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UNIVERSITÀ DEGLI STUDI DI TORINO

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Summary

- Glomeromycota have been considered the most ancient group of fungi capable of positively interacting with plants for many years. Recently, other basal fungi, the Endogone Mucoromycotina fungi, have been identified as novel plant symbionts, challenging the paradigm of Glomeromycota as the unique ancestral symbionts of land plants. Glomeromycota are known to host endobacteria and recent evidences show that also some Mucoromycotina contain endobacteria. In order to examine similarities between basal groups of plant-associated fungi, we tested whether Endogone contained endobacteria.

- Twenty-nine Endogone were investigated in order to identify Mollicutes-related endobacteria (Mre). Fruiting bodies were processed for transmission electron microscopy and molecularly investigated using fungal and Mre-specific primers.

- We demonstrate that Mre are present inside 13 out of 29 Endogone: endobacteria are directly embedded in the fungal cytoplasm and their 16S rDNA sequences cluster together with the ones retrieved from Glomeromycota, forming, however, a separate new clade.

- Our findings provide new insights on the evolutionary relations between Glomeromycota, Mucoromycotina and endobacteria, raising new questions on the role of these still enigmatic microbes in the ecology, evolution and diversification of their fungal hosts during the history of plant–fungal symbiosis.

Introduction

Arbuscular mycorrhizal fungi (AMF) have been considered as the oldest group of fungi capable of positively interacting with plants (Brundrett, 2002; Bonfante & Genre, 2008). On the basis of fossil reports (Taylor et al., 1995; Krings et al., 2007) and molecular analyses (Simon et al., 1993; Wang et al., 2010), Glomeromycota have been hypothesized to be crucial for the conquest of land by plants c. 450 Myr ago. However, some recent reports are suggesting that another basal group of fungi has been involved: Bidartondo et al. (2011) demonstrated that Endogone-like Mucoromycotina fungi are often associated with the gametophytes of basal groups of land plants. Mucoromycotina fungi have been found interacting, alone or together with Glomeromycota, with liverworts (Bidartondo et al., 2011) and hornworts (Desirò et al., 2013a). Field et al. (2014) showed Mucoromycotina fungi are mutualistic with liverworts. The associations between these ancient partners could represent the dawn of symbiosis between plants and fungi. In addition, Strullu-Derrien et al. (2014) observed that sections of Horneophyton lignieri, a fossil plant from the Devonian Rhynie Chert, contained fungal structures identified as belonging to two newly-named endophytes: (1) Palaeoglomus boullardii, which colonizes intracellularly the aerial axis of Horneophyton and forms arbuscule-like structures, and (2) Palaeoendogone gwynne-vaughaniae, which colonizes the corm of the plant with intercellular hyphae forming intracellular coil-like structures. These recent findings of Endogone-like fungi associated with the earliest branching extant land plants and with Devonian fossil plants support the hypothesis proposed by Bidartondo et al. (2011), claiming that both Mucoromycotina and Glomeromycota were associated with the first land plants, and challenging the paradigm of Glomeromycota as the exclusive ancient fungal symbionts of land plants (Selosse & Le Tacon, 1998; Wang & Qiu, 2006; Bonfante & Genre, 2008). This novel set of results broadens the range of interactions established by Endogone-like fungi: so far they were only described as mycorrhizal with members of Pinaceae (Bonfante & Scannerini, 1977; Chu-Chou & Grace, 1979, 1984). On the basis of genome sequence data from Rhizophagus irregularis (Tisserant et al., 2013; Lin et al., 2014), Glomeromycota are currently considered phylogenetically more related to Mucoromycotina than to Dykaria (Ascomycota and Basidiomycota) as previously stated (Schüßler et al., 2001). In addition to their distinctive biological features (obligate biotrophs, asexual and multinucleated), AMF have the peculiarity of possessing endobacteria inside their cytoplasm (Bonfante & Anca, 2009). Two bacterial types have been so far identified: Candidatus Glomeribacter gigasporarum (CaGg) is a rod-shaped, Gram-negative beta-proteobacterium, whose distribution is limited to the family Gigasporaceae (Bianciotto et al., 2003; Mondo et al., 2012), while the second typology identifies a group of more enigmatic bacteria, known as Mollicutes-related endobacteria (Mre), which show a wide distribution across Glomeromycota (Naumann et al., 2010). Interestingly, the two bacterial populations have also been simultaneously detected in some Gigaspora margarita (Glomeromycota) isolates hosting a newly described fungal microbiota (Desirò et al., 2014). Due to the morphological and phylogenetic similarities between Glomeromycota and Mucoromycotina, and the detection of endobacteria in Rhizopus microsporus (Mucoromycotina) (Partida-Martinez & Hertweck, 2005) as well as in Mortierella species (Mucoromycotina sensu Hibbett et al., 2007) (Sato et al., 2010; Kai et al., 2012; Bonito et al., 2013), we hypothesized that endobacteria were present in the ancient plant-associated Endogone. Our hypothesis was also supported by a report dating back to the 1970s that described bacteria-like organisms (BLOs) thriving inside the hyphae of Endogone flammicorona associated with Pinus strobus roots (Bonfante & Scannerini, 1977). By using a combination of

morphological, molecular and phylogenetic analyses we demonstrate that Mre are present inside the cytoplasm of Endogone fruiting body-forming spores, and their 16S rRNA gene sequences cluster together with the ones retrieved from AMF, forming, however, a separate new clade. Our results open new scenarios for these endobacteria as important components of ancient plant-associated fungi, probably representing a so far neglected marker not only of fungal but also of mycorrhizal evolution.

Materials and Methods

Fungal sampling and morphological analysis

Twenty-nine fungal fruiting bodies were investigated. Most of them (28) were obtained from public and private collections (Supporting Information Table S1), the only exception being a fresh sample (AD001) collected in October 2013 in an eastern white pine (Pinus strobus) forest located in Veglio, Italy (45°65000″N, 8°10000″E). Some spores were mounted in polyvinyl alcohol-lactic acid-glycerol (PVLG) (Koske & Tessier, 1983) and examined under a light microscope. The sporocarps were then cut in small fragments that were surfaced-sterilized as described in Lumini et al. (2007). Some of these fragments were processed for transmission electron microscopy by using cryomethods as described in Desirò et al. (2014). Ultrathin sections (70 nm thick) were cut with an ultramicrotome (Ultracut, Reichert and Jung, Vienna, Austria) and observed with a Philip CM10 transmission electron microscope (FEI, Hillsboro, OR, USA).

DNA extraction, amplification and clone library preparation

Five out of 29 fruiting bodies were molecularly investigated to confirm their morphological identification, while the remaining fungal fruiting bodies (24) had already been analyzed (Bidartondo et al., 2011; Desirò et al., 2013a). A small fragment of the inner part of each sporocarp was sampled and surface-sterilized as described earlier. Genomic DNA was extracted using a CTAB-based method (Doyle, 1991). The fungal 18S rRNA gene was amplified using HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) with the universal fungal primer combination NS1 (White et al., 1990) – EF3 (Smit et al., 1999). The cycling conditions were: an initial step at 95°C for 15 min, 35 cycles at 94°C for 55 s, 58°C for 50 s, 72°C for1min50 s and a final extension step of 72°C for 7min. When the NS1-EF3 primer pair did not succeed in amplifying, we applied semi-nested PCR using the forward primers NS3 (White et al., 1990) or EndAD1f (Desirò et al., 2013a). The semi-nested PCR cycling conditions were: an initial step of 95°C for 15 min, 27 cycles at 94°C for 40 s, 58°C (with NS3) or 63°C (with EndAD1f) for 30 s, 72°C for 1 min 20 s and a final extension step of 72°C for 7 min. The Mre 16S rRNA gene was amplified using Phusion High-Fidelity DNA polymerase (Finnzymes Oy, Espoo, Finland) with the primers 109F (Naumann et al., 2010) and 1387R (Marchesi et al., 1998). The cycling conditions were: an initial step at 98°C for 4min, 30 cycles at 98°C for 10 s, 58°C for 30s, 72°C for 55s and a final extension step of 72°C for 7min. When the 109F-1387R primer pair did not succeed, we applied a semi-nested PCR using the reverse primer 1184R (Naumann et al., 2010). The semi-nested PCR cycling conditions were as mentioned in Desirò et al. (2013b) but with 27 cycles. All PCR products from fungal and bacterial amplifications were cloned using the pGEM-t Easy Vector System (Promega, Madison, WI, USA) and then transformed into One Shot Top10 Chemically Competent Escherichia coli (Invitrogen,

Carlsbad, CA, USA). Fungal and bacterial clones were sequenced on an ABI 3730 capillary sequencer using BigDye v. 3.1 sequencing chemistry (Applied Biosystem, Foster City, CA, USA).

Bioinformatics

Fungal and bacterial sequences were assembled and curated in Mega 5 (Tamura et al., 2011), aligned with MUSCLE (Edgar, 2004) and used as queries to conduct BLAST (Altschul et al., 1997) searches against the INSD (Benson et al., 2008). To eliminate PCR-generated chimeras, all the retrieved sequences were submitted to the stringent three-steps-chimeracheck previously described in Desirò et al. (2014). Sequence similarity/sequence divergence was evaluated using MOTHUR (Schloss et al., 2009), treating gaps of any length as a single evolutionary event. The Mre 16S rDNA sequences were grouped into operational taxonomic units (OTUs) at the cut-off of 0.03 genetic distance value using MOTHUR. Phylogenetic reconstructions were conducted using one representative sequence for each OTU. Representative sequences were selected by using the 'get.oturep' command available in MOTHUR. Before phylogenetic reconstruction, the best-fit model of nucleotide substitution was estimated for both fungal and bacterial data set using jModelTest v. 2.1.5 (Darriba et al., 2012). Phylogenetic trees were inferred with MrBayes v. 3.2.2 (Ronquist et al., 2012), RAxML v. 7.2.8 (Stamatakis, 2006), PhyML v. 3.1 (Guindon et al., 2010) and PAUP v. 4.0 (Swofford, 2003). The Markov chain Monte Carlo was run for 10 million generations under the TrN+G and TIM2+G nucleotide substitution models for fungal and bacterial phylogenetic reconstruction, respectively. The GTR+CAT nucleotide substitution model together with the 'autoMR' option of automatic 'bootstopping' (Pattengale et al., 2010) were used for trees constructed with RAxML. A second maximum likelihood analysis performed with PhyML was carried out with 100 bootstrap replicates using the GTR+G nucleotide substitution model. Heuristic searches were carried out with 1000 random addition sequence replicates and tree bisection reconnection (TBR) branch swapping. Each tree shows the topologies that are supported by at least three of the four different phylogenetic analyses carried out, with ≥ 0.90 posterior probability or 75% bootstrap values.

Results

Fungal host morphology

Morphological observations were performed on the fresh sample AD001. The sporocarps of Endogone sp. AD001 are 2–5 9 1– 11 mm in size and appear as a dense agglomeration of spores intertwined with whitish hyphae (Fig. 1a–d). The spores presented a multi-layered wall (Fig. 1d) and a diameter ranging from 120 to 160 lm. When observed under a transmission electron microscope, the spores were surrounded by multiple layers of small-diameter hyphae which originate the connecting structure among the spores (Fig. 1e). The spores presented a cytology that resembled the phylogenetically-related Glomeromycota: the fungal wall was thick with a highly organized fibrillar structure (Fig. 1e), while the cytoplasm was rich in large electron-transparent lipid bodies and small nuclei which were linked by cytoplasmic threads (Fig. 1e,f).

Identification of fungal fruiting bodies

As a first step, we confirmed the morphological identification of five out of the 29 fungal fruiting bodies investigated, analyzing their 18S rRNA gene region. The specimens PERTH7648847 and PERTH7648049 clustered within a clade constituted by Endogone oregonensis and several unknown Endogone species (Fig. 2), while the specimens 9142, 2190 and AD001 clustered within a clade encompassing E. flammicorona and E. lactiflua (Fig. 2). Overall, 20 fruiting bodies represented five described species: E. aggregata, E. flammicorona, E. lactiflua, E. oregonensis and E. pisiformis, while the remaining nine fruiting bodies were only identified as Endogone. The DNA sequences are available in GenBank (KM594016–KM594020).

Identification of endobacteria

Thirteen out of 29 Endogone specimens harbored Mre: six E. lactiflua, four unknown Endogone species and three E. flammicorona (Table 1). No Mre were retrieved from E. aggregata, E. oregonensis or E. pisiformis. Sequencing of the 16S rRNA gene generated 178 sequences (Table 1). In order to eliminate potential PCR artifacts, the sequences were submitted to a stringent chimera screen which reduced their number to 165 sequences (Table 1). The rDNA Mre sequences retrieved from most of the Endogone specimens (8) showed intra-host sequence similarity values > 97% and, accordingly, grouped into a single OTU (Table 1). By contrast, 16S rDNA Mre sequences from the remaining samples (5) showed intra-host sequence divergence values up to 9.4% and a higher number of OTUs (Table 1). All the Mre sequences retrieved from Endogone showed sequence similarity values < 97% when compared with Mre sequences retrieved from AMF (Naumann et al., 2010; Desirò et al., 2013b, 2014): the closest sequences (with 94.4% of sequence similarity) were represented by members of Mre group A (Desirò et al., 2014); conversely, the farthest (with sequence divergence up to 21.8%) were members of Mre group B (Desirò et al., 2014). Furthermore, Mre sequences from Endogone showed up to 11.8% of sequence divergence among themselves. Notwithstanding this high variability, all new Mre sequences clustered with those obtained from AMF (Naumann et al., 2010; Desirò et al., 2013b, 2014), together into a single well-supported new monophyletic clade, identified as Mre group C, located within the Mre group A (Fig. 3). Representative DNA sequences are available in GenBank (KM593997–KM594015)

Localization of Mre in the cytoplasm of Endogone spores

To validate molecular detection of Mre, we used transmission electron microscopy to test the cytoplasmatic location of the endobacteria within Endogone spores. We used high pressure and freeze substitution preparation in order to properly preserve not only the fungal organelles but also the small endobacteria. Coccoid Mre were observed: they were directly embedded in the fungal cytoplasm (Fig. 1e–h), without any evidence of the fungal membrane which surrounds the rod-shaped endobacteria belonging to the Candidatus Glomeribacter gigasporarum taxon (Bianciotto et al., 1996). Similarly to the Mre detected in AMF (Scannerini & Bonfante, 1991; Naumann et al., 2010; Desirò et al., 2013b, 2014), they were 290–490 nm in size and possessed a homogeneus cell

wall-like envelope, which upon high pressure and freeze substitution was rather electron-transparent (Fig. 1g,h).

Discussion

With a combination of morphological, molecular and phylogenetic analyses we demonstrate the presence of Mre within spores of Endogone species from three continents. Our report is the first to describe and molecularly identify Mre in a group of plant-associated basal fungi which are different from Glomeromycota: the Mucoromycotina. These results agree with previous morphological observations reporting the presence of BLOs inside hyphae of Endogone flammicorona in symbiosis with Pinus strobus roots (Bonfante & Scannerini, 1977). Our findings offer new insights on the evolutionary relations between Glomeromycota, Mucoromycotina and endobacteria.

Genetic and phylogenetic features of Endogone-Mre

The new Mre were identified in 13 out of 29 Endogone, and among them, nine clustered in the E. flammicorona and E. lactiflua clade. However, Mre have not been detected in more than half of the studied Endogone (16). We hypothesize that this could be due to the state of preservation and the age of the samples; they are dry fruiting bodies and some were collected > 20 yr ago. Most of the fungal samples (8) showed a high level of intrahost Mre 16S rRNA gene sequence similarity. This is in contrast with the high level of intrasporal sequence divergence observed in Glomeromycota (Naumann et al., 2010; Desirò et al., 2014). However, a deeper analysis with a higher number of Mre sequences could increase the level of sequence divergence. On the contrary, despite the stringent removal of PCR-generated chimeras, the remaining samples (5), most of them morphologically identified as Endogone lactiflua, showed < 97% of intra-host Mre 16S rRNA gene sequence similarity. The identification of different Mre phylotypes in the same sporocarp suggests the presence of a microbiota resembling that of some spore isolates of Gigaspora margarita (Glomeromycota), where, together with the Candidatus Glomeribacter population, up to three different Mre phylotypes were detected (Desirò et al., 2014). The Mre sequences from spores of Endogone are distinct from Mre sequences previously retrieved from Glomeromycota (Naumann et al., 2010; Desirò et al., 2013b, 2014), reaching up to 21.8% of sequence divergence with Mre group B (Desirò et al., 2014). However, despite such high sequence divergence levels, all Mre sequences generated in this study clustered within the clade that encompasses Mre retrieved from liverwort-associated AMF (Desirò et al., 2013b) and Glomeromycota spore isolates (Naumann et al., 2010; Desirò et al., 2014). Interestingly, they cluster together in a well-supported new monophyletic clade, named Mre group C, located within the Mre group A. As a consequence of this temporary division into Mre groups, Mre group A was found to be paraphyletic. In conclusion, it seems that the more Mre sequences are obtained, the more diversity emerges, suggesting that a complete description of Mre biodiversity is far.

Bacteria as markers to assess fungal presence

Desirò et al. (2013b) did not detect a correlation between Mre and liverwort-associated AMF. A similar result arises from the Mre-Endogone association, where Mre sequences are spread all around the Mre group C regardless of the identity of their fungal host. Nevertheless, the different phylogenetic position within the Mre clade of Mollicutes endobacteria from different fungal hosts (i.e. Glomeromycota and Endogone Mucoromycotina fungi) suggests an additional use of these results: Mre could be used as a molecular marker to assess the presence of their fungal host in biodiversity studies. However, the absence of endobacteria does not imply the absence of their fungal host, since not all Glomeromycota and Mucoromycotina possess endobacteria. Irrespectively of this limitation, the idea of using endobacteria as markers for fungal detection could be extended at least to other three taxa: (1) the beta-proteobacterium associated with Mortierella elongate (Sato et al., 2010; Bonito et al., 2013), (2) Candidatus Glomeribacter gigasporarum which is exclusive of the Gigasporaceae family (Glomeromycota) (Bianciotto et al., 2003), and (3) Burkholderia rhizoxinica, intracellular symbiont of the pathogenic fungus Rhizopus microsporus (Partida-Martinez & Hertweck, 2005).

The hyphal highway: bacteria prefer coenocytic mycelium

The finding of endobacteria associated with Mucoromycotina fungi, like Rhizopus (Partida-Martinez & Hertweck, 2005) and Mortierella (Sato et al., 2010; Kai et al., 2012; Bonito et al., 2013), as well as with Glomeromycota (Naumann et al., 2010; Desirò et al., 2013b, 2014), raised the hypothesis that fungal endobacteria prefer coenocytic mycelia (Desirò et al., 2014). Our results support this hypothesis: Endogone also possesses a coenocytic mycelium where the absence of transverse septa may facilitate the movement of bacteria along 'hyphal highways'. The presence of endobacteria both in Glomeromycota and Mucoromycotina, together with their morphological similarities (Bonfante & Scannerini, 1977), and the relatedness of their mitochondrial (Lee & Young, 2009; Pelin et al., 2012) and nuclear (Tisserant et al., 2013; Lin et al., 2014) genomes, offers a novel common element between these two lineages of basal fungi.

Mre as passengers of fungi: a hundred million years journey

The distribution of endobacteria along closely related lineages of basal fungi raises questions about the origin and evolution of interactions among plants, fungi and bacteria. Redecker et al. (2000) described fossil spores from Ordovician sediments that resembled modern AMF spores, dating back the origin of Glomeromycota to c. 460 Myr ago. Starting from this paleontological evidence and due to the widespread distribution of Mre across Glomeromycota, Naumann et al. (2010) hypothesized that Mre were living in AMF for > 400 Myr, placing their origin before the split of the earliest branching Glomeromycota lineages. However, the finding of Mre within Endogone moves the origin of this group of bacteria further back in time, at least to the split between Glomeromycota and Mucoromycotina, whose common ancestor probably already hosted Mre. These results raise new questions on the role of these still enigmatic endobacteria in the ecology, evolution and diversification of their fungal hosts and, as a consequence, in the history of plant–fungal symbioses. The idea that mycorrhizas are the result of three-partite interactions is now based on broader

observational and experimental evidence. We now need to know the partners' reciprocal roles and the benefits gained through inter-domain networks.

Acknowledgements

The authors wish to thank J. Trappe, A. Montecchi, P. McGee, C. Walker and Oregon State University Herbarium who generously provided fungal specimens, and M. Palenzona for his help in hunting Endogone fruiting bodies in the field. The authors also wish to thank three anonymous referees for their helpful comments. Financial support was provided by the University of Torino (local grant ex 60% to P.B.). M.I.B. was supported by a grant from NERC.

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Supporting Information

Additional supporting information may be found in the online version of this article.

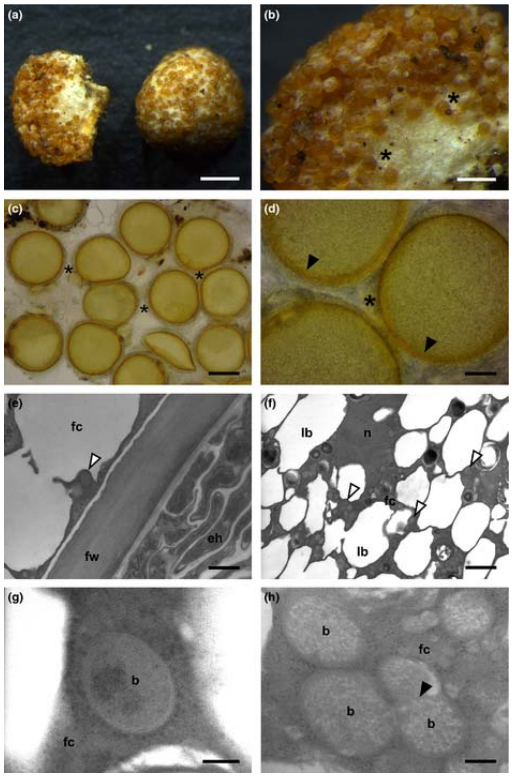

Figure 1. Fruiting body of Endogone sp. AD001 as seen in stereo (a, b), bright-field (c, d) and transmission electron microscopy (e–h). (a–d) The fruiting body is a dense agglomerate of orange-brown spores with a multi-layered wall (arrowhead) and connected by whitish hyphae (asterisk). (e, f) The spores were surrounded by multiple layers of small-diameter hyphae (eh) which originate the connecting structure among them; the fungal wall (fw) was thick with a highly organized fibrillar structure, while the cytoplasm (fc) is rich in large electron-transparent lipid bodies (lb) and small nuclei (n). (g, h) Mre (b) are directly embedded in the fungal cytoplasm (fc). The central constriction (arrowhead) suggests that endobacteria are engaged in cell division. Bars: (a) 1 mm; (b) 0.36 mm; (c) 87 µm; (d) 31 µm; (e) 0.64 µm; (f) 1 µm; (g) 0.11 µm; (h) 0.14 µm.

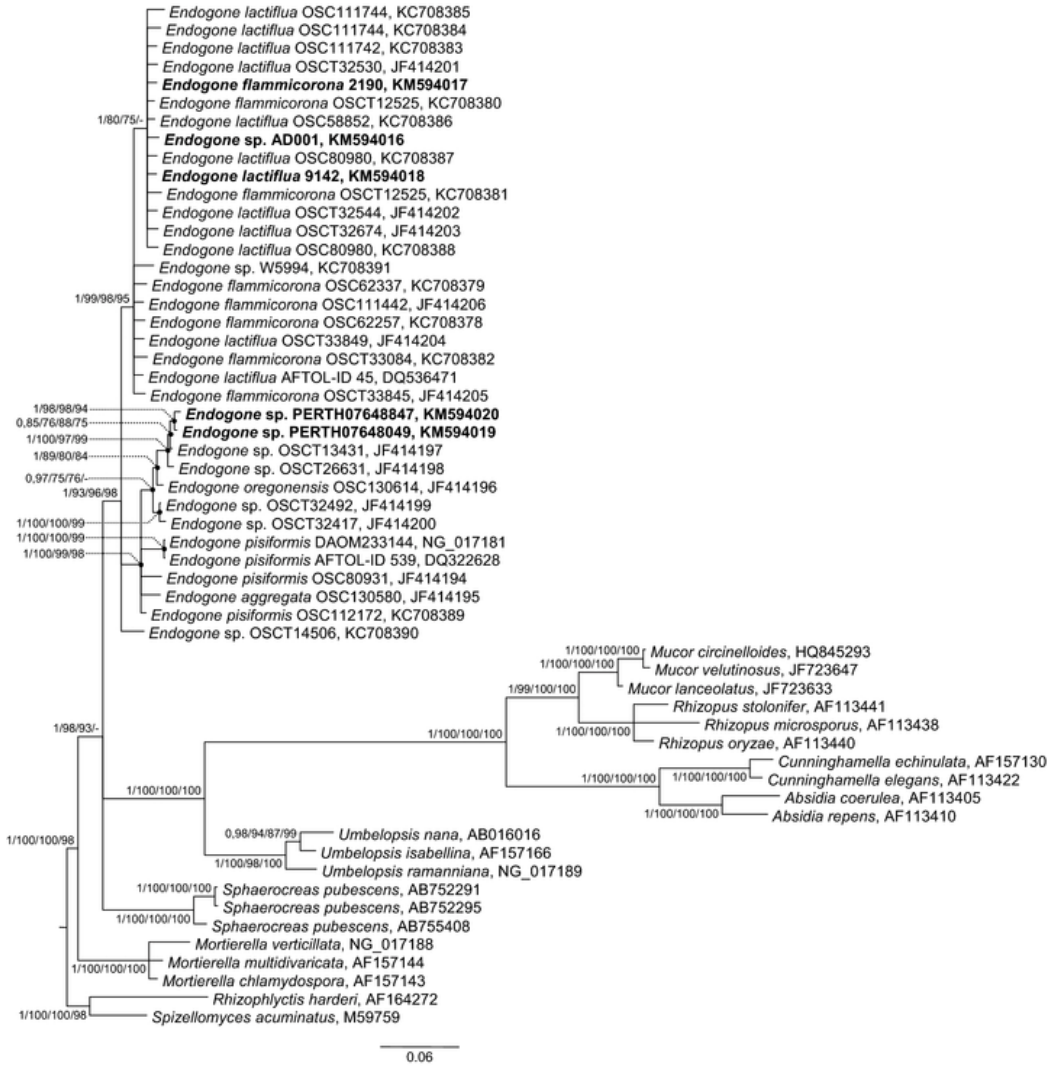

Figure 2. Phylogenetic placement of the partial 18S rRNA gene sequences retrieved from Endogone. The DNA sequences generated in this work are in bold. The five new fungal specimens are located in the clade encompassing Endogone identified in previous studies (Bidartondo et al., 2011; Desirò et al., 2013a): the specimens PERTH7648847 and PERTH7648049 clustered together with E. oregonensis and several unknown Endogone species, while 9142, 2190 and AD001 clustered within a clade constituted by E. flammicorona and E. lactiflua. Support values are from Bayesian/maximum likelihood with RAxML/maximum likelihood with PhyML/maximum parsimony analyses. Dashes instead of numbers imply that the topology was not supported in the respective analysis.

Figure 3. Phylogenetic placement of representative Mollicutes-related endobacteria (Mre) partial 16S rRNA gene sequences retrieved from Endogone. The DNA sequences generated in this work are in bold. The Mre sequences retrieved from Endogone cluster together with those retrieved from Glomeromycota spore isolates (Naumann et al., 2010; Desirò et al., 2014) and liverwort-associated arbuscular mycorrhizal fungi (AMF) (Desirò et al., 2013b), and they are grouped into a new monophyletic clade, identified as Mre group C, located within the Mre group A. The number of sequences included in each operational taxonomic unit (OTU) is in brackets. Support values are from Bayesian/maximum likelihood with RAxML/maximum likelihood with PhyML/maximum parsimony analyses. Dashes instead of numbers imply that the topology was not supported in the respective analysis.

Table 1. Mollicutes-related endobacteria (Mre) sequences generated from *Endogone*

Endogone specimen[1]	Collection site[2]	Retrieved sequences[3]	Sequences after chimera screen[4]	OTU number[5]	Sequences in[6]			Intra-host sequence similarity (%)[7]
					OTU1	OTU2	OTU3	
Endogone flammicorona 2190	Italy	7	7	1	7	–	–	97.5
Endogone flammicorona OSC111442	Oregon, USA	15	15	1	15	–	–	99.3
Endogone flammicorona OSC62257	Idaho, USA	16	16	1	6	–	–	99.5
Endogone lactiflua 9142	England, UK	8	6	2	4	2	–	92.4
Endogone lactiflua OSC111742	Oregon, USA	16	12	2	6	6	–	91.7
Endogone lactiflua OSC111744	Oregon, USA	16	16	3	14	1	1	93.1
Endogone lactiflua T32530	Mexico	16	16	1	16	–	–	99.4
Endogone lactiflua T32544	Mexico	15	15	1	15	–	–	99.3
Endogone lactiflua T32674	Mexico	15	11	2	9	2	–	90.6
Endogone sp. AD001	Italy	8	5	1	5	–	–	99.5
Endogone sp. T26631	Australia	15	15	1	15	–	–	99.2
Endogone sp. T32417	Mexico	16	16	2	14	2	–	92.6
Endogone sp. T32492	Mexico	15	15	1	15	–	–	99.5

[1] *Endogone* specimen. [2] Collection site. [3] Number of Mre retrieved sequences. [4] Number of sequences after the removal of PCR-generated chimeras. [5] Number of operational taxonomic units (OTUs) and [6] their related sequences. [7] The lowest values of sequence similarity between two representative sequences of different OTUs of the same sample.