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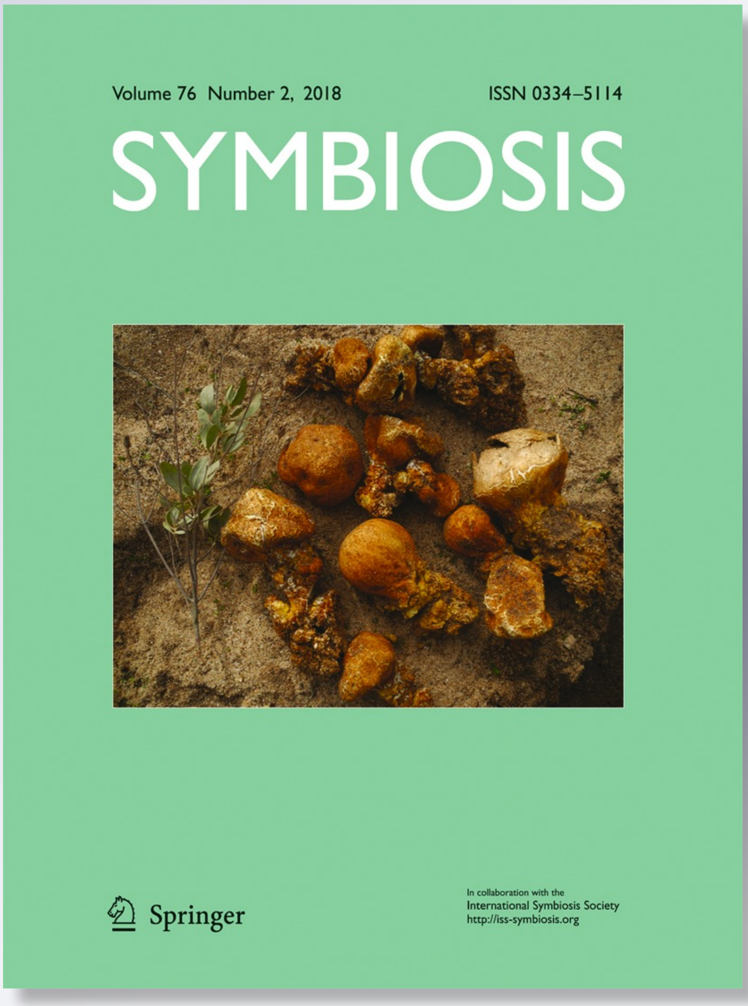

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Scleroderma meridionale ectomycorrhizae on *Halimium halimifolium*: expanding the Mediterranean symbiotic repertoire

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Abstract

Scleroderma is a gasteroid genus in the *Boletales* (Basidiomycota), with a cosmopolitan distribution. Species of *Scleroderma* establish ectomycorrhizal (ECM) symbiosis with a range of coniferous and non-coniferous trees and shrubs, both in temperate and tropical regions, with little tendency to host specificity, a feature that might have facilitated the wide distribution of the genus. With the contribution of confocal laser scanning microscopy, we describe the morpho-anatomical features of the ectomycorrhizae formed by *Scleroderma meridionale* on *Halimium halimifolium*, a cistaceous plant belonging to a small group of woody shrubs occurring in open vegetation types in the Mediterranean region. The mycobiont and host plant identity in ECM was verified through molecular tools. Mycorrhizal system is very small, up to 1.9 mm, mostly coralloid to irregularly pinnate. The mantle surface is felty, whitish with silver patches. Differentiated rhizomorphs occur infrequently. Mantle surface is characterized by a network of branched hyphae organized in hyphal boundles. Hyphae are frequently covered by granules or warts. These characters, except for the presence of granules, are similar to those reported for the only two naturally-occurring *Scleroderma* ECM described so far, i.e. *S. bovista* on *Populus* and *S. citrinum* on *Betula* and *Pinus*. On the other side, the peculiarity of *S. meridionale* + *Halimium* ECM is the particularly small dimension of mycorrhizal system, a character shared with ECM formed by *Cistaceae*. At the best of our knowledge, this is the first description of an ectomycorrhiza on *Halimium*, a plant whose mycorrhizal biology deserves to be explored in greater detail.

Keywords Confocal laser scanning microscopy · Molecular phylogeny · Maquis shrubland · *Cistaceae* · *Sclerodermatаceae* · *Boletales*

1 Introduction

Scleroderma is a gasteroid genus in the *Boletales* (Basidiomycota), with a cosmopolitan distribution. About 25 species are currently recognized (Kirk et al. 2008), although much work on the delimitation of taxa and their phylogenetic relationships is still ongoing (Phosri et al. 2009; Wilson et al. 2012; Rusevska et al. 2014), and so is the identification of new species (Baseia et al. 2016; Crous et al. 2016). Species of *Scleroderma* establish ectomycorrhizal (ECM) symbiosis with a range of coniferous and non-coniferous trees and shrubs, both in temperate and tropical regions, with little tendency to host specificity (Jeffries 1999; Mrak et al. 2016). It has been suggested that the ectomycorrhizal generalist habit has facilitated the wide distribution of *Scleroderma* and other Sclerodermatineae groups (Wilson et al. 2012). In addition to field-based observations, the relatively easy laboratory synthesis of *Scleroderma* ectomycorrhizae with a number of hosts and the frequent occurrence of such symbiotic interactions in plant nurseries (hereafter defined as semi-natural ectomycorrhizal symbioses) have been reported (Richter and Bruhn 1989; Ingleby 1999; Jeffries 1999; Rinaldi et al. 2008; Comandini et al. 2012b; Wilson et al. 2012; Mrak et al. 2016), indirectly confirming the broad host-range of the genus and

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supporting its use in many studies focused on the inoculation of trees for reforestation programs (Jeffries 1999). Despite its importance as a prominent ectomycorrhizal genus, surprisingly only very little information is available on the features of naturally-occurring *Scleroderma* mycorrhizae. Detailed descriptions are available only for *Scleroderma bovista* Fr. on *Populus alba* (Jakucs and Agerer 1999), and for *S. citrinum* Pers. on *Betula pendula* and *Pinus sylvestris* (Waller et al. 1993). In other cases, *Scleroderma* ectomycorrhizae were observed in natural settings, but the mycobiont species remained undetermined and/or the morphological description of relevant features was too preliminary to allow comparisons (e.g., Meotto et al. 1994; Lee et al. 1997; Buée et al. 2005).

The genus *Halimium* is in the family *Cistaceae*. The genus comprises 13 accepted species (http://www.theplantlist.org/1.1/browse/A/Cistaceae/Halimium/), all of them being evergreen or semi-deciduous small to large shrubs, with yellow or white flowers with three locules in each ovary (Thorogood 2016). Distribution coincides with the Mediterranean basin, and, like in the very close genus *Cistus*, the centre of diversity is in the western part of the floristic region (Civeyrel et al. 2011). As *Cistus*, *Halimium* species are mainly found in open vegetation types, like matorral shrublands and garrigues, but also occur at the verges of woods, in degraded forest areas, abandoned fields, pasturelands, and also on coastal sandy soils (Zunzunegui et al. 2009). So far, *Halimium* mycorrhizal biology has received little attention. However, it is known that the genus may form both ectomycorrhizae and vesicular arbuscular mycorrhizae (Camprubi et al. 2011; Buscardo et al. 2012; Beddiar et al. 2015). This dual mycorrhizal status is shared with *Cistus* (Comandini et al. 2006) and several other genera of host plants, such as *Populus*, *Salix*, *Alnus*, and *Eucalyptus* (Smith and Read 1997). In the case of *Cistus* and *Eucalyptus*, mycorrhizal plasticity may represent an adaptive trait to the cyclical pattern of accumulation and loss of organic resources that occur in regions prone to disturbance events, such as wildfires (Smith and Read 1997), and this possibly also applies to *Halimium* (Buscardo et al. 2012).

In the present study, we describe the naturally-occurring ectomycorrhizae formed by *Scleroderma meridionale* Demoulin & Malençon [MycoBank #323250] on *Halimium halimifolium* (L.) Willk. (Jaguarzo blanco, Ciste jaune, Cisto giallo), as based on samples collected in Sardinia, Italy. Ectomycorrhizae were characterized following a combined morpho-anatomical and molecular approach, and the identity of the plant host was verified through molecular tools. At the best of our knowledge, this is the first description of an ectomycorrhiza on *Halimium*.

Fig. 1 Basidiomes of *Scleroderma meridionale*, from a coastal area in Sardinia, Italy

2 Materials and methods

2.1 Study site and fungal collections

Basidiomes of *Scleroderma meridionale* (Fig. 1) were harvested in a sandy area close to Gonnesa (*39°15′18” N, 8°24′ 32″ E,* 98 m asl), about 70 km west of Cagliari; basidiomes were identified in the field on the basis of published descriptions of macroscopic and microscopic characters (Brotzu and Colomo 2009). Specimens were collected from under *Halimium halimifolium* (Fig. 2) in a pure stand. Identity of the plant host in the ectomycorrhizae was confirmed through molecular tools (see below). Soil cores (about 20 × 20 × 20 cm) were excavated from beneath basidiomes and immersed overnight in water, and ectomycorrhizal roots were carefully separated under a dissecting microscope. Several tips were immediately transferred into 50% EtOH and stored at −20 °C for subsequent DNA analysis. Reference material for basidiomes (ACR-2014-4, Hal-BP-92, Hal-BP-94) and ectomycorrhizae (ACR-2014-4-E, Hal-BP-92-E, Hal-BP-94-E) is deposited in CAG, at the collection of the Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy.

2.2 Microscopy

Mantle preparations of fresh ectomycorrhizae were fixed on microscope slides with polyvinyl lactophenol for light microscopy. Observations were made with a Zeiss Axioplan 2 bright field microscope and a Leica MZ 6 stereomicroscope. Images were acquired with a Leica DFC290 digital camera. For longitudinal sections (2.5 μm thick), ectomycorrhizae were embedded in LR White resin (Multilab Supplies, Surrey, UK), cut with a Leica Ultracut R ultramicrotome and stained with toluidine blue in 1% sodium borate for 15 s at 60 °C. For confocal laser scanning microscopy, fixed ectomycorrhizae (4%

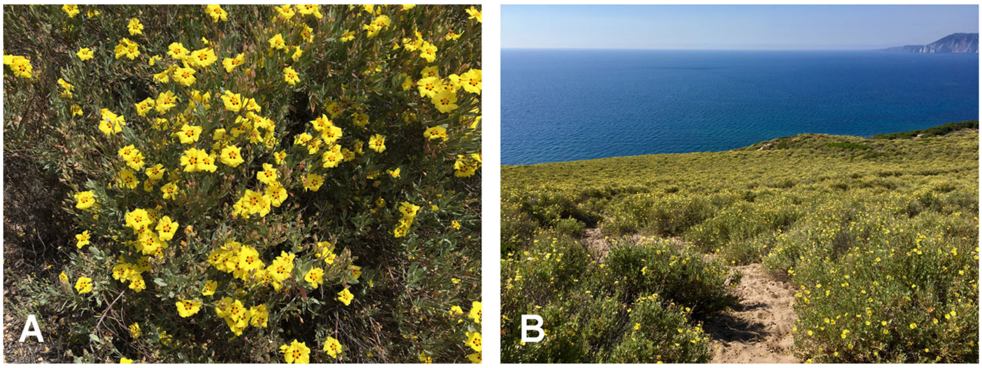

Fig. 2 *Halimiun halimifolium*, *Cistaceae*. **A**. Plant in blossom. **B**. View of ECM collection site

glutaraldehyde) were mounted in Vectashield Antifade Mounting Medium (Burlingame, CA) and then examined by TCS SP5 Leica confocal microscopy (Leica Microsystems, Mannheim, Germany) equipped with an inverted microscope DMI 6000 CS (Objective HCX PL APO CS 40 × 1.3 oil) and a VIS Argon laser. The laser excitation wavelength was fixed at 488 nm. The general methodology and terminology used to characterize ectomycorrhizae followed Agerer (1986, 1987–2012, 1991, 1995). Munsell Soil Colour Charts (2000) were used as reference for the description of the colours of ectomycorrhizae. ECM descriptions are based on 20 root tips from five samples collected under *S. meridionale* basidiomes.

2.3 Molecular analyses and phylogenetic inference

Characterization of ectomycorrhizae using a molecular approach was based on PCR amplification and sequencing of the complete internal transcribed spacer (ITS) regions in nuclear ribosomal DNA (Gardes and Bruns 1993). Genomic DNAs of the basidiomes were isolated from 20 mg of each dried sample using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and the ITS amplifications were performed following the protocol reported by Leonardi et al. (2005). A direct PCR approach was applied to identify ECM tips isolated from soil samples as described by Iotti and Zambonelli (2006). Three ECM tips were selected as PCR targets and directly amplified using ITS1F/ITS4 primers pair (White et al. 1990; Gardes and Bruns 1993). Two microliters of 20 mg/ml BSA (bovine serum albumine) solution (Fermentas, Vilnius) were added to each reaction tube to prevent PCR inhibition (Leonardi et al. 2013). The amplified products were purified using the QIAquick PCR Purification Kit (Qiagen, Milan, Italy) and sequenced by Eurofins Genomics service (Ebersberg, Germany). Sequences are deposited in GenBank under accession numbers MG264160 and MG367369 for basidiomes and ECMs, respectively. To confirm the identity of the host shrub roots, the plastid trnL region of ECM root tip DNA was amplified using primers pair trnC/trnD following Tedersoo et al. (2008). The chloroplast trnL region obtained by PCR amplification of DNA extract from *H. halimifolium* leaves was used as positive control (see supplementary materials, Fig. 1S). The ITS regions of the nuclear rDNA obtained were compared with those present in international nucleotide sequence databases using the BLASTN search (Altschul et al. 1990).

Based on these results and on the outcomes of recent phylogenetic studies on *Scleroderma* (Phosri et al. 2009; Rusevska et al. 2014; Mrak et al. 2016), sequences were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) and UNITE (http://unite.ut.ee/) databases for a comparative phylogenetic analysis. After excluding the ambiguous regions at the 5′ and 3′ ends of the chromatograms, sequences were edited using BioEdit v. 7.2.5 (Hall 1999) and aligned with MAFFT programme (https://mafft.cbrc.jp/alignment/software/) using the E-INS-i aligning strategy (Katoh et al. 2005). Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). Model with the lowest AICc value (Akaike Information Criterion, corrected; Nei and Kumar 2000) was chosen to describe the best substitution pattern. The maximum likelihood analysis was based on 1000 bootstrap replicates using a Tamura 3-parameter model with a gamma distributed rates among sites. *Pisolithus arhizus* (FM213365) was chosen as outgroup taxon.

3 Results

3.1 Descriptions of ectomycorrhizae

Morphological characters: Mycorrhizal systems 0.5–1.9 mm long (up to 3.5 mm the coralloid systems), coralloid to irregularly pinnate, with 0–3 orders of ramification, mostly dense and short. *Main axis* 0.1–0.2 mm in diameter. *Mantle surface* smooth, white (sometimes yellowish) with silver patches. *Mycorrhiza* light yellowish brown (HUE 10YR 6/4) with whitish parts (Fig. 3). *Unramified ends* sometimes tortuous, but mostly bent, 0.1–0.5 (−0.7) mm long and 0.1–0.2 mm in diameter. *Hyphal strands* abundant in the soil, most of them sticking to mantle surface. *Rhizomorphs* present but infrequent, rounded, connected to the mantle at a restricted point, or in ‘flat angle’; color lighter than mantle surface. *Sclerotia* were not observed.

Anatomical characters of mantle in surface views: *Outer mantle layer* (Figs. 4A, 5A). Loosely plectenchymatous, sometimes arranged net-like, in some other parts hyphae irregularly

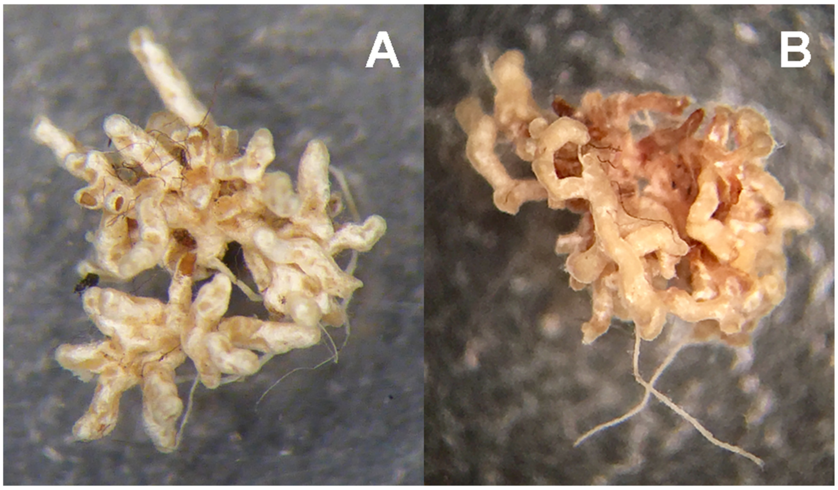

Fig. 3 **A**-**B**. Habitus of *Scleroderma meridionale* ectomycorrhizae with *Halimiun halimifolium*

arranged. Hyphae present several incrustations or warts that hamper an easy and clear observation of structures. However, at the margins structures are easier to observe. Hyphae are 3–4 (−4.5) μm thick, swollen in some parts, especially close to the septa, hyphal walls less than 1 μm thick; clamps have been observed, although rather infrequently, but not in all samples. Hyphae frequently branched, sometimes rounded apical ends are observable. *Middle mantle layer* (Figs. 4B, 5B) more densely plectenchymatous, structure very similar to the outer mantle layer but here hyphae are much more branched than in the outer part, and are also more densely interwoven and compact. Hyphae still possessing incrustations. Sometimes hyphal cells can also be observed. *Inner mantle layer* (Figs. 4C, 5C) transitional type, formed by rectangular hyphal cells, 3–5 μm long, and by hyphal cell-like structures 3–3.5 μm in diam.

Anatomical characters of emanating elements. Rhizomorphs (Fig. 4D). Hyphal strands are abundant in soil cores and they often stick to mantle surface, so that sometimes they can be confused with rhizomorphs. Rhizomorphs are present, albeit infrequently, and come generally from restricted points of the mantle surface, although they can also be connected to the mantle surface at flat angles. They are very thin, up to 1 mm thick and compact. Hyphae are abundantly covered by warts that make the observations of the thicker parts of the rhizomorphs very difficult. However, in their thinner parts, rhizomorphs display a differentiated structure with thicker vessel-like central hyphae up to 8 μm in diameter. Peripheral hyphae showing the same characteristics observed in the outer mantle layer. *Emanating hyphae* lacking. *Cystidia* lacking.

Anatomical characters, longitudinal section (Fig. 5D). *Mantle* (15–) 20–40 (−45) μm thick, two main layers discernible: outermost layer, 10–20 μm thick, formed by loose hyphae that in section display as rounded/rectangular cells 2–3 ×

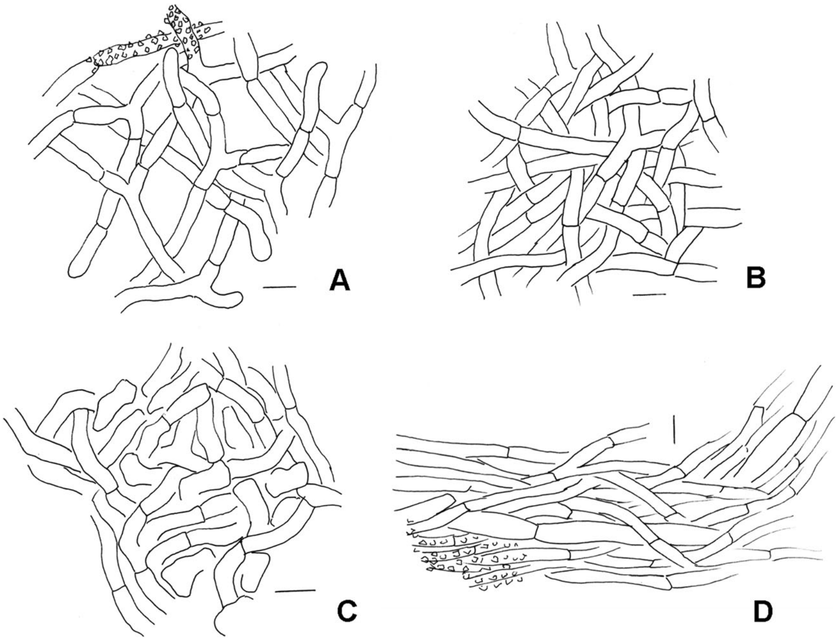

Fig. 4 Anatomical characters of *Scleroderma meridionale* + *Halimium* ectomycorrhizae. **A**. Outer mantle layer showing a loosely plectenchymatous structure. Hyphae present abundant incrustations or warts. **B**. Middle mantle layer, more densely plectenchymatous. Hyphae still possessing incrustations (not shown, to make pattern more discernible). **C**. Inner mantle layer with transitional structure, both hyphae and hyphal cells are observable. **D**. Differentiated rhizomorphs, were hyphae are also covered with warts. Bars = 5 μm

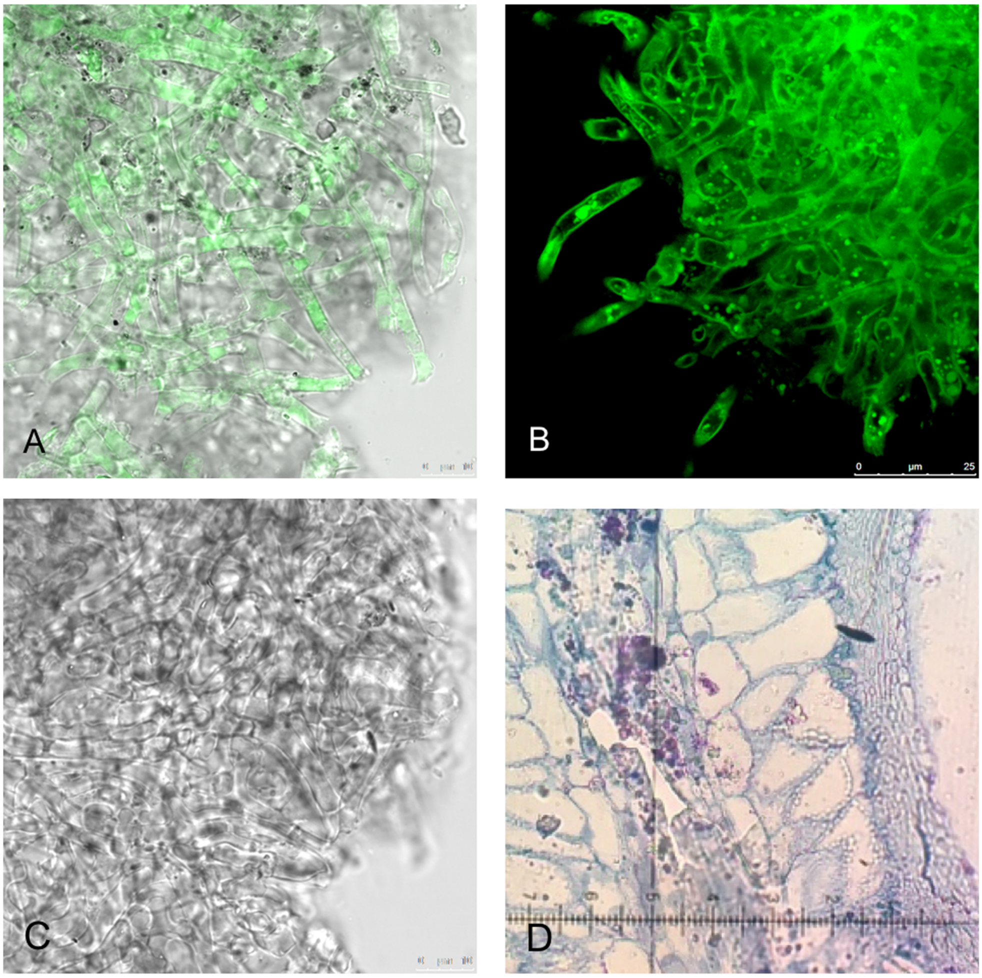

Fig. 5 Anatomical characters of *Scleroderma meridionale* + *Halimium* ectomycorrhizae. **A**. Outer mantle layer viewed with confocal laser scanning microscopy (CLSM), loosely plectenchymatous structure, hyphae arranged net-like. **B**. Plectenchymatous middle mantle layer viewed with CLSM. More compact structure, hyphae more branched, incrustations are visible. **C**. Inner mantle layer with transitional type structure, viewed with CLSM. **D**. Longitudinal section viewed with optical microscope, mantle with two main layers discernible (on the right side of the image, the outermost layer formed by loose hyphae that in section display as rounded/rectangular cells, and an underlying layer, closer to cortical cells, which presents a more compact structure), Hartig net surrounding a single row of cortical cells. Bars: in **A**-**C**, measure is stated in the image (10 µm in **A** and **C**, 25 µm in **B**); in **D**, each notch corresponds to 2.5 µm

2–3 (−4) µm; underlying layer, about 15–20 (−25) µm thick, presenting a more compact structure with hyphal cells possessing the same characteristics observed in the outermost layer. *Tannin cells* not observed. *Cortical (epidermal) cells* 1–2 rows, radially rectangular 25–45 (−50) × 15–20 µm, oriented obliquely. *Hartig net* peri-epidermal, made of one row of rounded, 2–3 µm diam hyphal cells, palmetti-type, lobes 1–1.5 µm wide, surrounding 1 row of cortical cells. Table 1 reports the main anatomical and morphological features of *Scleroderma meridionale* + *Halimium* ECM, as compared to those reported for the natural and semi-natural *Scleroderma* ECMs described so far.

3.2 Molecular and phylogenetic analyses

The ITS sequence of the basidiomes of *Scleroderma meridionale* matches exactly that of the ectomycorrhizae found on *Halimium halimifolium* roots, thus confirming the identity of the collected mycorrhizal structures. The amplifications produced a fragment of 587 bp containing the complete ITS1–5.8S-ITS2 sequence. The identity of the host plant was confirmed through analysis of the plastid trnL region of ECM root tip DNA compared to that of *H. halimifolium* leaves (Fig. 1S, supplementary materials). To build a multiple sequence alignment for phylogenetic analysis, ITS sequences with the best score resulting from BLASTN search were retrieved from GenBank. Additional ITS sequences representative of other *Scleroderma* major clades were also imported (Table 2). As an outgroup, *Pisolithus arhizus* (FM213365) was chosen. The ITS data set comprised 37 taxa and 733 characters, and contains 304 (41.47%) variable sites. Here we confirm that sequences identified as *S. citrinum* (AY935514) and *S. meridionale* (EU718121) correspond to *S. meridionale* and *S. septentrionale*, respectively, as also reported by Rusevska et al. (2014). A tree was constructed using the Maximum Likelihood method and is shown in Fig. 6. Nodes with bootstrap values lower than 70% were eliminated. Gaps were treated by selecting the "partial deletion" option in MEGA. The phylogenetic analysis shows that all the sequences attributed to *S. meridionale* cluster in a clade together with *S. septentrionale* and *S. laeve* as reported before by Rusevska et al. (2014) and Mrak et al. (2016), but the low bootstrap values statistically do not support the sister species hypothesis within the clade.

Table 1 Comparison of the main anatomical and morphological features reported for natural and semi-natural* *Scleroderma* ECMs

Species (ref.)	*S. areolatum* Mrak et al. 2016	*S. citrinum* Waller et al. 1993	*S. bovista* Jakucs and Agerer 1999	*S. sinnamariense* Ingleby 1999	*S. meridionale* this study
Occurrence	semi-natural	natural	natural	semi-natural	natural
Colour	silver white	silver white	silver white	chrome-yellow	silver white
SV	lpl	lpl	lpl	lpl	lpl
ML	dpl	dpl	dpl	ps	dpl
IV	pl	pl	pl	pl	pl
RHIZ	frequent, diff	diff	rare, hd	hd	rare, diff
Hyphae	clampless, branched, inflated	clamps, branched, inflated	clamps, branched	clamps, branched, incrustations	rare clamps, swollen, warts, branched

SV, surface view; ML, middle layer; IV, inner view; RHIZ, rhizomorphs; pl, plectenchymatous; lpl, loosely plectenchymatous; dpl, densely plectenchymatous; ps, pseudoparenchymatous; diff, differentiated; hd, highly differentiated. *Ectomycorrhizal interactions spontaneously occurring in plant nurseries

4 Discussion

Scleroderma meridionale is characterized by its almost globose fruit body and long pseudostipe, which is usually immersed deep in the soil, and by its smooth to finely furfuraceous peridium of an intense sulphur yellow color, which becomes brighter in the pseudostipe (Fig. 1). Frequently found in sandy, dry habitats, it was originally described on the basis of collections from southern Portugal, continental France, Corsica and Morocco (Demoulin and Malençon 1970), but it occurs in all the Mediterranean basin, including Greece, Macedonia and Turkey (Rusevska et al. 2014; Dimou et al. 2016). It is also reported from North America, from Florida to Arizona (Guzmán and Ovrebo 2000, http://www.mushroomexpert.com/scleroderma_septentrionale.html), although the identity of these collections still awaits a molecular confirmation. *S. meridionale* closely resembles *S. septentrionale* Jeppson, which is also found on sand dunes and banks but has a more northern distribution (North and Central Europe, North America from Oregon to Massachusetts), and the two species can be distinguished both morphologically and molecularly (Guzmán and Ovrebo 2000, Jeppson and Piątek 2005, Phosri et al. 2009, Rusevska et al. 2014, this study). Based on field observations, *S. meridionale* has been reported as associated to both evergreen and deciduous species of *Quercus* and to *Pinus* (Phosri et al. 2009; Dimou et al. 2016), but also to *Cistus salviifolius* (Comandini et al. 2006). In Sardinia, it is fairly common in coastal areas, under pine forests but also in more open vegetation (Brotzu and Colomo 2009).

In recent years, we have been carrying out a long-term study on the ectomycorrhizal biology of several fungal genera occurring in selected ecosystems in Europe and the Neotropics (Comandini et al. 1998; Comandini and Rinaldi 2001; Eberhardt et al. 2000; Nuytinck et al. 2004; Comandini et al. 2004, 2012a; Flores Arzú et al. 2012; Roy et al. 2017). Despite its importance as a prominent ectomycorrhizal genus (Jeffries 1999), surprisingly only very little information is available on the features of naturally occurring *Scleroderma* mycorrhizae. Detailed descriptions are available only for *S. bovista* on *Populus alba* (Jakucs and Agerer 1999), and for *S. citrinum* on *Betula pendula* and *Pinus sylvestris* (Waller et al. 1993). Moreover, a detailed description of *S. areolatum* ECM on *Fagus sylvatica* obtained from a tree nursery (semi-natural, i.e. spontaneously formed, not an artificial inoculation) – together with a detailed discussion of all *Scleroderma* ECMs observed until this study – has been recently published (Mrak et al. 2016). Table 1 reports the main anatomical and morphological features reported for the described natural and semi-natural *Scleroderma* ECMs, including *S. meridionale* + *Halimium*. All these are whitish, silvery white, except those formed by *S. sinnamariense* that are chrome-yellow as the fruit body. A plectenchymatous outer mantle is also a common feature to all the described *Scleroderma* ECMs and hyphae are branched and irregularly arranged or forming ring-like hyphal bundles. Also the presence of swollen or inflated hyphae, observable in different parts of the mantle and rhizomorphs, is a trait shared among ECMs formed by this fungal genus. Rhizomorphs are present in all *Scleroderma* ECMs, however their structure and abundance differ among the species. The presence of clamps connection is not uniform, and seems to be related to the taxonomic position of the species considered (Mrak et al. 2016). The ectomycorrhizae of *S. meridionale* described in this work share the main features present in the other *Scleroderma* ECMs, although some pecurialities have been observed (Table 1). The presence of granules or warts, that at times makes it difficult to clearly observe *S. meridionale* ECMs mantle and rhizomorph structures, is a character observed also in *S. sinnamariense* ECM which is, however, not so closely taxonomically related. In our case, the abundance of warts in *S. meridionale* could be due to the sandy soil, whose tiniest particles might stick to the hyphae. At difference from most of the described *Scleroderma* ECMs, in *S. meridionale* the

Table 2 List of *Scleroderma* sequences used in this study. Beyond the information obtained from GenBank, sequences extracted from UNITE database are also reported

Taxon name	UNITE taxon name	GenBank accession number	UNITE Hypothesys species code	Country	Reference
Scleroderma citrinum	*Scleroderma meridionale*	AY935514	SH186878.07FU	Spain	Ruiz-Díez et al. 2006
Scleroderma meridionale		HF933239	SH186878.07FU	Macedonia	Rusevska et al. 2014
Scleroderma meridionale		HF933238	SH186878.07FU	Macedonia	Rusevska et al. 2014
***Scleroderma meridionale* (basidiome)**		MG264160	SH186878.07FU	Italy	this study
***Scleroderma meridionale* (ectomycorrhiza)**		MG367369	SH186878.07FU	Italy	this study
uncultured Sclerodermatineae	*Scleroderma sp.*	KX499290	SH642977.07FU	Costa Rica	unpublished
Scleroderma laeve		EU718120	SH186879.07FU	?	Wilson et al. 2011
Scleroderma sp.	*Scleroderma laeve*	EU718125	SH186879.07FU	Malaysia	Wilson et al. 2011
Scleroderma suthepense	*Scleroderma laeve*	NR132871 (=JX205215)	SH186879.07FU	Thailand	Kumla et al. 2013
Scleroderma sp.	*Scleroderma septentrionale*	FM213342	SH186875.07FU	USA	Phosri et al. 2009
Scleroderma cf. *septentrionale*	*Scleroderma septentrionale*	FM213337	SH186875.07FU	USA	Phosri et al. 2009
Scleroderma cf. *bovista*	*Scleroderma septentrionale*	FM213339	SH186875.07FU	USA	Phosri et al. 2009
Scleroderma septentrionale		FM213338	SH186875.07FU	USA	Phosri et al. 2009
Scleroderma meridionale	*Scleroderma septentrionale*	EU718121	SH186875.07FU	USA	Wilson et al. 2011
Scleroderma bovista		KU739419	SH186874.07FU	Spain	unpublished
uncultured *Scleroderma*	*Scleroderma bovista*	KM522823	SH186874.07FU	New Zealand	unpublished
Scleroderma bovista		HF933242	SH186874.07FU	Serbia	Rusevska et al. 2014
Scleroderma sp.	*Scleroderma bovista*	FM213341	SH186874.07FU	Hungary	Phosri et al. 2009
Scleroderma bovista	*Scleroderma bovista*	MG973282	SH186874.07FU	Italy	unpublished
Scleroderma bovista	*Scleroderma bovista*	MG973283	SH186874.07FU	Italy	unpublished
Scleroderma bovista		FM213340	SH186874.07FU	Spain	Phosri et al. 2009
Scleroderma septentrionale	*Scleroderma sp.*	FM213336	SH186877.07FU	Sweden	Phosri et al. 2009
Scleroderma michiganense		FM213346	SH186876.07FU	USA	Phosri et al. 2009
Scleroderma michiganense		FM213347	SH186876.07FU	USA	Phosri et al. 2009
Scleroderma michiganense		FM213348	SH186876.07FU	USA	Phosri et al. 2009
Scleroderma citrinum		EU784414	SH186873.07FU	UK	Brock et al. 2009
Scleroderma citrinum		EU784413	SH186873.07FU	UK	Brock et al. 2009
Scleroderma citrinum		FM213344	SH186873.07FU	USA	Phosri et al. 2009
Scleroderma areolatum		FM213352	SH182459.07FU	USA	Phosri et al. 2009
Scleroderma verrucosum	*Scleroderma areolatum*	EU784416	SH182459.07FU	UK	Brock et al. 2009
Scleroderma verrucosum		EU784415	SH182460.07FU	UK	Brock et al. 2009
Scleroderma cepa	*Scleroderma verrucosum*	FM213354	SH182460.07FU	Spain	Phosri et al. 2009
Scleroderma verrucosum	*Scleroderma verrucosum*	MG973284	SH182460.07FU	Italy	unpublished
Scleroderma verrucosum	*Scleroderma verrucosum*	MG973285	SH182460.07FU	Italy	unpublished
Scleroderma polyrhizum		FM213349	SH179759.07FU	USA	Phosri et al. 2009
Scleroderma polyrhizum		FM213350	SH179759.07FU	USA	Phosri et al. 2009
Pisolithus arhizus		FM213365	SH177625.07FU	Spain	Phosri et al. 2009

rhizomorphs are quite rare, while abundant are the hyphae strands produced by the fungus. Another feature of *S. meridionale* + *Halimium* ECM is the small dimension of mycorrhizal system, which is a character tipically depending on the host plant; indeed, this trait is shared with the ECMs formed by other *Cistaceae* (Nuytinck et al. 2004; Comandini and Rinaldi 2008; Leonardi et al. 2016).

So far, only a handful of recent studies have dealt with the contingent of ECM fungi linked to *Halimium*. Two species of the newly described genus *Tubariomyces* (*T. hygrophoroides*;

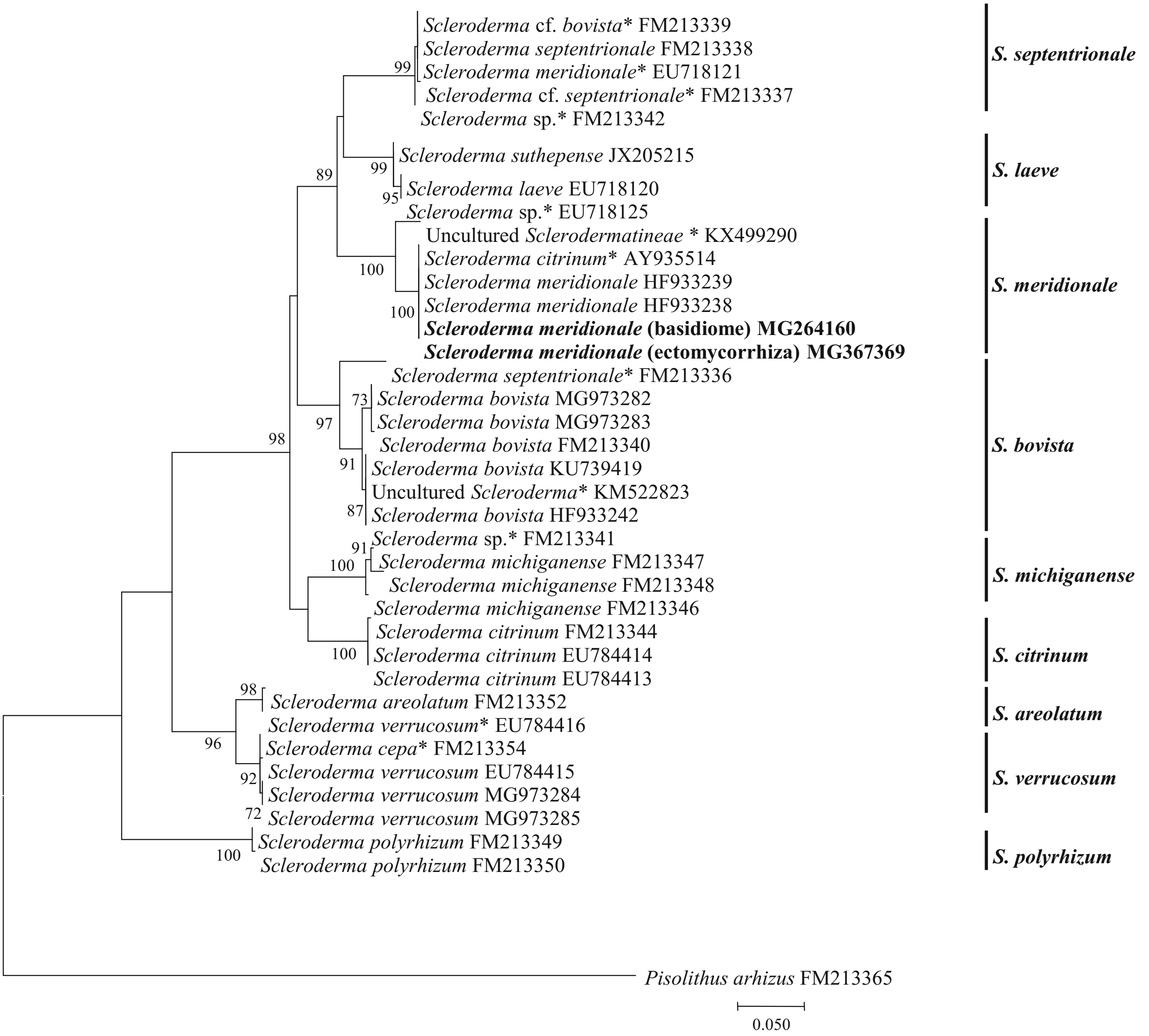

Fig. 6 Maximum Likelihood tree obtained from the alignment of ITS nuclear rDNA region sequences. Maximum Likelihood phylogenetic analysis was inferred from the ITS nrDNA sequences of *Scleroderma* specimens retrieved from GenBank, included in Table 2. The tree displays the relationships of basidiomes and ectomycorrhizae of *Scleroderma meridionale* associated with *Halimium halimifolium*. Sequences obtained during this study are indicated in bold. Bootstrap values ≥70% are indicated on the nodes of branches. The tree is drawn to scale, with branch length measured from the number of substitutions per site. *Pisolithus arhizus* was included as outgroup. *Taxon name in GenBank

T. inexpectata), *Inocybaceae*, have been reported from *H. halimifolium* stands in Corsica (Moreau et al. 2007; Alvarado et al. 2010). A survey of several plots dominated by *Halimium lasianthum* in northwestern Spain revealed that these were particularly productive for the edible and economically valuable porcini *Boletus edulis* and *B. aereus*, with considerable yields being associated with mature plants older than 3 years (Oria-De-Rueda et al. 2008). Brotzu and Peintner (2009) have described a new, striking variety of *Cortinarius cedretorum* as associated with *H. halimifolium* in coastal sand dunal habitats in Sardinia, Italy. In central Portugal, Buscardo et al. (2012) investigated the ECM networks shared between understorey shrubs and pine trees in a *Pinus pinaster*-dominated forest, with the ultimate goal of checking the influence of fire return interval length on such shared ECM communities and on and the potential facilitation offered by the ECM networks for pine regeneration. Fungal symbionts on the roots of *Halimium ocymoides* and *H. lasianthum alyssoides* were identified using molecular techniques, revealing the presence of several ECM fungal taxa. These included *Amanita citrina*, *Cenococcum geophilum*, *Hebeloma cistophilum*, *Lactarius hepaticus*, *Rhizopogon luteolus*, *R. roseolus*, *Russula densifolia*, *R. drimeia*, *Terfezia sp.*, *Thelephora terrestris*, *Tomentella terrestris*, *Tomentellopsis* sp. (Buscardo et al. 2012). Interestingly, some of the listed mycobionts are considered to be host-specific, namely *H. cistophilum* of *Cistus*, and *Rhizopogon* spp. and *L. hepaticus* of *Pinus*, and, consistently, were detected also on the roots of these plant hosts in the same area; in total nine ECM fungal species were in common between *P. pinea* and *Halimium* spp. (Buscardo et al. 2012). In a study of a dune system in Corsica dominated by *H. halimifolium*, sporocarps of several ECM fungi were collected: *Amanita cistetorum*, *A. curtipes*, *A. muscaria* var. *inzengae*, *A. pantherina*, *Boletus edulis*, *Coltricia perennis*, *Cortinarius scobinaceus*, *Hebeloma cistophilum*, *Inocybe halophila*, *Lactarius pseudoscrobiculatus*, *Leccinum corsicum* (now *Leccinellum c.*), *Russula amoenicolor*, *R. littoralis*, *R. tyrrhenica*, *Scleroderma meridionale*, *Thelephora terrestris* (Moreau et al. 2007). Scrolling this list, it is evident that *Halimium* is apparently able to establish mycorrhizal symbiosis with fungal species considered to be *Cistus*-specific mycobionts, such as *A. cistetorum* and *L. corsicum* (in addition to the already quoted *H. cistophilum*). This is not surprising, considering the taxonomic proximity of the two host plants, and the fact that *Cistus* and *Halimium* share in many instances the same habitats. However, other factors can be recruited to explain *Halimium* ECM diversity, at least on the local basis. "La plupart des espèces semblent provenir de la chênaie verte voisine, ou s'être maintenues après une disparition du chêne, peutêtre initialement présent sur le site," wrote the authors (Moreau et al. 2007). A more recent analysis of ECM networks in Corsica, largely based on sporocarp occurrence, has confirmed that although the ECM contingent of *Halimium* is rather small, it is widely shared (in different proportions) with all other ECM host plants considered in the survey, namely *Cistus*, *Quercus*, *Castanea*, *Fagus*, *Corylus*, *Populus*, *Salix*, *Alnus*, *Betula, Abies*, *Pinus* (Taudiere et al. 2015). The study also showed that, within *Cistaceae*, *Cistus* spp. hosts significantly more "specialist" fungal species than *Halimium* (Taudiere et al. 2015). These observations, coupled to our introductory study of the morpho-anatomical details of *Halimium* ECMs, suggest that the mycorrhizal biology of this Mediterranean host plant deserves to be explored in greater detail.

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