N-Atlantic Ocean	NO	Topsent, 1900
<i>P. modestus</i> (Pulitzer-Finali, 1978)	Tylostyles	(400–1050) x (8–14)	NO	N-Atlantic Ocean Mediterranean Sea	NO	Pulitzer-Finali, 1978
<i>P. incrustans</i> (Hansen, 1885)	Tylostyles	(225– 320.7 –477) x (7.38– 10.02 –15.11)	NO	N-Atlantic Ocean Mediterranean Sea	NO	Present paper
<i>P. ferrer-hernandezii</i> (Boury-Esnault & Lopes, 1985)	Tylostyles	(135.3– 510.1 –965.6) x 2.7– 8 –20.7	NO	N-Atlantic Ocean	NO	Boury-Esnault & Lopes, 1985
<i>P. aquaedulcioris</i> (Annandale, 1914)	Tylostyles, subtylostyles, oxeas (rare bent centrotyloses)	330 x 5	YES	Bay of Bengal S-Atlantic Ocean	YES	Annandale, 1914
<i>P. collaris</i> (Annandale, 1924)	Tylostyles	≥800	NO	Laut Banda Laut Jawa Wallacea	YES	Annandale, 1924
<i>P. lacustris</i> (Annandale, 1915)	Tylostyles Subtylostyles	(560–580) x 8	YES	Bay of Bengal Indian Ocean	YES	Annandale, 1915

single foraminal aperture and collar closer to the distal part of the suboval theca (430 μm , total length with collar) (Fig. 4). **Foramen** with well-developed, thin-walled, transparent collar (ca. 80 μm in length) (Fig. 4). **Inactive resting bodies** present in the same specimens with no foraminal aperture and no collar, but bearing a small area apparently devoid of cells closer to the distal part of the longer axis of the suboval theca (Fig. 4).

Habitat and topographic distribution. The population of *Protosuberites mereui* **sp. nov.** occurs as scattered *facies* (up to 1 m^2 covered area) in mixohaline pool water within the totally dark main tunnel of the estuarine/anchialine southern branch of the Bue Marino Cave. In this part of the cave, salinity varies from 24 ‰ up to 39.6 ‰, depending on the point, depth and date of sampling. Substrata range from vertical limestone walls and large boulders to artificial substrata (plastic) at 0.5–1 m of depth. The new species share the same microhabitat with the serpulid *Ficopomatus enigmaticus* (Fauvel, 1923) and sometime with unidentified haplosclerid sponges. The kamptozoan *Barentsia gracilis* M. Sars, 1835 was strictly associated with the suberitid sponges.

Geographic range. *Protosuberites mereui* **sp. nov.** is only known from the type locality Bue Marino Cave in the central-eastern Sardinian Karst, western Tyrrhenian Sea.

Etymology. The species is dedicated to the Sardinian speleologist and photographer Luigi Mereu, who died prematurely during the exploration of a terrestrial cave, in recognition of his key contribution in the present research.

Molecular analyses

The concatenated analysis consisted of 3990bp (551 bp of *COI*, 2320 bp of *18S*, and 1119 bp of *28S*) including 23 sequences *i.e.* 19 belonging to 6 species of *Protosuberites* and 4 outgroups (*Suberites domuncula*, *S. pagurorum*, *Prosuberites laughlini*, and *P. longispinus*). For *P. denhartogi* and *P. mereui* **sp. nov.** we sequenced the gene *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 (Table 1).

The phylogenetic reconstruction of the concatenated set of genes recovered unequivocally a sister relationship between *Protosuberites* sp. ‘Panama’ and *P. mereui* **sp. nov.** with 0.99 of posterior probability and 98% of bootstrap (Fig. 5). The clade containing both *Protosuberites* sp. ‘Panama’ and *P. mereui* **sp. nov.** showed a sister relationship with *P. denhartogi* with posterior probability and bootstrap values of 1 and 100%, respectively (Fig. 5). *Protosuberites incrustans* (Hansen 1885) and *Protosuberites* sp. ‘Thailand’ formed a robustly supported clade that was sister to the clade containing *P. mereui* **sp. nov.**, *Protosuberites* sp. ‘Panama’, and *P. denhartogi*. Finally, *P. ectyoninus* (Topsent 1900) appeared as the earliest divergent clade within the *Protosuberites* species group analysed here (Fig. 5).

Genetic distances calculated for the *COI* gene were always larger than 2% between the *Protosuberites* species compared in this study (Table 4). The genetic distance calculated between *P. mereui* **sp. nov.** and the closest species, *Protosuberites* sp. ‘Panama’, was the lowest (2.9 ± 0.8 ; Table 4), while the distance between *Protosuberites* sp. ‘Panama’ and *P. ectyoninus* was the highest found (6.8 ± 1.1 ; Table 4).

TABLE 4. Genus *Protosuberites*. Average *COI* *p*-distances (lower left corner) and their standard deviation (upper right corner) computed between the different species included in the phylogenetic analyses. Values of *p*-distance between *Protosuberites mereui* **sp. nov.** and its analyzed closest relative are shown in bold.

	<i>P. ectyoninus</i>	<i>P. incrustans</i>	<i>P. sp.</i> ‘Thailand’	<i>P. denhartogi</i>	<i>P. mereui</i> sp. nov.	<i>P. sp.</i> ‘Panama’
<i>P. ectyoninus</i>	-	1.1	1.1	0.9	1.1	1.2
<i>P. incrustans</i>	5.6	-	0.3	0.9	0.8	1.2
<i>P. sp.</i> ‘Thailand’	5.6	5	-	0.9	0.9	1
<i>P. denhartogi</i>	5.5	5.8	5.8	-	1	1
<i>P. mereui</i> sp. nov.	5.7	3.4	3.9	6.4	-	0.8
<i>P. sp.</i> ‘Panama’	6.8	4.4	4.4	6.3	2.9	-

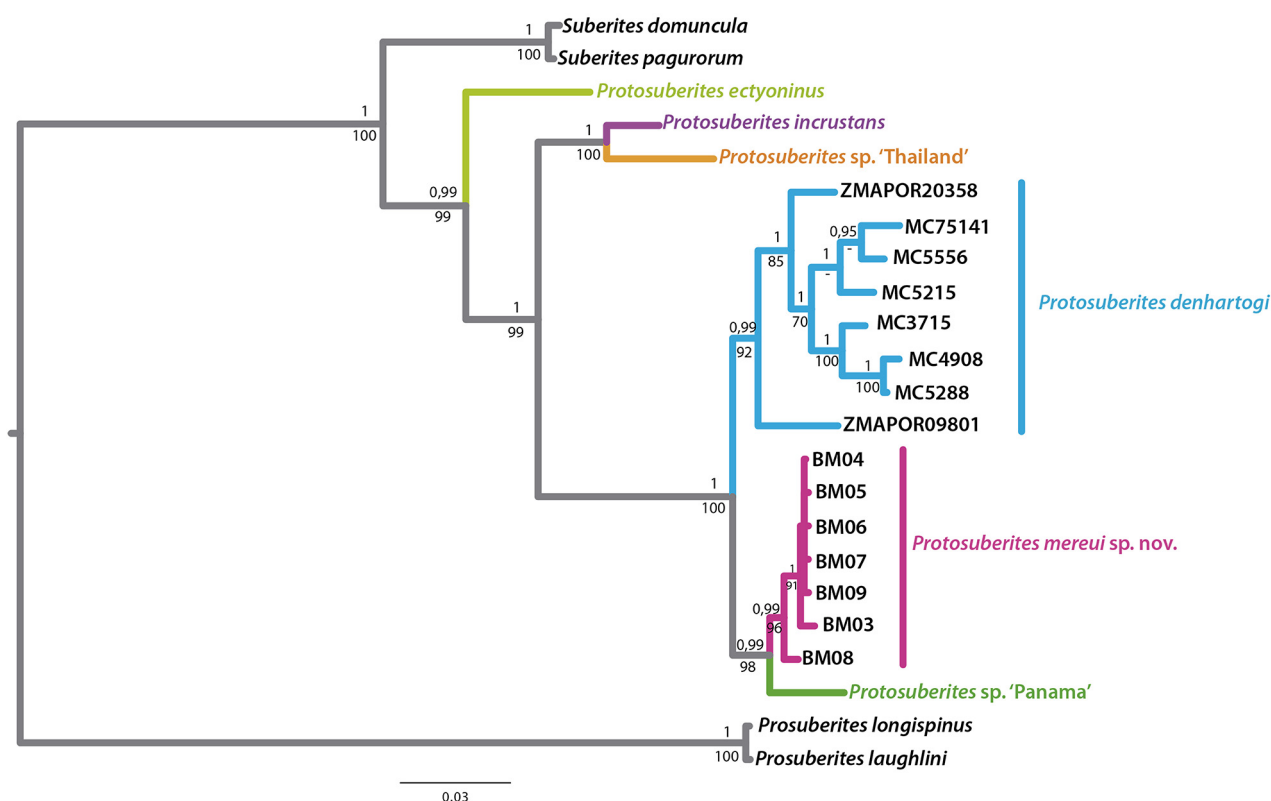


FIGURE 5. Genus *Protosuberites*. Phylogenetic tree obtained using a concatenated analysis of *COI*, *18S*, and *28S* and analyzed by both Bayesian inference (BI) and maximum likelihood (ML) methods. Numbers over branches indicate posterior probabilities and below bootstrap values. Only posterior probabilities over 0.95 and bootstrap values over 70 are indicated.

Discussion

Protosuberites mereui **sp. nov.** occurring in the Bue Marino cave is assigned to the genus *Protosuberites* Swartschewsky *sensu* van Soest (2002) on the basis of its skeletal architecture *i.e.* erected tylostyles either single or in tufts/bundles. The new species is morphologically similar to *P. denhartogi*, which shows a North-Eastern Atlantic geographic range and *P. prototipus* Swartschewsky, 1905, which is endemic to the Black Sea, but it differs in several characters. Tylostyle morphometries of *P. mereui* **sp. nov.** partially match those of *P. denhartogi* (van Soest 1977; van Soest & de Kluijver 2003) and those of *P. epiphytum* from the Mediterranean (Pulitzer-Finali 1983), now identified as *P. denhartogi*. However, the peculiar small slender tylostyles with a sinuous shaft and a typical mushroom-like head of *P. mereui* **sp. nov.** have never been reported for any *Protosuberites* of 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). In addition, *P. mereui* **sp. nov.** shares with *P. aquaedulcioris* and *P. lacustris* the ability to produce resting bodies (Annandale 1915), and the spicule sizes overlap in many instances (see Table 3). However, *P. aquaedulcioris* and *P. lacustris* have a spicular complement of subtylostyles and resting bodies without aperture (Annandale 1914, 1915) and *P. aquaedulcioris* also possesses bent centrotylotes or oxeas (Annandale 1915). Another species from brackish water with similarities to *P. mereui* **sp. nov.** is *P. collaris*, but the latter is not reported to produce resting bodies and the tylostyles are sensibly larger (Annandale 1924; Table 3).

Perhaps one of the most interesting features of *P. mereui* **sp. nov.** is its peculiar habitat in mixohaline waters of a Mediterranean cave. Indeed, the southern branch of the Bue Marino Cave is an estuarine-anchialine cave where the physical-chemical parameters, such as water salinity, change dramatically during the year. This cave-dwelling habitat preference is only shared by another congeneric species, *P. geracei* (van Soest & Sass 1981), to our knowledge the only Suberitidae reported from an estuarine/anchialine cave so far (Manconi *et al.* 2012), for which resting body are not reported. Only 3 species of the genus *Protosuberites* were reported till now from brackish water *i.e.* *Protosuberites aquaedulcioris* (Annandale, 1914), *Protosuberites lacustris* (Annandale, 1915), and

Protosuberites collaris (Annandale, 1924) from coastal basins of the Indian Ocean. Interestingly, *P. aquaedulcioris* and *P. lacustris*, as well as a few other species of the family Suberitidae, e.g. *Suberites sericeus* Thiele, 1898 are able to produce gemmule-like resting bodies (Annandale 1915; Simpson & Fell 1974; Manconi & Pronzato 2007, 2016). As for morphology of resting bodies, while the spongin theca in *S. sericeus*, *P. aquaedulcioris*, and *P. lacustris* is reported as multilayered without an aperture (foramen), the cave-dwelling *P. mereui* **sp. nov.** is characterised by gemmules with sublayered compact spongin in the theca and a structurally organized foraminal aperture ornate by a conspicuous long collar. Gemmulation of sponges is interpreted as a successful adaptive strategy to resist harsh environmental conditions (Simpson & Fell 1974; Pronzato & Manconi 1994, 1995; Manconi & Pronzato 2002, 2007, 2015, 2016). In summary, the presence of resting bodies in the brackish species of a preeminent marine genus supports the idea of their marine origin, with the consequent adaptive changes that the species suffered to cope with drastic shifts in salinity.

Protosuberites mereui **sp. nov.** seems to be close to *P. denhartogi* from a morphological point of view (van Soest 1977; van Soest & de Kluijver 2003), however, our phylogenetic analyses confirm its generic status but shows a slightly different scene. Our molecular results (Fig. 5) indeed reveal that *P. mereui* **sp. nov.** forms a clade with *Protosuberites* sp. ‘Panama’, which is characterized by tylostyles in combination with trichodragmas (Nichols 2005), and those two are sister to *P. denhartogi*. Such results could be indicating a high plasticity of the morphological characters of tylostyles (Chombard *et al.* 1998) and the possibility that these might be affected by environmental factors (McDonald 2002). Therefore, convergent evolution could explain how species relatively far from a phylogenetic point of view show a high similarity in morphotraits. The genetic distance (*p*-distance) calculated between *P. mereui* **sp. nov.** and *Protosuberites* sp. ‘Panama’ 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 *COI p*-distances at around 1.6% distinguished a cryptic species closely related to *Tedania* (*Tedania*) *ignis* (Wulff 2006). Slightly larger mean *COI p*-distances have been reported in the cryptic species within *Astrosclera willeyana* Lister, 1900 (4.1% in Wörheide 2006), *Cliona* aff. *celata* Grant, 1826 (6.2% to 8.4% in Xavier *et al.* 2010), and *Scopalina blanchensis* Blanquer & Uriz 2008 (13–24% in Blanquer & Uriz 2007).

In summary, both morphological and molecular analyses confirm unanimously *Protosuberites mereui* **sp. nov.** as a new species from a Western Mediterranean estuarine-anchialine cave. Therefore, the presence of resting bodies with a foraminal aperture in *P. mereui* **sp. nov.** combined with the presence of very rare small, slender tylostyles with a sinuous shaft and a typical mushroom-like head represent the exclusive diagnostic traits for the new species, never reported for any cave-dwelling *Protosuberites* recorded in the Mediterranean Sea (see Pouliquen 1972; Pulitzer-Finali 1983; Corriero *et al.* 2000).

Further studies including sequencing of other *Protosuberites* species with resting bodies will help us understand whether cryptobiosis has appeared several times in the evolutionary history of the genus and family.

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