## Mollicutes-related Endobacteria thrive inside Liverwort-associated Arbuscular Mycorrhizal Fungi

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**Mollicutes-related endobacteria thrive inside liverwort-associated arbuscular mycorrhizal fungi**

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*Summary*

Arbuscular mycorrhizal fungi (AMF) can host Gram-positive endobacteria (BLOs) in their cytoplasm. These have been identified as Mollicutes-related microbes based on an inventory of AMF spores from fungal collections. Bacteria-like organisms (BLOs) of unknown identity have also been reported in the cytoplasm of AMF associated with liverworts, the earliest-diverged extant lineage of land plants. A combination of morphological, molecular and phylogenetic analyses revealed that three samples of two liverwort species (Conocephalum conicum and Lunularia cruciata) growing spontaneously in a botanical garden harboured AMF belonging to Glomerales, and these, in turn, hosted coccoid BLOs. 16S rDNA sequences from these BLOs clustered with the Mollicutes sequences identified from the spore collections but revealed the presence of novel phylotypes. Electron microscopy and fluorescence in situ hybridization (FISH) confirmed the presence of BLOs inside the cytoplasm of AMF hyphae colonizing the liverwort thalli. The high genetic variability of BLOs in liverwort–AMF associations thriving in the same ecological niche raises questions about the mechanisms underlying such diversity.

*Introduction*

Arbuscular mycorrhizae (AMs) are the most widespread terrestrial form of symbiosis (Fitter, 2005): they involve 70–90% of all land plants (Smith and Read, 2008) and a group of soil microbes, the arbuscular mycorrhizal fungi (AMF), that form the ancient, monophyletic phylum of Glomeromycota (Schüßler et al., 2001; Schüßler and Walker, 2010; Oehl et al., 2011; Krüger et al., 2012). In addition to their obligate biotrophy, the unique biological features of AMF (Bonfante and Genre, 2010) include the presence of endobacteria in their cytoplasm. Bacterial endosymbionts are widespread in the animal kingdom, while examples are more limited in fungi (Partida-Martinez and Hertweck, 2005; Bonfante and Anca, 2009), with AMF endobacteria representing one of the most thoroughly investigated cases. Two types of bacteria-like organisms (BLOs) are known in AMF. A rod-shaped, Gram-negative BLO (Bonfante et al., 1994), today known as Candidatus Glomeribacter gigasporarum, is common in several Gigasporaceae (Bianciotto et al., 2003). A second type of BLO, coccoid and surrounded by a homogeneous cell wall (MacDonald et al., 1982; Scannerini and Bonfante, 1991), has recently been identified as a novel bacterial taxon clustering with the Mollicutes (Naumann et al., 2010) and proposed as sister to a clade encompassing the Mycoplasmatales and Entomoplasmatales. On the basis of its sequenced genome (Ghignone et al., 2012), Candidatus Glomeribacter is suggested to be nutritionally dependent on its fungal host, with the possibility that it supports the fungus with essential factors like vitamin B12 (Lumini et al., 2007). In contrast, at the moment no function has been associated with the coccoid BLOs: they have been so far detected in 17 out of 28 investigated AMF samples from culture collections, including members of Glomerales, Diversisporales and Archaeosporales (Naumann et al., 2010). In most of these AMF, endobacteria display a conspicuous variability in 16S rDNA sequence.

Since the extensive investigation by Naumann and colleagues (2010) was performed exclusively in AMF spores from culture collections, the question whether Mollicutes-related bacteria are identifiable in wild AMF samples remains open. The presence of endobacteria in Glomeromycota was first documented by electron microscopy (Mosse,

1970; Gerdemann and Trappe, 1974; Scannerini and Bonfante, 1991; Bonfante et al., 1994). Reports of coccoid BLOs in AMF associated with higher plants are somewhat erratic. In contrast, BLOs are commonly detected in AMF from bryophytes such as the hornworts Anthoceros (Schüßler, 2000) and Phaeoceros (Ligrone,1988), as well as a diversity of thalloid and leafy liverwort taxa (Ligrone and Lopes, 1989; Carafa et al., 2003; Ligrone et al., 2007).

Because of the regular occurrence of BLOs in their fungal associates, liverwort gametophytes are a particularly favourable material to verify whether Mollicutes-related endobacteria do occur in AMF living in natural conditions. In this study we utilized the gametophyte thallus of two liverwort species living spontaneously in the Botanical Garden of the University of Turin. To test whether Mollicutes-related bacteria occur in environmental AMF, we detected BLOs in fungal endophytes of these plants by electron microscopy, identified the fungi involved as members of several AMF lineages, and developed a targeted molecular analysis to test the mollicutean affinity of their endobacteria.


*Results*

*AMF colonization of liverworts*

Three liverwort species were identified in a humid area of the Botanical Garden of Turin University. Marchantia polymorpha, subspecies ruderalis, did not harbour any endophyte, in agreement with Ligrone and colleagues (2007), while two out of three samples of Lunularia cruciata (L2, L3) and one out of two samples of Conocephalum conicum (C6) showed extensive fungal colonization in precise anatomical regions of the thallus (Fig. 1). Fungal colonization in C. conicum was detected in a half-moon-shaped area of the hyaline parenchyma underlying the chlorenchyma (Fig. 1A, B, E and F), while in L. cruciata it was limited to the lower part of the inner parenchyma (Fig. 1C and D), immediately above the ventral epidermis. AMF present in the sampled liverworts were fairly uniform in their development and morphology: hyphae preferentially penetrated the thallus through the rhizoids, crossed the epidermis and started to branch and proliferate inside the cells of the internal parenchyma where they developed arbuscule-like structures and coils (Fig. 1F and G).
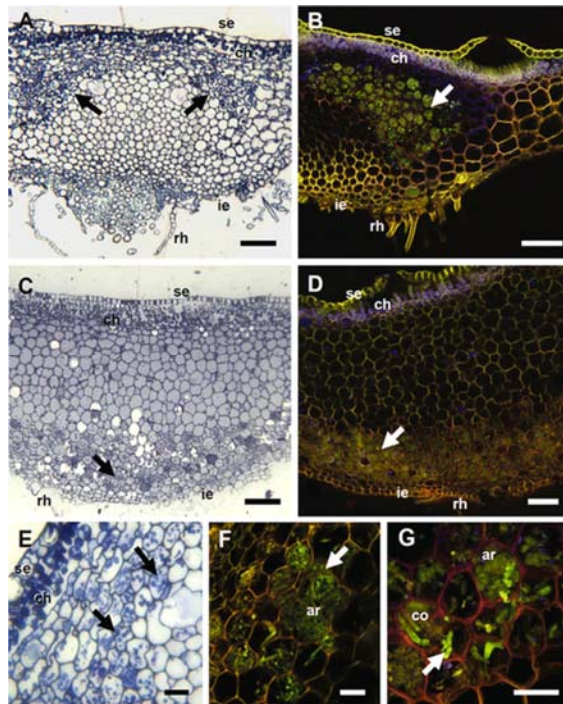
*Figure 1.* Patterns of fungal colonization detected in liverwort thalli. Transverse section of the gametophyte thallus in C. conicum (A, B, E, F) and L. cruciata (C, D, G) as seen in bright-field (A, C, E) and confocal (B, D, F, G) microscopy. Fungal endophytes colonize the internal hyaline parenchyma (arrows), forming coils (co) and arbuscule-like (ar) structures, whereas the ventral and dorsal epidermis (ie, se), and the chlorenchyma (ch) are never colonized. In confocal images a diffuse orange signal due to sample fixation outlines the liverwort cell walls, whereas chlorophyll autofluorescence is false-coloured in blue. rh: rhizoids. Scale bars: (A, B) 145 µm; (C, D) 85 µm; (E–G) 30 µm.

## BLOs can be detected in AMF thriving in liverwort thalli

When examined by transmission electron microscopy, the colonizing hyphae revealed a 4–6 µm diameter and the absence of septa, two features that discriminate AMF from other endophytes known to colonize liverwort thalli (Pressel et al., 2010; Bidartondo et al., 2011). In the internal parenchyma, intracellular fungal structures displayed an arbuscule- or coil-like morphology (Fig. 2A and Fig. S1A) and were constantly surrounded by a perifungal membrane of host origin (Fig. 2A and B). This is a ubiquitous feature of all AM interactions and indicates that the symbiotic interface maintains a remarkably constant pattern irrespectively of the ploidy status of the host plant, haploid in liverworts and diploid in higher plants (Bonfante and Genre, 2008). Collapsed and degenerated hyphae, as well as cell re-colonization events were frequently observed, suggesting that thallus colonization, in the specimens examined, had already reached an advanced stage (Fig. S1B–D).
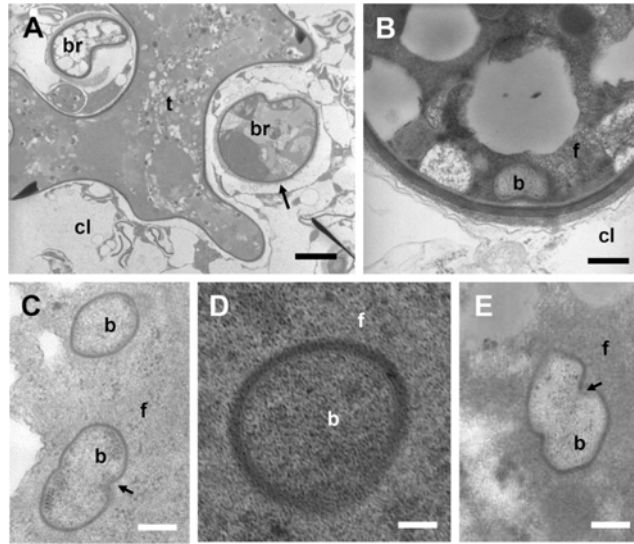
*Figure 2.* Electron microscopy of a parenchyma cell in the thallus of L. cruciata colonized by an AMF-like fungal endophyte (A) and of BLOs within fungal hyphae (B, C, D, E). (A) The intracellular fungus forms a large trunk hypha (t) that branches profusely (br), forming an arbuscule-like system. (A) The intracellular hypha shows an arbusculated-like morphology, forming a main truck (t) from which many branches derive (br); liverwort cell (cl); (B, C, D and E) BLOs (b) are in direct contact with the fungal cytoplasm (f), and lack outlining membranes. The central constriction (arrow) suggests bacterial division. Liverwort cell (cl). Scale bars: (C) 0.24 μm; (C, E) 0.18 μm; (D) 0.06 μm.

A more detailed ultrastructural analysis was performed in L. cruciata (L3), searching for BLO presence in the colonizing AMF hyphae, as described in previous reports (Ligrone and Lopes, 1989; Carafa et al., 2003; Ligrone et al., 2007). BLOs were detected inside the cytoplasm of intracellular AMF hyphae (Fig. 2B). No evidence of a peribacterial membrane was found (Fig. 2C–E). The homogeneous bacterial cell wall was typical of Gram-positive bacteria and often displayed a central constriction suggestive of cell division events (Fig. 2C and E).

*AMF associated to liverworts belong to the Glomerales group*

A molecular analysis of the fungal 18S rDNA was performed to identify the fungal host of the bacteria and cross-check with the list of AMF already known to contain endobacteria (Naumann et al., 2010). We applied the AMF primers AML1 and AML2 (Lee et al., 2008) to the total DNA extracted from thalli and expected to include plant, fungal and bacterial DNA. After sequencing, we identified 80 sequences related to Glomeromycota: 29 from C. conicum (C6), 30 from L. cruciata (L3) and 21 from L. cruciata (L2) (Table 1).

*Table 1*. Number of AMF and BLO sequences retrieved from C. conicum (C6), L. cruciata (L2) and L. cruciata (L3) and subdivided in OTUs.

| Sample name | AM fungi | | | BLOs | | |
|---|---|---|---|---|---|---|
| | *C. conicum* C6 | *L. cruciata* L2 | *L. cruciata* L3 | *C. conicum* C6 | *L. cruciata* L2 | *L. cruciata* L3 |
| Total sequences | 29 | 21 | 30 | 25 | - | 39 |
| OTU1 | 23 (*Glomus* gr Ab) | 9 (*Glomus* gr A) | 28 (*Glomus* gr B) | 2 | - | 5 |
| OTU2 | 3 (*Glomus* gr Aa) | 4 (*Glomus* gr B) | - | 5 | - | 2 |
| OTU3 | 3 (*Glomus* gr B) | 2 (*Glomus* gr Ab) | - | 5 | - | 4 |
| OTU4 | - | 6 (*Glomus* gr Aa) | - | 4 | - | 4 |
| OTU5 | - | - | - | 2 | - | 2 |
| OTU6 | - | - | - | - | - | 2 |
| OTU7 | - | - | - | - | - | 3 |
| Singletons | - | - | 2 (*Glomus* gr Ab) | 7 | - | 17 |
| Total OTUs | 3 | 4 | | 12 | - | 24 |

The number of sequences for each OTU, the number of singletons and the total amount of OTUs is shown. All the retrieved AMF sequences cluster inside the *Glomerales*. Most sequences from C6 and L3 samples are grouped in OTU1, whereas L2 sequences are more variable. BLO OTUs are numerous in *C. conicum* (C6) and *L. cruciata* (L3) whereas BLO amplification was not successful for *L. cruciata* (L2). More than half of the BLO OTUs of each sample are singletons, suggesting a high level of sequence variability.

Arbuscular mycorrhizal fungi sequences from each sample were grouped into separate OTUs with at least 97% sequence similarity (Table 1). On this basis, 3 OTUs were assembled for C. conicum (C6), 3 OTUs for L. cruciata (L3), including two singletons, and 4 OTUs for L. cruciata (L2). Sequence analysis indicated that all thalli were associated to Glomerales, in particular Glomus group Aa, Ab and B (sensuSchüßler et al., 2001) (Table 1 and Fig. 3).
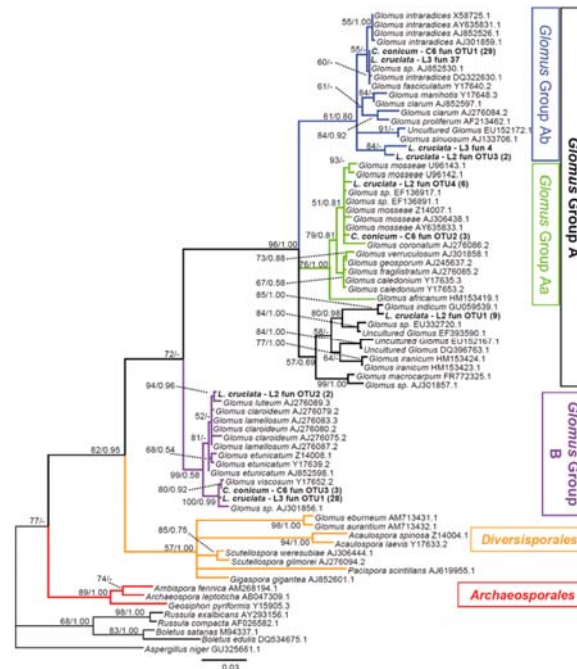


*Figure 3*. Phylogenetic tree based on the 800 bp 18S rDNA, showing the position of the AMF retrieved from liverworts within the Glomeromycota clade; Basidiomycota and Ascomycota sequences were used as outgroups. The tree encompasses different subclades that are in line with Schüßler and colleagues (2001) classification, and are shown in different colours. Branch tip labels: AMF phylotype names from L. cruciata (L2), L. cruciata (L3) and C. conicum (C6) are bold black. The support values given are from Quartet Maximum Likelihood/Bayesian analyses. Dashes instead of numbers imply that

the topology was not supported in the respective analysis. A representative sequence was selected for each OTU. The number into brackets indicates the whole sequence number for OTU.

*Identification of BLOs in environmental AMF lineages*

Having verified the presence of BLOs in L. cruciata (L3) fungal endophytes (Fig. 2) and identified the latter as AMF mostly belonging to Glomus group B, we moved on to 16S rDNA-based identification of the endobacteria in all of the liverwort L. cruciata and C. conicum samples where AMF taxa had been detected.

PCR amplification by means of primers specifically designed for Mollicutes-related AMF endobacteria (Naumann et al., 2010) gave a positive result for DNA from L. cruciata L3 and C. conicum C6 samples but no amplification signal was shown in the L. cruciata (L2) (Table 1). Sequencing led to 25 sequences from C. conicum (C6) and 39 from L. cruciata (L3); blast analysis related them all to the AMF Mollicutes endobacteria. The high number of OTUs, and especially the relatively large number of singletons (Table 1), pointed out a high level of variability. In line with Naumann and colleagues (2010), this was also supported by clustalw analysis, which demonstrated up to 20% sequence divergence within each sample and up to 21% divergence between the two liverwort samples.
Regardless of this important variability, all liverwort-BLO sequences were related to those obtained from the 17 samples from AMF spore collections (Naumann et al., 2010).

*FISH experiments confirm BLO localization inside the cytoplasm of liverwort AMF*

The 16S rDNA sequences obtained from the previous experiment allowed us to design probes for BLO detection in sectioned liverwort thalli through FISH experiments, in order to confirm the occurrence of endobacteria in the AMF cytoplasm and validate results of molecular analyses. To this aim, we first set up a FISH protocol for AM to identify the fungal hosts. The probe AML2ADf, designed on the basis of the reverse primer AML2 (Lee et al., 2008) and whose specificity was tested in PCR, targeted the AMF structures (coils and arbuscules) with a strong fluorescent signal in the thalli from Lunularia (L2), (L3) and Conocephalum (C6), as shown in Figs 4A and B and 5A and C–E. The FISH signal intensity was highest in the hyphae and arbuscules that had been cut by the microtome blade, which directly exposed the fungal cytoplasm to the probe.
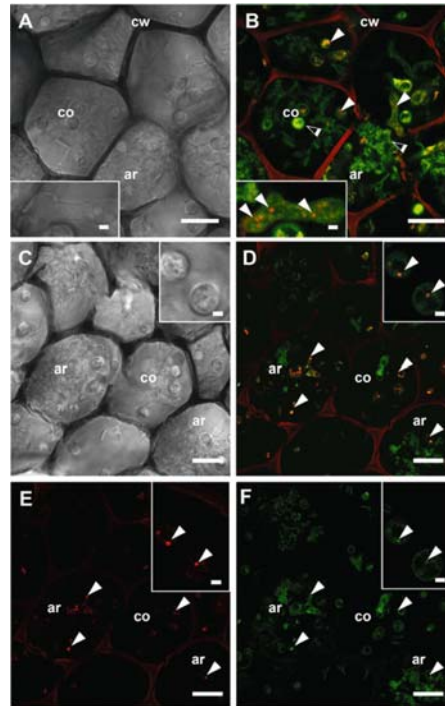
*Figure 4.* FISH of transverse sections of the thallus in L. cruciata (A, B) and C. conicum (C–F). A and C. Bright-field images of liverwort parenchyma colonized by AM showing fungal coils (co) and arbuscules (ar). Liverwort cell walls (cw) are marked by a weak reddish autofluorescence due to the fixative. B. Double labelling of BLOs with the BLO-specific probe BLOsADf2 (red), and fungal hyphae, arbuscules and coils (black arrowheads) with the AMF-specific probe AML2ADf (green); in this image, where the red and green channels are overlayed, BLOs are visualized as fluorescent orange coccoid spots (arrowheads) inside the green fungal cytoplasm. The corresponding red and green channel images are presented in Fig. S2. The inset shows a higher magnification of one intracellular hypha harbouring several BLOs. D. Double labelling of BLOs with both the eubacterial 16S probe EUB338 (green) and the BLO-specific probe BLOsADf2 (red); orange coccoid spots (arrowheads) are visible inside the fungus (which displays a weak green autofluorescence). Inset shows a higher magnification of two transversely cut hyphae containing the labelled BLOs. E and F. Red and green channel images of picture D are presented in (E) and (F), to highlight the BLO labelling with both probes. Bars: 15 μm, 1.5 μm in insets.
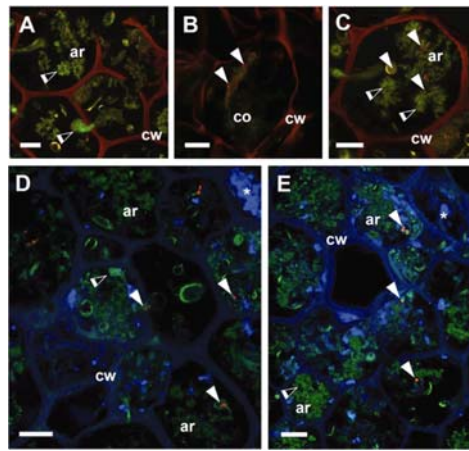
*Figure 5*. FISH of transverse sections of the thallus in L. cruciata L2 (A) and L3 (B, D) and C. conicum (C, E). Liverwort parenchyma cells are colonized by AMF arbuscules (ar) and coils (co). Green fluorescence from the fungal-specific probe AML2ADf (A, C, D, E) highlights the fungal cytoplasm (black arrowheads). Red fluorescence from the BLO-specific probe BLOsADf2 labels BLOs as red fluorescent coccoid spots (arrowheads). BLOs were not detected in Lunularia L2 thalli (A). In contrast, they were regularly visualized inside the fungal hyphae in both Lunularia L3 (B, D) and Conocephalum C6 (C, E). Background autofluorescence derived from sample fixation highlights the liverwort cell walls and senescent fungal structures (asterisks) in diffuse red (A–C) and blue (D, E). Scale bars: 10 μm.

The BLO-specific probe, designed to cover the 16S rDNA variability revealed by sequence analysis, produced a specific FISH signal in Lunularia L3 and Conocephalum C6 samples (Figs 4B, D and E and 5B–E) but, in agreement with PCR results, not in Lunularia (L2) (Fig. 5A). The fluorescent signal of the BLOsADf2 probe was always localized in trunk hyphae or larger arbuscular hyphae (Figs 4 and 5). Importantly, BLOsADf2 signal colocalized with the general bacterial probe EUB338 (Amann et al., 1990), as shown in Fig. 4D and F. No fluorescence was detected with the probe ApisP2a, used as a negative control (Fig. S3). Control hybridization with nonsense probes, as well as pre-treatment with RNase, did not generate any FISH signal. A weak background autofluorescence, likely deriving from the use of aldehydic fixatives, was visible in all samples as a broad spectrum emission in both plant and fungal walls. In conclusion, the set up of novel FISH protocols for both AMF and their endobacteria validated the endocellular location of Mollicutes-related BLOs in two out of three liverwort samples.
Phylogenetic analysis of the BLOs versus their environmental AMF hosts

Phylogenetic analysis highlighted a heterogeneous distribution of the bacterial sequences inside the Mollicutes-related BLO clade (Fig. 6) although the topology of the resulting phylogenetic tree is not always well supported. This was partially expected due to the relative low number and high variability of available sequences. Most of the sequences were placed in the lower, not well supported, part of the phylogenetic tree almost all sequences from C. conicum (20 out of 25) were located in this part, while sequences from L. cruciata were scattered throughout the tree. BLO sequences from liverworts mainly clustered with Mollicutes-related sequences from Glomerales and from Glomus versiforme spores, located in the better supported part of the tree, or with the sequences retrieved from Geosiphon pyriformis, in the lower part of the tree. Furthermore, clustalw analysis revealed that half of the newly obtained sequence types (37 out of 64) were highly similar (at least 95% of similarity) to known sequences from different cultured AMF samples, like Glomus caledonium

(Glomerales, Glomus group Ab), G. claroideum, G. etunicatum (Glomerales, Glomus group B), Ambispora appendicula and G. pyriformis (Archaeosporales).
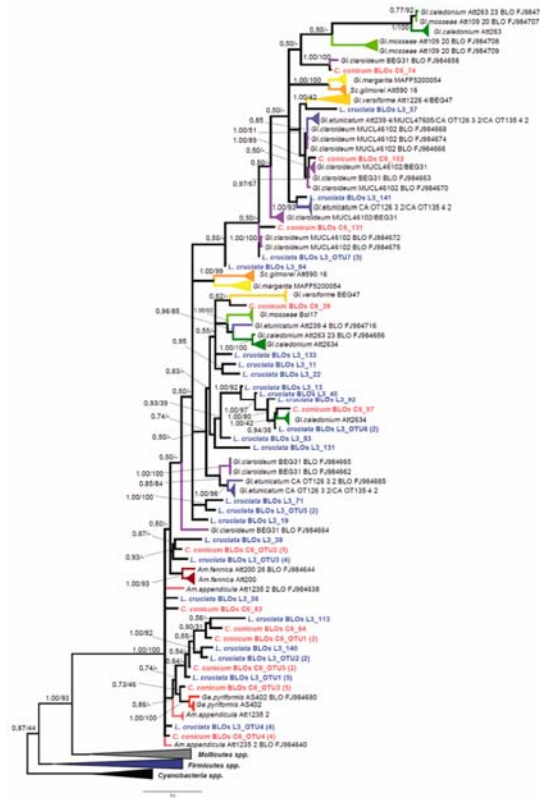


*Figure 6.* Phylogenetic tree based on 1075 bp 16S rDNA, showing the relative position of BLOs phylotypes from liverworts and from AMF spores within the Mollicutes clade (grey) and Firmicutes (blue) clade (Naumann et al., 2010); Cyanobacteria (black) sequences were used as the outgroups. BLO sequences retrieved from spore collections and present in data bank are grouped and coded in different colours according to the associated AMF species (red-pink: Archaeosporales; orange-yellow: Diversisporales; green: Glomus group Aa; violet: Glomus group B). Branch tip labels: BLO sequence names from L. cruciata (L3) and C. conicum (C6) are respectively blue and red. Support values given are from Bayesian/maximum likelihood analyses. Dashes instead of numbers imply that the topology was not supported in the respective analysis. The lower part of the tree was collapsed to a polytomy; the backbone of the tree is not well supported and only the internal ramifications have well-supported values. A representative sequence was selected for each OTU. The number into brackets shows the number of sequences included in each OTU. Mollicutes, Firmicutes and Cyanobacteria sequences used to build the phylogenetic tree were retrieved from GenBank.

All the remaining sequences (27 out of 64), including most singletons, showed a similarity level lower than 95% with known BLO sequences retrieved from AMF collections.

Altogether, all 16S rDNA sequences resulted to be distant from the Mollicutes-related sequences obtained from Diversisporales species as well as from Glomus mosseae isolates (up to 22% sequence divergence).

In conclusion, clustalw and phylogenetic analyses of the liverwort-BLO sequences indicated a low AMF host specificity under environmental conditions (Fig. 6) and showed the presence of novel BLO phylotypes relative to BLO sequences from AMF spores.

*Discussion*

Mycorrhizal symbioses are often described as tripartite associations, due to the presence of endobacteria living inside the cytoplasm of many AMF (Bonfante and Anca, 2009). A Gram-negative bacterium has consistently been associated with AMF belonging to the Gigasporaceae family and identified as a Burkolderia-related taxon with a reduced genome (Ghignone et al., 2012); in addition Gram-positive endobacteria identified as a novel Mollicutes-related group, have been reported in AMF spores from different culture collections (Naumann et al., 2010). The present investigation demonstrates that Gram-positive endobacteria are also present in AMF colonizing the thallus of liverworts growing spontaneously in a botanical garden. Their ribosomal sequences cluster with the Mollicutes-related sequences retrieved from AMF spore collections (Naumann et al., 2010). A novel protocol, based on the use of fungal- and bacterial-specific probes for FISH experiments, was developed; this method allowed us to validate the molecular identification and to provide direct evidence for the presence of Mollicutes-related endobacteria inside the cytoplasm of AMF associated with liverworts.

*Bacterial sequences from AMF-colonized liverworts are Mollicutes-related*

In agreement with previous reports (Ligrone and Lopes, 1989; Carafa et al., 2003; Ligrone et al., 2007), BLOs were detected inside hyphae colonizing the liverworts C. conicum and L. cruciata. Their morphology is substantially the same as that previously described in AMF from field samples (Scannerini and Bonfante, 1991; Schüßler, 2000), germinating mycelia (Kuga et al., 2008) and spore collections (Naumann et al., 2010). This latter investigation demonstrated that 17 out of 28 AMF isolates contained endobacteria, whose 16S ribosomal sequences clustered in a well-supported monophyletic clade, falling into the wall-less clade of Mollicutes.
Most of the Mollicutes-related sequence types identified in this study (37 out of 64) showed a high similarity with sequences already retrieved from AMF spores, while the rest were characterized by novel polymorphisms. Distance identity values of 0.03 are usually thought to differentiate the species level, 0.05 the genus level, 0.10 the family/class level and 0.20 the phylum level (Schloss and Handelsman, 2004). In this work, the presence of sequences with 0.21–0.20 distance values (79–80% of identity) suggests a relatively high diversity; however, the bacterial species definition remains a difficult issue, since there are strains of the same bacterial species that are known to differ in up to 35% in their gene content (Konstantinidis and Tiedje, 2005).

The AMF species known to contain BLOs (Naumann et al., 2010) fall within different glomeromycotean groups including early-diverging and more derived lineages such as Ambispora, Archaeospora and Geosiphon. This suggests that either BLOs were acquired very early in glomeromycotean evolution or were transmitted horizontally between separate fungal lineages. Vertical transmission is in fact expected to reduce genetic variability by generating bottlenecks in the spread of endosymbiont populations within individual host lineages (Frank, 1996), leading to a hypothesis which is not in line with the observed variability of BLOs in cultured spores (Naumann et al., 2010) and in the field AMF.

*Mollicutes-related endobacteria and AMF identity in environmental samples*

One of the aims of the present investigation was to detect the correlation, if any, between the Mollicutes-related endobacteria and the liverwort-associated AMF, on the basis of their ribosomal sequences.

The primers we used, AML1 and AML2, allowed the detection of AMF belonging to Glomus group A and B (Glomerales). In this context, the results revealed that one sample of L. cruciata (L3) mainly harboured AMF sequences related to Glomus group B, while the majority of AMF sequence types retrieved from one sample of C. conicum (C6) corresponded to Glomus group Ab with a few related to Glomus group Aa and B (Table 1). Altogether, these results highlight a limited degree of variability in the liverwort-hosted AMF lineages belonging to Glomerales, even if a primer bias cannot be excluded.

Looking at the sequence position inside the bacterial 16S-based phylogenetic tree and the identity values obtained from clustalw analysis, a clear correlation between the AMF taxa and the bacteria hosted inside their cytoplasm was not found. The sequences retrieved from liverwort- AMF seem to be, in percentage, more similar to the Mollicutes-related sequences identified in Archaeosporales spores and, to a lesser extent, in Glomerales (Glomus group B). Since the fungal primer pair used in this work is expected to detect Archaeosporales members (Lee et al., 2008) with the exception of Archaeospora trappei, we cannot fully exclude that this specific taxon is present and it acts as a niche for many BLOs. At the same time, the bacterial sequences retrieved in this work appear to be very far from those originated from Diversisporales and G. mosseae spores.

*Conclusions*

Mollicutes-related endobacteria have been detected for the first time in AMF-colonized thalli of liverworts. The bacterial sequences consistently cluster with those derived from endobacteria living in fungal spores and obtained from established collections originating from four continents (Naumann et al., 2010). FISH experiments validate the morphological observations demonstrating that Mollicutes-related bacteria are localized inside the cytoplasm of AM hyphae.

Although it was not the first aim of our investigation, we have demonstrated, in agreement with Bidartondo and colleagues (2011), that the association of liverworts with AMF is not restricted to Glomus group A, as previously suggested (Russell and Bulman, 2005; Selosse, 2005); in contrast it includes members from the whole Glomerales order, which host Mollicutes-related endobacteria. While confirming the presence of Mollicutes-related endobacteria in AMF endophytes of field-collected plants, our experiments revealed that the same AMF lineage may host a diversity of BLOs. It is known that AMF harbour hundreds of haploid nuclei, but it is still unclear whether such nuclei are genetically similar (Pawlowska and Taylor, 2004) or divergent (Kuhn et al., 2001; Hijri and Sanders, 2005). Irrespectively of the genetic structure of these coexisting nuclei (Corradi and Bonfante, 2012), as additional extranuclear genomes, BLOs further increase the genetic complexity of environmental AMF and demonstrate how the plant-associated microbiome is complex and still largely undiscovered.

*Experimental procedures*

*Environmental samples and sample preparation*

Thalli of L. cruciata, M. polymorpha subspecies ruderalis and C. conicum were collected from May to June 2008 in a humid meadow in the Botanical Garden of Turin University, Italy, a protected area which was established in 1729. Liverworts were identified according to Paton (1999) and Schumacker and Váňa (2005). Three samples (each of them consisting of three to eight adjacent thalli) were collected for L. cruciata (L1, L2, L3), one for M. polymorpha (M4) and two for C. conicum (C5, C6). Lunularia cruciata and C. conicum were sampled respectively in three and two sampling sites in the front garden along the south-western wall with 30 m distance between each sampling point, while the M. polymorpha thalli were collected in the arboretum behind the garden.
The samples were washed to remove soil and separated into two parts. One part was used for molecular and one for morphological analyses (see below).

*Light and electron microscopy*

For the detection of fungal colonization in bright-field microscopy, samples were clarified with KOH 10% (w/v), stained with Cotton blue in lactic acid (w/v) (Lumini et al., 2007) and observed using a Nikon Eclipse E400 optical microscope. Other thalli were chemically fixed for transmission electron microscopy according to Ligrone and colleagues (2007). In this case, semithin (1 µm) and ultrathin sections (70 nm) were cut with an Ultracut ultramicrotome (Reichert and Jung). Semithin sections were stained with 1% toluidine blue, 1% sodium tetraborate in water and observed at the bright-field microscope. Ultrathin sections were counterstained with uranyl acetate and lead citrate. Ultrastructural analyses were performed using a CM10 transmission electron microscope (Philips, the Netherlands).

*DNA extraction, amplification and clone library analysis*

Molecular analyses were performed on the thallus parts that were not used for morphology. Each thallus was separated into venation and 'leafy' parts, the latter being used as a negative control since it was never colonized by AMF. The material was surface-sterilized by means of sonication and incubation in a solution containing 3% Chloramine T (w/v), 0.03% Streptomycin (w/v) and a drop of Tween20 for 10 min, washed once with sterile, DNA free water, and then with a sterilizing solution followed by three washes with sterile, DNA free water. The material was stored at −80°C and lyophilized prior to further processing. DNA was extracted with a DNA Easy Plant Mini Kit (Qiagen, Milan, Italy).
The fungal 18S rDNA was PCR-amplified with the general AMF primers, AML1 and AML2 (Lee et al., 2008). They are expected to detect all the Glomeromycota lineages with the exception of A. trappei (Lee et al., 2008). The PCR was performed in a final volume of 20 µl containing 1× reaction buffer with 1.5 mM MgCl2, 200 µM each dNTP, 500 nM each primer, 1 U of HotStar Taq (Qiagen, Milan, Italy), 40–100 ng of template DNA. The cycling conditions were an initial step of 95°C for 15 min, 40 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 7 min.

The bacterial SSU rDNA of the Mollicutes-related endobacteria was amplified with the specific primers 109F (2:1 mixture of 109F-1 ACGGGTGAGTAATRCTTATCT and 109F-2 ACGAGTGAGTAATGCTTATCT) and 1184R (2:1:1 mixture of 1184R-1 GACGACCAGACGTCATCCTY, 1184R-2 GACGACCAAACTTGATCCTC and 1184R-3 GATGATCAGACGTCATCCTC) (Naumann et al., 2010; Supporting information), which are able to cover a region of about 1075 bp. These primers were designed to specifically target Mollicutes endobacteria from Glomeromycota. The PCR mixes contained Phusion HF Mastermix $1\times$ (Finnzymes, Vandaa, Finland), 500 nM of each primer, and 0.5–2 µl of template DNA, in a final volume of 20 µl. The cycling conditions were an initial step of 99°C for 5 min, 35 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 45 s and a final extension step at 72°C for 7 min.

The PCR products were purified using Wizard SV Gel and a PCR Clean-up System (Promega, Milan, Italy), cloned in a pGEM-t Easy Vector System (Promega, Milan, Italy) and then transformed into Top10 chemically competent Escherichia coli (Invitrogen, Milan, Italy) according to the manufacturer's instructions. One hundred colonies were selected from each cloned PCR fungal fragment (150 colonies for bacterial fragment) for further analyses. The colonies were screened for insert length by PCR. The PCR products were digested with the restriction enzymes to produce restriction fragment length polymorphism (RFLP) profiles, with HinfI and Hsp92II (for fungal 18S rDNA fragments) and HaeIII and RsaI (for bacterial 16S rDNA fragments) (1 U, 3–4 h at 37°C) (Promega, Milan, Italy). The RFLP patterns were used to select clones for plasmid extraction: one to three clones were selected for each RFLP pattern. A modified heat-lysis protocol (Ganguly et al., 2005) was applied to extract plasmids: colonies were plated on 9 cm2 solid LB Agar supplemented with Ampicillin, grown at 37°C overnight, collected with a pipette tip from the agar surface, suspended in 50 µl of sterile, DNA-free water and lysed at 95°C for 10 min. The lysates were collected by centrifugation. The vector inserts were sequenced from both ends in the sequencing service at the Department of Biology, Genomics Service Unit (GSU) of Ludwig-Maximilian-University, Munich, Germany.

*FISH experiments*

Seven to eight portions of each liverwort sample harbouring internal fungal structures were processed for FISH analyses in confocal microscopy. Samples were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS), incubated for 1–2 h at room temperature and washed four times in PBS. After embedding in 8% agarose, samples were cut into 100 µm thick sections, using a Mod. 1000 vibratome (Bal-Tec, Balzers, Liechtenstein).

All FISH probes, labelled at their 5′-end with Cy3 or FITC were purchased from Primm (Milan, Italy). A specific 18S rRNA oligonucleotide probe was designed to target AM fungi associated with liverworts (AML2ADf 5′-GAACCCAAACACTTTGGTTTCC-3′). The AML2ADf probe was designed on the basis of the AML2 primer (Lee et al., 2008), the reverse primer used in the PCR experiments to detect AM fungi. A specific 16S rRNA oligonucleotide probe was then designed to target Mollicutes-related bacteria (BLOsADf2 5′-ATCCRTAGACCTTCMTCCTTC-3′) expected to be present in the cytoplasm of liverworts-associated AM fungi. The oligonucleotide sequences were further checked for their suitability with the software Ribosomal Database Project (Cole et al., 2009). In addition, the eubacterial probe EUB338 (Amann et al., 1990) was used as a non-

specific bacterial probe and ApisP2a (Koga et al., 2003), which targets the genus Buchnera, was used as negative control. As additional negative controls, we developed non-EUB338, non-AML2ADf and non-BLOsADf2 probes, corresponding to the reverse complements of EUB338, AML2ADf and BLOsADf2 respectively (Table 2).

Table 2. Oligonucleotide probes used for FISH analysis.

| Probe | Nucleotide sequence | Fluorochrome |
|-------|---------------------|--------------|
| AML2ADf | 5'-GAACCCAAACACTTTGGTTTCC-3' | FITC |
| Non-AML2ADf | 5'-GGAAACCAAAGTGTTTGGGTTC-3' | FITC |
| BLOsADf2 | 5'-ATCCRTAGACCTTCMTCCTTC-3' | Cy3 |
| Non-BLOsADf2 | 5'-GAAGGAKGAAGGTCTAYGGAT-3' | Cy3 |
| EUB338 | 5'-GCTGCCTCCCGTAGGAGT-3' | FITC |
| Non-EUB338 | 5'-ACTCCTACGGGAGGCAGC-3' | FITC |
| ApisP2a | 5'-CCTCTTTGGGTAGATCC-3' | FITC |

The sections then underwent a pre-hybridization treatment with proteinase K (1 µg ml−1) for 10 min. Subsequently, the sections were washed for 5 min at room temperature, once in PBS, twice in PBS containing 1% Tween20 and once in PBS. Prior to hybridization, additional negative controls were prepared treating the samples with RNase (40 µg µl−1) for 1 h at 37°C.

The hybridization was carried out in 30–50 µl of hybridization buffer [10× saline-sodium citrate (SSC) buffer, Denhardt's solution 0.5%, 50 ng ml−1 probes]; the final volume was adjusted based on the tissue sizes. The probes were hybridized at a stringency of 35% formamide for 2 h at 46°C. After hybridization, the sections were washed in 2× SSC for 10 min and 0.1× SSC for 10 min. After the washes, samples were mounted in microscope slides with 1,4-diazabicyclo[2.2.2]octane (DABCO) antifade and stored in the dark at 4°C until confocal microscopy observations. Each sample was hybridized using one of the three FITC-labelled probes (AML2ADf, EUB338 and ApisP2a), together with the Cy3-labelled BLO-specific probe BLOsADf2. These three separated hybridizations were also performed on the negative controls: three separately hybridizations after RNase treatment and three hybridizations with the nonsense probes, for a total of nine hybridizations for each samples.

*Confocal microscopy*

Samples were observed using a Leica TCS-SP2 confocal microscope equipped with 10× dry objective and 40× water immersion objective (Leica HCX Apo 0.80). FITC fluorescence of AML2ADf, EUB338 and ApisP2a probes was excited at 588 nm and imaged with an emission window at 500–540 nm. Cy3 fluorescence for the BLOsADf2 probe was excited at 546 nm and imaged at 550–600 nm. Fixative-derived autofluorescence was imaged at 600–700 nm, using either one of the excitation wavelengths. All images were captured at 1024 × 1024 pixels.

*Bioinformatic analyses*

Sequences were assembled, curated and aligned using Mega5 (Tamura et al., 2011), BioEdit 7.0.9 (Hall, 1999) and MUSCLE (Edgar, 2004) and then processed to blast

searches (Altschul et al., 1997) to compare them against the INSD (Benson et al., 2008). Similarities between sequence types were estimated with clustalw (Thompson et al., 1994). The bacterial 16S and fungal 18S sequences were separately grouped into operation taxonomic units (OTUs) and rarefaction curves were produced using MOTHUR software (Schloss et al., 2009). The OTUs were created using 97% of identity (OTUs0.03). Phylogenetic analyses were conducted using only one representative sequence for each OTU. Phylogenetic trees were inferred with RaxmlGUI 1.1 (for BLOs tree) (Silvestro and Michalak, 2011) or Tree-Puzzle 5.2 (for AMF tree) (Strimmer and Von Haeseler, 1996) and with MrBayes (for BLOs and AMF tree) (Huelsenbeck and Ronquist, 2001), using jModelTest 0.0.1 (Posada, 2008) for the choice of best-fit models of nucleotide substitution (Posada, 2008).

The tree shows only the topologies which are supported by at least one of the two phylogenetic analysis methods (maximum likelihood and Bayesian) and with $> 50\%$ bootstrap or $> 0.5$ posterior probability values. Others are collapsed to polytomies. Dashes instead of numbers indicate that the topology was not supported in the respective analysis.

Even if more recent data for systematic and phylotaxonomy of Glomeromycota have been recently published (Schüßler and Walker, 2010; Oehl et al., 2011; Krüger et al., 2012), this work follows Schüßler and colleagues (2001) classification in order to allow a direct comparison between the novel data set and those previously obtained (Naumann et al., 2010).

*Nucleotide sequence accession numbers*

The sequences analysed in this study have been submitted to the GenBank databases under the Accession Nos JN791098–JN791241.

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