The ISME Journal (2014) 8, 257–270

www.nature.com/ismej

ORIGINAL ARTICLE

Detection of a novel intracellular microbiome hosted in arbuscular mycorrhizal fungi

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Arbuscular mycorrhizal fungi (AMF) are important members of the plant microbiome. They are obligate biotrophs that colonize the roots of most land plants and enhance host nutrient acquisition. Many AMF themselves harbor endobacteria in their hyphae and spores. Two types of endobacteria are known in Glomeromycota: rod-shaped Gram-negative *Candidatus* Glomeribacter gigasporarum, *Ca*Gg, limited in distribution to members of the Gigasporaceae family, and coccoid Mollicutes-related endobacteria, Mre, widely distributed across different lineages of AMF. The goal of the present study is to investigate the patterns of distribution and coexistence of the two endosymbionts, *Ca*Gg and Mre, in spore samples of several strains of *Gigaspora margarita*. Based on previous observations, we hypothesized that some AMF could host populations of both endobacteria. To test this hypothesis, we performed an extensive investigation of both endosymbionts in *G. margarita* spores sampled from Cameroonian soils as well as in the Japanese *G. margarita* MAFF520054 isolate using different approaches (molecular phylotyping, electron microscopy, fluorescence *in situ* hybridization and quantitative real-time PCR). We found that a single AMF host can harbour both types of endobacteria, with Mre population being more abundant, variable and prone to recombination than the *Ca*Gg one. Both endosymbionts seem to retain their genetic and lifestyle peculiarities regardless of whether they colonize the host alone or together. These findings show for the first time that fungi support an intracellular bacterial microbiome, in which distinct types of endobacteria coexist in a single cell.
The ISME Journal (2014) **8,** 257–270; doi:10.1038/ismej.2013.151; published online 5 September 2013
Subject Category: Microbial population and community ecology
Keywords: arbuscular mycorrhizal fungi; fungal microbiome; endobacteria; fluorescence *in situ* hybridization; phylogenetic analysis; quantitative real-time PCR

Introduction

The discovery that the human body can be described as a complex ecosystem where human cells interact with trillions of bacteria and other microbes has represented a scientific revolution. The human microbiome, that is, the microbial communities and the genetic information they contain, cooperate with the human genome to regulate crucial physiological processes ranging from digestion to obesity and immunity (Methé *et al*., 2012). Similarly, plants rely on microorganisms living both in their tissues and in the rhizosphere, creating a network of mutual relationships (Porras-Alfaro and Bayman, 2011; Berendsen *et al*., 2012; Bulgarelli *et al*., 2012: Lundberg *et al*., 2012). To date, most of the work on plant-associated microbes focused almost exclusively on bacteria (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012), even though eukaryotes such as fungi are also crucial components of the plant microbiome. They not only thrive in the rhizosphere, but also colonize plant tissues, exhibiting a range of lifestyles, including mutualism, parasitism and commensalism (Porras-Alfaro and Bayman, 2011).

Among plant-associated microbiota, arbuscular mycorrhizal fungi (AMF) are the most widespread: they belong to an ancient monophyletic phylum, the Glomeromycota (Schüßler *et al*., 2001), and have a key role in nutrient cycling and plant health due to their capacity for improving the mineral nutrition of plants (Smith and Read, 2008). AMF display many unusual biological features. In addition to their obligate biotrophy (Bonfante and Genre, 2010), many of them harbor endobacteria in their cytoplasm

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Received 6 May 2013; revised 17 July 2013; accepted 22 July 2013; published online 5 September 2013

(Bonfante and Anca, 2009). Bacterial endosymbionts are widespread among animals (Wernegreen, 2012; McFall-Ngai *et al.*, 2013) and in particular the ones living in insect tissues have been investigated in depth (Ferrari and Vavre, 2011). In contrast, examples of endobacteria living inside the fungal cells are much more limited (Bianciotto *et al.*, 2003; Partida-Martinez and Hertweck, 2005; Lackner *et al.*, 2009; Naumann *et al.*, 2010; Kai *et al.*, 2012).

The endobacteria of Glomeromycota are the most thoroughly investigated bacterial endosymbionts of fungi, having been discovered in the early 1970s on the basis of electron microscope observations (Mosse, 1970). Two types of endosymbionts are known in AMF: (i) a rod-shaped, Gram-negative beta-proteobacterium (Bonfante *et al.*, 1994), *Candidatus* Glomeribacter gigasporarum (*Ca*Gg), common in several species of the family Gigasporaceae (Bianciotto *et al.*, 2003; Mondo *et al.*, 2012), and (ii) a coccoid bacterium displaying a homogeneous Gram-positive-like wall structure (MacDonald *et al.*, 1982; Scannerini and Bonfante, 1991), which represents a currently undescribed taxon of Mollicutes-related endobacteria (Mre) with a wide distribution across Glomeromycota (Naumann *et al.*, 2010).

The *Ca*Gg genome sequence (Ghignone *et al.*, 2012) revealed that Glomeribacter endobacteria are nutritionally dependent on the fungal host and have a possible role in providing the fungus with essential factors like vitamin B12 (Ghignone *et al.*, 2012). Phenotypic consequences of *Ca*Gg removal from the host include important morphological changes as well as reduced proliferation of host presymbiotic hyphae. Yet, the host is not obligately dependent on the bacteria (Lumini *et al.*, 2007; Mondo *et al.*, 2012). These features suggest that Glomeribacter endobacteria are mutualistic associates of AMF (Lumini *et al.*, 2007). Comparisons of host and symbiont phylogenies indicate that, while *Ca*Gg is a heritable endosymbiont (Bianciotto *et al.*, 2004), it also engages in recombination and host switching, which play an important role in stabilizing this 400-million-year-old association (Mondo *et al.*, 2012). In contrast, information on the coccoid Mre is much more limited. Based on the 16S rRNA gene sequences, this novel lineage is sister to a clade encompassing the Mycoplasmatales and Entomoplasmatales (Naumann *et al.*, 2010). The Mre have been detected in 17 out of 28 investigated AMF samples from culture collections, including members of Archaeosporales, Diversisporales, Glomerales (Naumann *et al.*, 2010), as well as in mycorrhizal thalli of liverworts (Desirò *et al.*, 2013). In most of the AMF hosts and irrespectively of the AMF identity, these endobacteria displayed a conspicuous variability in their 16S rRNA gene sequence. Collectively, these observations indicate that *Ca*Gg is a stable associate of Gigasporaceae, whereas the lifestyle of the Mre and the nature of their association with Glomeromycota are uncertain. Furthermore, the interaction between the two endosymbionts remains unclear, that is, it is not known whether the presence of one endosymbiont in the host leads to the exclusion of the other one.

The goal of the present study is to investigate the patterns of distribution and coexistence of the two endosymbionts, *Ca*Gg and Mre, in isolates of one host species, *Gigaspora margarita* WN Becker & IR Hall. Previous electron microscopy observations revealed that the strain of *G. margarita* MAFF520054 harbored a Gram-positive-like endobacterium (Kuga *et al.*, 2008), whereas molecular analyses indicated the presence of *Ca*Gg (E Lumini, personal communication, ref. seq. AM886455; Long *et al.*, 2009). Based on these observations, we hypothesize that some AMF could host populations of both endobacteria. To test this hypothesis, we performed an extensive investigation of both endosymbionts in *G. margarita* spores sampled from Cameroonian soils as well as in *G. margarita* MAFF520054 from Japan using different approaches. We found that a single AMF host can harbor both types of endobacteria, with Mre populations being more abundant, variable and prone to recombination than the *Ca*Gg ones. These findings show for the first time that fungi support an intracellular bacterial microbiota, in which distinct types of endobacteria coexist in a single cell.

Materials and methods

All the details of the experimental procedures are available in the Supplementary Text S1.

Sampling and sample preparation

Twelve soil samples were collected from three locations in Cameroon (Table 1). Trap cultures with *Sorghum* and *Vigna* were established using autoclaved sand mixed with the sampled soils. The Japanese isolate *G. margarita* MAFF520054 was provided by NIAS Genebank and propagated in pot cultures with *Trifolium*.

The spores were recovered from pot cultures by wet sieving (Gerdemann and Nicolson, 1963) and surface sterilized (Lumini *et al.*, 2007). The spore samples were morphologically identified as *Gigaspora margarita* following Bentivenga and Morton (1995).

DNA extraction, amplification and clone library analysis

DNA extractions were performed by crushing either individual spores or groups of five or ten spores according to Lumini *et al.* (2007). Three fragments of the fungal ribosomal gene cluster, namely 18S, ITS and 28S, were amplified.

The *Ca*Gg 16S rRNA gene was specifically amplified with the newly designed primers CaGgADf (5′-AGATTGAACGCTGGCGGCAT-3′) and CaGgADr (5′-ATGCGTCCTACCGTGGCCATC-3′), while the Mre 16S rRNA gene was amplified as described in

Table 1 List of the spore samples studied in this work

Sample	*Origin*	*AM species*	*Endobacteria*
CM2	Cameroon: Nkolbisson, Yaoundé	*Gigaspora margarita*	Mre + *Ca*Gg
CM3	Cameroon: Nkolbisson, Yaoundé	*Gigaspora margarita*	Mre
CM9	Cameroon: Nkolbisson, Yaoundé	*Gigaspora margarita*	Mre + *Ca*Gg
CM21	Cameroon: Nkolbisson, Yaoundé	*Gigaspora margarita*	Mre + *Ca*Gg
CM23	Cameroon: Nkoemvone, Ebolowa	*Gigaspora margarita*	Mre + *Ca*Gg
CM27	Cameroon: Nkoemvone, Ebolowa	*Gigaspora margarita*	Mre + *Ca*Gg
CM46	Cameroon: Nkoemvone, Ebolowa	*Gigaspora margarita*	Mre + *Ca*Gg
CM47	Cameroon: Maroua	*Gigaspora margarita*	Mre + *Ca*Gg
CM49	Cameroon: Nkolbisson, Yaoundé	*Gigaspora margarita*	Mre + *Ca*Gg
CM50	Cameroon: Nkoemvone, Ebolowa	*Gigaspora margarita*	Mre + *Ca*Gg
CM51	Cameroon: Nkoemvone, Ebolowa	*Gigaspora margarita*	Mre + *Ca*Gg
CM52	Cameroon: Nkoemvone, Ebolowa	*Gigaspora margarita*	Mre
MAFF520054	Japan: Saitama	*Gigaspora margarita*	Mre + *Ca*Gg

Collection sites, fungal species and endobacteria typology are shown for each sample.

Desirò *et al.* (2013). Fungal and bacterial PCR amplicons were transformed and the obtained clone libraries were analyzed.

Bioinformatics
Sequences were assembled and curated in Mega v. 5.2 (Tamura *et al.*, 2011), aligned with MAFFT (Katoh *et al.*, 2002) or MUSCLE (Edgar, 2004) and then examined for chimerism. Sequence similarity/divergence was evaluated using MOTHUR (Schloss *et al.*, 2009). Nucleotide diversity (π) was calculated in DNAsp v. 5.10.01 (Librado and Rozas, 2009). The *Ca*Gg and Mre 16S rRNA gene sequences were grouped into operational taxonomic units at the cut-off of 0.03 genetic distance value using MOTHUR. Phylogenetic analyses were conducted using one representative sequence for each OTU. The Genetic Algorithm for Recombination Detection (Kosakovsky Pond *et al.*, 2006), was used to identify recombination breakpoints in 16S rRNA genes of *Ca*Gg and Mre.

Ultrastructural analysis
Single *G. margarita* spores from CM23 and CM47 samples were processed by using high-pressure freezing followed by freeze substitution. Single spores floating in water were transferred in the cavity of an aluminum carrier with a pipette. Excess of water was drawn off with filter paper and the space was filled with 1-Hexadecene. The sandwich was completed with a flat specimen carrier and frozen in a HPM 100 high-pressure freezing machine (Leica Microsystems, Wetzlar, Germany) (McDonald *et al.*, 2010). Samples were then freeze substituted, resin embedded and processed for transmission electron microscopy.

FISH experiments and confocal microscopy
Sterilized spores of the samples CM21, CM23, CM47, CM52 and *G. margarita* BEG34 were fixed as described in Naumann *et al.* (2010). The Mre-specific probe BLOsADf2 (Desirò *et al.*, 2013), together with a newly designed *Ca*Gg-specific 16S rRNA probe (CaGgADf1 5′-CTATCCCCCTCTACAGGAYAC-3′), were used to label the endobacteria. In addition, the eubacterial probe EUB338 (Amann *et al.*, 1990) and the *Buchnera*-specific probe ApisP2a (Koga *et al.*, 2003) were used. Spores were observed using a Leica TCS-SP2 confocal microscope (Leica Microsystems).

Quantification of the bacterial populations
The sample CM23 (containing both Mre and *Ca*Gg) was selected for the relative quantification of the two bacterial populations by real-time quantitative PCR (qPCR). Briefly, the 16S rRNA gene sequences obtained for both *Ca*Gg and Mre were used to design two distinct qPCR primer pairs. Template plasmids containing the target DNA sequences were constructed to generate a standard curve as an external standard. The number of target DNA sequences present in each PCR mixture was calculated by comparing the crossing points of the samples with those of the standards.

Results

Identity of AMF
To confirm the morphological identification of AMF originating from Cameroon and Japan as *Gigaspora margarita*, we analyzed their 18S, ITS and 28S rRNA gene regions. These analyses revealed that all the fungi could be identified as *G. margarita* (Figure 1 and Supplementary Figure S3). As expected, the 18S rRNA gene analysis led to an unresolved, polytomic phylogeny (not shown), whereas a better resolution was provided by the ITS region (Figure 1) and the 28S rRNA gene (Supplementary Figure S3). DNA sequences are available in GenBank (KF378653-KF378691).

Identity of endobacteria
Bacterial 16S rRNA gene sequences were PCR-amplified from single AMF spores using primers

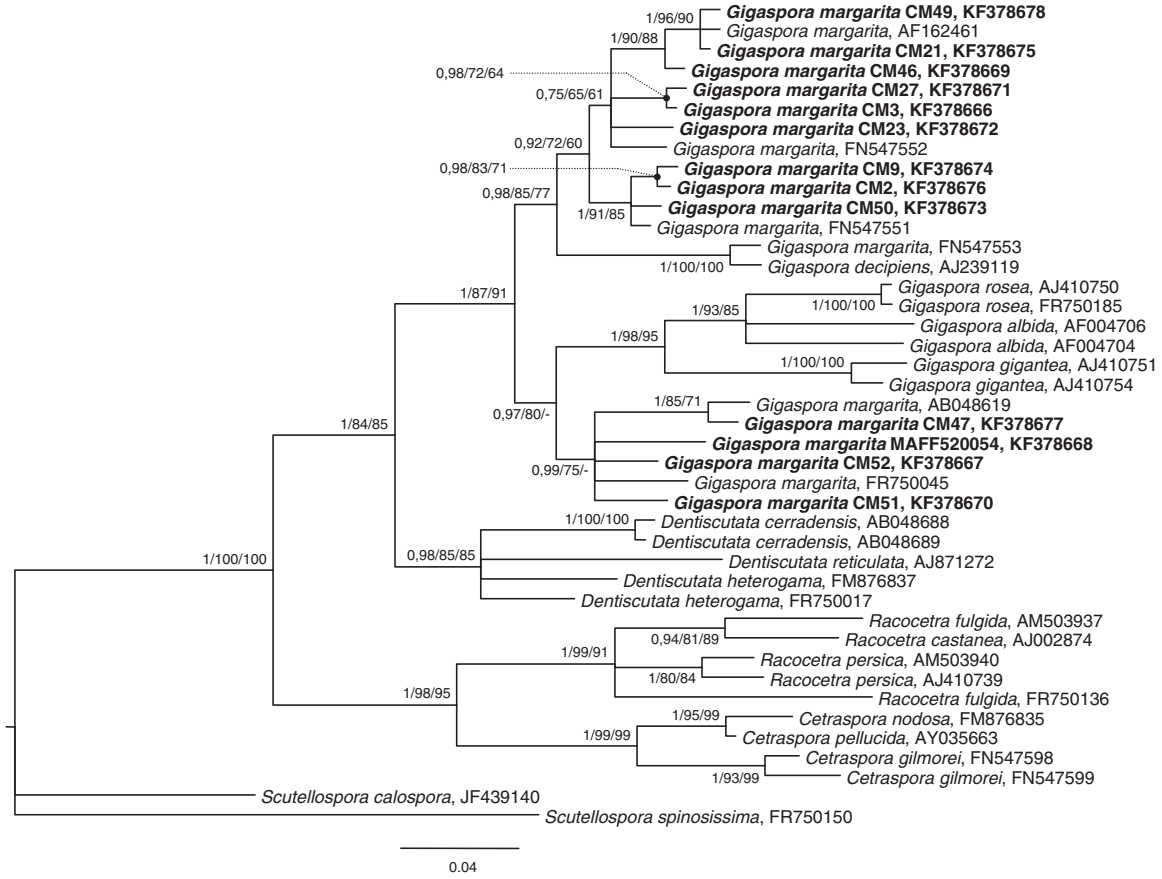

Figure 1 Phylogenetic placement of Cameroonian and Japanese spore samples inside the Gigasporaceae tree. The fungal phylogeny was inferred from ITS1, 5.8S rRNA gene and ITS2 sequences. The DNA sequences retrieved in this work are in bold. All the thirteen spore samples are located inside the *Gigaspora* clade, close to *Gigaspora margarita*. Supported values are from Bayesian/maximum likelihood/maximum parsimony analyses. The partitioned Bayesian analysis was performed with JC, K80 + G, and HKY + G nucleotide substitution models for ITS1, 5.8S and ITS2 regions, respectively. The maximum likelihood analysis was performed with GTR + CAT nucleotide substitution model. Dashes instead numbers imply that the topology was not supported in the respective analysis.

specific for *Ca*Gg and Mre (Naumann *et al.*, 2010) to detect endosymbiont presence. Most samples harbored both types of endobacteria with the exception of the *G. margarita* samples CM3 and CM52, which contained only Mre (Table 1). The absence of *Ca*Gg in the samples CM3 and CM52 was confirmed by real-time qPCR (data not shown), which can detect up to 10 bacterial cells (Salvioli *et al.*, 2008).

In order to faithfully describe the microbiome contained inside the AMF spores and to capture all of the bacterial biodiversity, a more extensive analysis was performed on pools of 10 spores from four Cameroonian samples (CM21, CM23, CM47, CM50) and from the Japanese isolate.

The RFLP analysis of *Ca*Gg 16S rRNA gene sequences revealed a single RFLP profile for each 10-spore sample, suggesting a limited intra-sample variability that was further confirmed by sequence analysis. The obtained sequences (~1460 bp) were grouped into operational taxonomic units at 97% of sequence similarity and, as expected, a single OTU for each sample was obtained (Table 2). Phylogenetic analyses of *Ca*Gg sequences retrieved from spore samples showed that they clustered with other *Ca*Gg sequences available in GenBank (Figure 2).

Table 2 *Candidatus* Glomeribacter gigasporarum sequences generated from the selected *Gigaspora margarita* spore samples

G. margarita[a] *sample*	*Retrieved*[b] *sequences*	*OTU*[c] *number*	*Sequences in*[d] *OTU1*
CM21	3	1	3
CM23	4	1	4
CM47	5	1	5
CM50	4	1	4
MAFF520054	4	1	4

[a]Gigaspora margarita spore sample.
[b]Number of *Candidatus* Glomeribacter gigasporarum generated sequences.
[c]Number of OTUs and [d]their related sequences.

Sequencing of the Mre 16S rRNA gene clones generated a total of 118 sequences (Table 3). To eliminate potential PCR artefacts expected in amplifications from complex templates such as Mre populations (Naumann *et al.*, 2010), the obtained

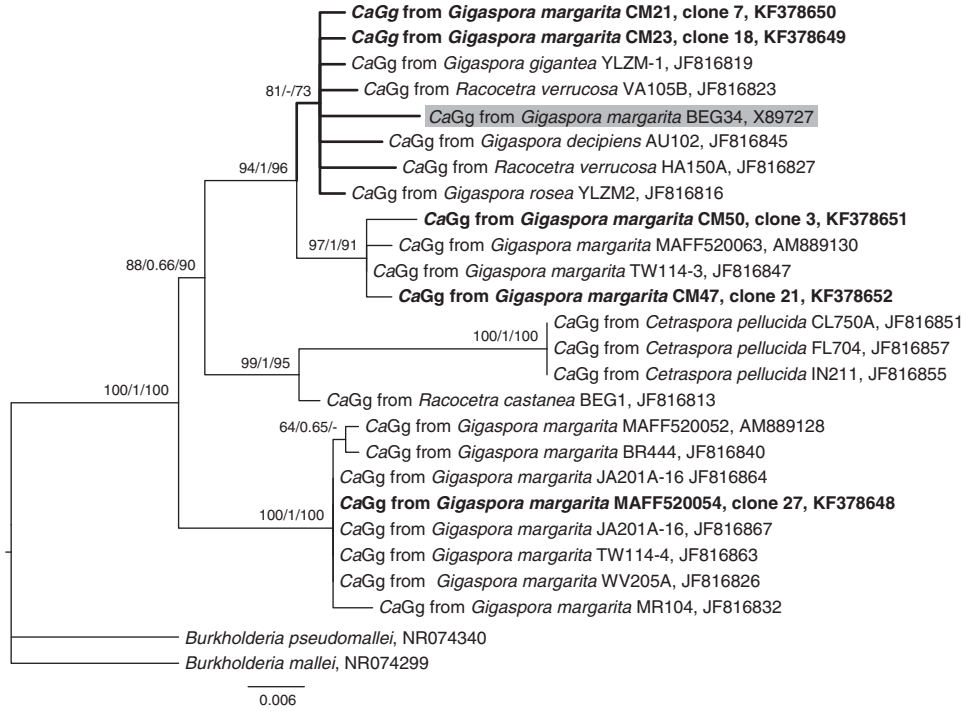

Figure 2 Phylogenetic placement of representative *Candidatus* Glomeribacter gigasporarum partial 16S rRNA gene sequences retrieved from spores of AMF. The DNA sequences retrieved in this work are in bold. The tree encompasses several *Ca*Gg groups. Sequences from *Gigaspora margarita* sample CM47 and CM50 cluster in a group sister to the one (with thickened branches) including *Ca*Gg from *G. margarita* BEG34 isolate (highlighted in gray) and from the Cameroonian CM21 and CM23 samples. The 16S rRNA gene sequences from the Japanese sample MAFF520054 are located in a different and more basal position inside the tree, together with other *Ca*Gg sequences retrieved from worldwide *G. margarita* isolates. Cameroonian isolates showed 97–100% sequence similarity with Gigasporaceae isolates (that is, *Gigaspora decipiens, Gigaspora gigantea, G. margarita*, including the isolate BEG34, *Gigaspora rosea, Racocetra castanea* and *Racocetra verrucosa*), which are located in the upper part of the tree. By contrast, *Ca*Gg sequence similarity, in particular of the samples CM47 and CM50, decreased to 96% relative to *Ca*Gg sequences retrieved from other worldwide isolates of *Cetraspora pellucida* and *G. margarita*, including the *G. margarita* isolate MAFF520054. Supported values are from maximum likelihood/Bayesian/maximum parsimony analyses. The maximum likelihood and Bayesian analyses were performed with GTR + G and TIM3 + G nucleotide substitution models, respectively. Dashes instead numbers imply that the topology was not supported in the respective analysis.

Table 3 Mollicutes-related endobacteria sequences generated from the selected *Gigaspora margarita* spore samples

G. margarita[a] *sample*	*Retrieved*[b] *sequences*	*Sequences after*[c] *chimera screen*	*OTU*[d] *number*	*Sequences in*[e]			*Nucleotide diversity (π)*[f] *up to*	*Sequence*[g] *divergence (%)*
				OTU1	*OTU2*	*OTU3*		
CM21	14	14	2	13	1	—	0.1764	20
CM23	38	7	3	3	2	2	0.1882	20
CM47	31	12	3	5	4	3	0.1608	17
CM50	10	10	1	10	—	—	—	—
MAFF520054	25	9	3	4	4	1	0.1590	17

[a]Gigaspora margarita spore sample.
[b]Number of Mollicutes-related endobacteria generated sequences.
[c]Number of sequences after the chimera removal.
[d]Number of OTUs and [e]their related sequences.
[f]The highest values of nucleotide diversity and [g]sequence divergence between two representative sequences of different OTUs of the same sample.

sequences (1049–1087 bp) were submitted to a rigorous chimera screen, which reduced the total amount to 52 sequences (Table 3). They were grouped into operational taxonomic units at 97% sequence similarity (Table 3). Most of the sequences (48 out of 52) showed sequence similarity values lower than 97% when compared with the Mre sequences obtained from GenBank, suggesting the presence of novel phylotypes (Table 3).

Despite the high variability, all retrieved Mre sequences clustered together with those obtained in previous studies (Naumann *et al.*, 2010; Desirò *et al.*, 2013) (Figure 3). Moreover, because the resulting phylogenies presented here are better supported and

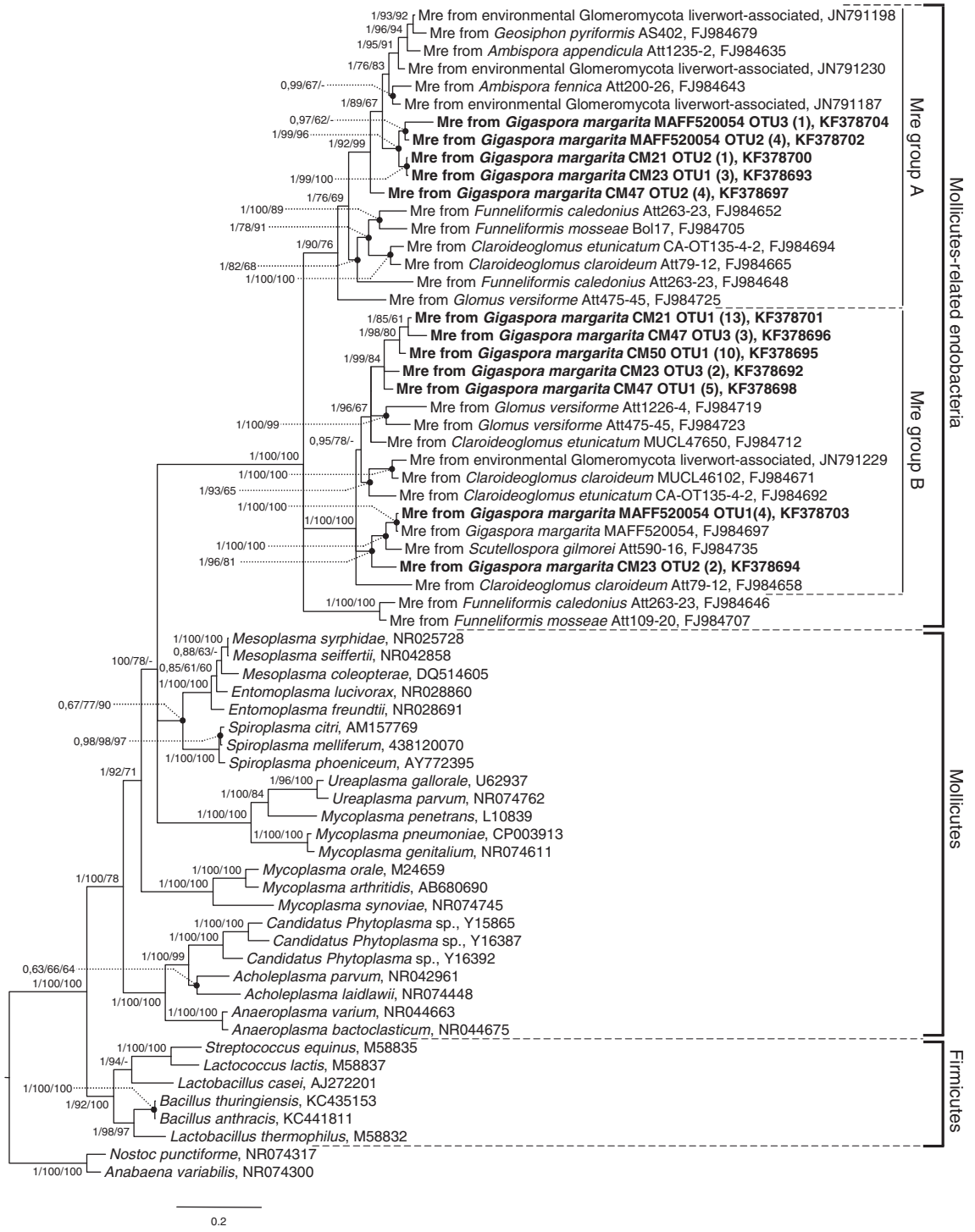

Figure 3 Phylogenetic placement of representative Mollicutes-related endobacteria partial 16S rRNA gene sequences retrieved from AM spores within the Mollicutes clade. The DNA sequences retrieved in this work are in bold. The tree encompasses at least two main and well-supported groups (Mre group A and B), which also include sequences retrieved in previous experiments from AM spore collection (Naumann *et al.*, 2010) and AMF liverworts-associated (Desirò *et al.*, 2013). The number of sequences included in each OTU is in brackets. Supported values are from Bayesian/maximum likelihood/maximum parsimony analyses. The Bayesian and maximum analyses were performed with GTR + G and GTR + CAT nucleotide substitution models, respectively. Dashes instead of numbers imply that the topology was not supported in the respective analysis.

resolved than those constructed in previous works (Naumann *et al.*, 2010; Desirò *et al.*, 2013), we conclude that there are at least two distinct and well-supported Mre clades, identified as Mre group A and group B (Figure 3), and that the level of sequence divergence among sequences clustering in the same

Mre group reached up to 15 and 16% in Mre group A and B, respectively. Overall, in all the samples, with the only exception of CM50, *Ca*Gg showed a high level of intra-host sequence similarity, whereas Mre revealed high levels of intra-host sequence diversity.

Representative DNA sequences are available in GenBank (KF378648-KF378652, KF378692-KF378705).

Recombination detection

To explore the underlying causes of differences in sequence evolution patterns between *Ca*Gg and Mre, we used the Genetic Algorithm for Recombination Detection (Kosakovsky Pond *et al.*, 2006) to look for evidence of recombination in 16S rRNA genes of the two endosymbionts associated with AMF from Cameroon and Japan. No evidence of recombination was detected in the *Ca*Gg sequences. In contrast, in the Mre data set, we found that the AIC_C score of 8529.9 for the best-fitting model allowing for different topologies of the alignment segments defined by recombination breakpoints was lower than the AIC_C score of 8819.4 for the model that assumed the same topology for all segments, indicating that a multiple tree model is preferable over a single tree model. Using the KH test, one breakpoint at the alignment position 479 was identified as resulting in significant topological incongruence between segments ($P<0.001$, Supplementary Figure S4).

Localization of the two bacterial morphotypes in AMF cells: high pressure/freeze substitution and transmission electron microscopy

We used electron microscopy to confirm the cytoplasmic location of both types of endobacteria. To ensure proper preservation of endosymbiont cells and fungal organelles, which could be jeopardized by the very thick fungal cell wall (12–16 μm, Lumini *et al.*, 2007), we used high pressure and freeze substitution specimen preparation. On the basis of the previous molecular analyses, two isolates of *G. margarita* (CM23 and CM47) were selected for this experiment. When inspected under the transmission electron microscope, CM23 and CM47 presented both the rod-shaped and coccoid bacteria in the same area of their cytoplasm (Figure 4). The rod-shaped *Ca*Gg were 330–550 × 960–1050 nm in size, with a layered, Gram-negative type cell wall (Figures 4a and b) and were located inside a vacuole-like organelle (Figure 4a), consistent with reports from earlier studies (Bianciotto *et al.*, 1996, 2003). The vacuole revealed an electron-dense matrix, which was identified as of protein origin (Bonfante *et al.*, 1994) (Figure 4a). In other cases, the matrix was reduced in size and the bacterium was more closely surrounded by a membrane of fungal origin (Figure 4b). In contrast, the coccoid Mre were directly embedded in the fungal cytoplasm (Figures 4a and c). They were consistently smaller, 300–600 nm in size, with a homogeneous, Gram-positive-like cell wall (Figure 4c).

Localization of the two endosymbionts in AMF spores: FISH

To further validate our molecular and morphological observations of the *Ca*Gg and Mre coexistence in *G. margarita,* we performed fluorescence *in situ* hybridization (FISH) experiments in samples CM21, CM23, CM47 and CM52. *G. margarita* BEG34 was used as negative control, as Mre have never been found in this isolate (Naumann *et al.*, 2010).

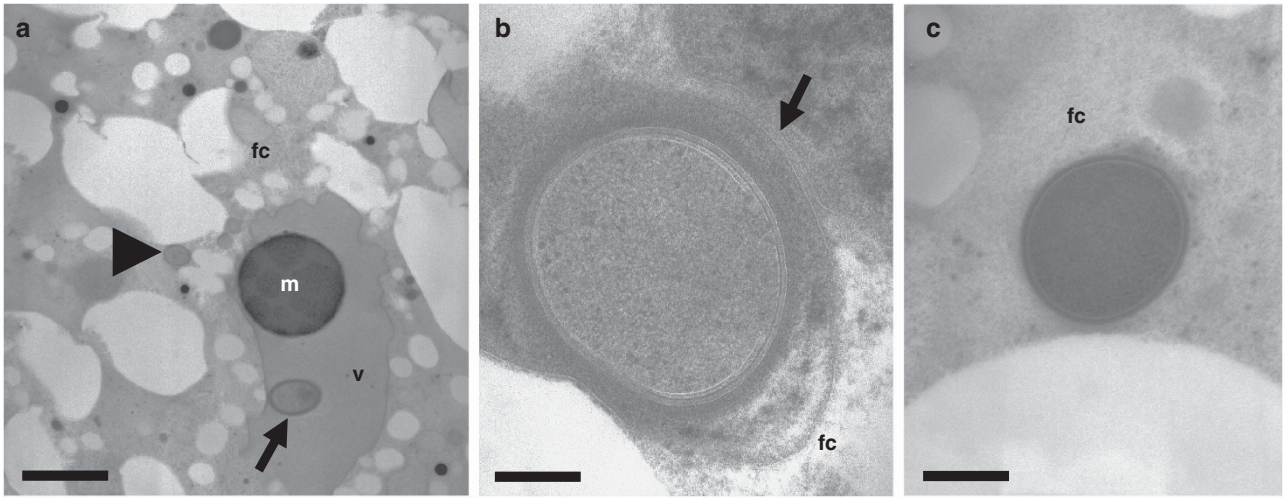

Figure 4 Electron microscopy of *Gigaspora margarita* sample CM23. (**a**) The two bacterial types, *Candidatus* Glomeribacter gigasporarum (arrow) and Mollicutes-related endobacteria (arrowhead) are present in the same district of the sporal fungal cytoplasm (fc). The rod-shaped type is constantly located inside a vacuole-like organelle (v). The vacuole reveals an electron-dense matrix (m), identified as of protein origin. (**b**) Sometimes *Ca*Gg (here cut in a transversal section) is more closely surrounded by a membrane of fungal origin (arrow). (**c**) The Mre is directly embedded in the fungal cytoplasm. Scale bars, (**a**) 1.5 μm; (**b**) 0.26 μm; (**c**) 0.17 μm.

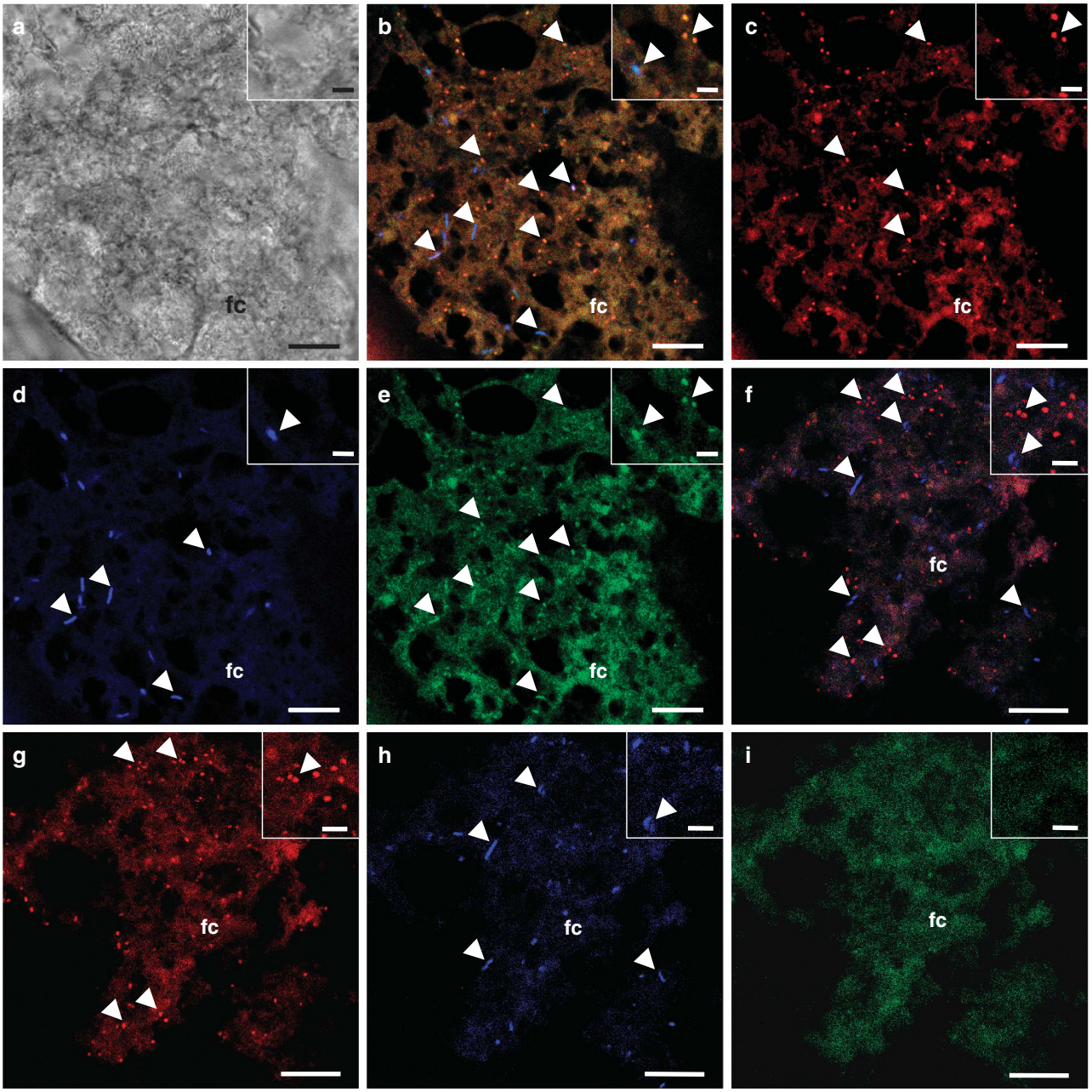

Figure 5 FISH on crushed spores of *Gigaspora margarita* samples CM21 (**a-e**) and CM23 (**f-i**). (**a**) Bright-field image of the fungal cytoplasm (fc) trapped in a drop of agarose. (**b**) Triple labelling of the endobacteria with the Mollicutes-related endobacteria-specific probe BLOsADf2 (red), the *Candidatus* Glomeribacter gigasporarum-specific probe CaGgADf1 (blue) and the universal bacterial probe EUB338 (green); bacteria are seen as coccoid or rod-shaped fluorescent spots (arrowheads); in this image, where red and green or blue and green channels are overlaid, bacteria are visualized as fluorescent orange or light blue spots inside the brown cytoplasm. The corresponding red, blue and green channels are shown in (**c-e**). (**f**) Triple labelling of the endobacteria with the Mre-specific probe BLOsADf2 (red), the CaGg-specific probe CaGgADf1 (blue) and the Buchnera-specific probe ApisP2a (green) used as negative control; bacteria are seen as coccoid or rod-shaped fluorescent spots (arrowheads). The corresponding red and blue channels are shown in (**g**, **h**). (**i**) No presence of non-specific fluorescent signal is detected. The insets show the magnification of some Mre and *Ca*Gg cells surrounded by the fungal cytoplasm. Scale bars: 12 μm, 3 μm in the insets.

We used two probes: CaGgADf1, which was designed to specifically detect *Ca*Gg, and BLOsADf2 (Desirò *et al.*, 2013), which targeted entire Mre variability contained in our spore samples. In agreement with PCR results, we did not observe any *Ca*Gg signal in CM52, where *Ca*Gg have never been detected by PCR amplification of 16S rRNA gene. Similarly, we did not observe any Mre signal in BEG34. On the contrary, the two specific probes produced simultaneous FISH signals in the spores where the presence of both bacterial types was expected (Figure 5). Image analysis on 16 confocal microscope images from spore samples containing the two bacterial

populations revealed that Mre were 1.62–3.15 times more abundant than *Ca*Gg (Supplementary Table S1).The fluorescent signals were located in the fungal cytoplasm and never on the spore surface. Importantly, the fluorescent signal of the probes BLOsADf2 (Desirò *et al.*, 2013) and CaGgADf1 was always co-localized with the fluorescence given by the universal bacterial probe EUB338 (Amann *et al.*, 1990) (Figures 5b–e). No fluorescent signal was detected with the negative control probe ApisP2a (Koga *et al.*, 2003) (Figure 5i). Pre-treatment with RNase, as well as control hybridization with nonsense probes, did not provide any FISH signal. A weak autofluorescence of the fungal cytoplasm, probably derived from the use of aldehydic fixatives, was visible in all spore samples. Hence, FISH experiments, validating the PCR results, confirmed the simultaneous presence of Mre and *Ca*Gg in some *G. margarita* samples.

Mre and CaGg abundance in AMF cells: real-time qPCR

To further examine differences in Mre and *Ca*Gg abundance suggested by FISH experiments, we used real-time qPCR to quantify the bacterial populations present in the *G. margarita* sample CM23 that was previously shown to contain both Mre and *Ca*Gg endobacteria. The 16S rRNA gene was used as a target gene, but while in the *Ca*Gg genome the 16S rRNA gene is present in a single copy (Ghignone *et al.*, 2012), in Mre one or at most two *rRNA* gene copies are expected based on the comparison with the closest microbes already sequenced (Fraser *et al.*, 1995; Glass *et al.*, 2000; Jaffe *et al.*, 2004; Minion *et al.*, 2004; Vasconcelos *et al.*, 2005; Bai *et al.*, 2006).

The accuracy of qPCR primers of *Ca*Gg and Mre was confirmed by assessing the melting profile generated by each primer pair (Supplementary Figure S2). Subsequently, we quantified the relative abundance of the two bacterial endosymbionts on the basis of the 16S rRNA gene sequences. In *G. margarita* CM23, we found that Mre were always more abundant than *Ca*Gg, and the bacterial ratio was maintained fairly constant irrespective of the size of the batches considered (that is, one, five or ten spores) (Table 4).

The qPCR analysis of the bacterial 16S rRNA gene sequences revealed that Mre are 5.17–6.12 times more abundant than *Ca*Gg in the *G. margarita* CM23 spores, assuming that a single copy of the 16S rRNA gene is present in the Mre genome. This value should be reduced to 2.59–3.06 times if two copies of the 16S rRNA are present in Mre genome instead (Table 4). This finding is consistent with our FISH observations, which suggested that Mre were more abundant than *Ca*Gg in *G. margarita* spores.

Table 4 Quantification of Mollicutes-related endobacteria and *Candidatus* Glomeribacter gigasporarum detected with real-time qPCR

Batch	*Biological replicates*	*Mre average*	*Mre standard deviation*	*CaGg average*	*CaGg standard deviation*	*Ratio*
A						
1	7	927	729	179	140	5.17
5	6	3963	1608	648	400	6.12
10	5	10752	3266	1897	1121	5.67
B						
1	7	463	364	179	140	2.59
5	6	1982	804	648	400	3.06
10	5	5376	1633	1897	1121	2.83

The quantification was performed for batches of 1, 5 and 10 spores considering at least five biological replicates. The ratio is obtained by dividing the number of Mollicutes-related endobacteria for that of *Candidatus* Glomeribacter gigasporarum. We observe high variability in the quantification of 16S rRNA gene sequences of both types of endobacteria when single spores are analyzed, with this variation being reduced when batches of multiple spores are considered. This pattern is consistent with previous observations that *Ca*Gg abundance in individual spores can vary greatly (Jargeat *et al.*, 2004). (A) The qPCR analysis revealed that Mre are 5.17–6.12 times more abundant than *Ca*Gg in the *Gigaspora margarita* CM23 spores, assuming that a single copy of the 16S rRNA gene is present in the Mre genome. (B) The value should be reduced to 2.59–3.06 times if two copies of the 16S rRNA gene are present in Mre genomes.

Discussion

A combination of morphological, molecular and phylogenetic analyses demonstrates that *Gigaspora margarita* spores host a complex microbiome consisting of rod-shaped and coccoid bacteria. The two bacterial groups are very distinct not only in their phylogenetic placement, that is, *Ca*Gg is closely related to Burkholderiaceae, whereas the coccoid endobacteria are related to the Gram-positive Mollicutes, but also in their genetic features.

Sharing the same host and revealing intra-host diversity

Notwithstanding the endobacteria share the same fungal host, a relevant difference in genetic diversity patterns between them was revealed. While *Ca*Gg shows a high level of intra-host sequence similarity, the Mre are characterized by high levels of intra-host sequence diversity. One of the underlying causes of differences in sequence evolution patterns between *Ca*Gg and Mre may be differences in their lifestyle. For example, in Mre, we found evidence of recombination, which was not apparent in *Ca*Gg. This finding was supported by some genomic features of *Ca*Gg genome: notwithstanding its high repetitive DNA (15%), *Ca*Gg contains a low number of active insertion sequences, which are considered important determinants for recombination (Ghignone *et al.*, 2012). Indeed, a recent study of *Ca*Gg, using a set of four marker genes, revealed that recombination is not entirely absent from the *Ca*Gg evolutionary

history and, together with host switching, may have an important role in evolutionary stability of *Ca*Gg association with Glomeromycota (Mondo *et al.*, 2012). Detecting evidence of recombination in a single gene of Mre sampled in the present study may suggest that Mre engage in more frequent recombination than *Ca*Gg. Interestingly, cryptic prophage remnants have been detected in the genome of the Mre-related phytoplasma, leading to the suggestions that these genetic elements may have had important roles in generating phytoplasma genetic diversity (Wei *et al.*, 2008).

Phylogenetic divergence patterns of the co-existing endobacteria

The extensive phylogenetic analyses performed on the endobacteria thriving in the cytoplasm of five spore samples and their comparison with data from previous investigations (Bianciotto *et al.*, 1996, 2000, 2003; Mondo *et al.*, 2012) confirmed that the 16S rRNA gene sequences of *Ca*Gg were relatively conserved, irrespective of the geographic origin of the fungal host. However, our careful analyses showed that the sequence similarity between *Ca*Gg from *G. margarita* MAFF520054 isolate and the already sequenced *Ca*Gg from *G. margarita* BEG34 was below the critical level of 97%. In fact, although this distinction is controversial (Rossello-Mora, 2003), it is generally accepted that sequences with similarity greater than 97% are typically assigned to the same species and those with similarity greater than 95% to the same genus (Stackebrandt and Goebel, 1994; Everett *et al.*, 1999; Gevers *et al.*, 2005). Consequently, further work is needed to resolve whether *Ca*Gg from *G. margarita* MAFF520054 and *G. margarita* BEG34, which show sequence similarity lower than 97% and a different location inside the *Ca*Gg phylogenetic tree, represent distinct taxa.

In contrast to *Ca*Gg and despite the stringent removal of chimeric sequences, the 16S rRNA gene sequences of Mre turned out to be highly variable inside at least four out of five spore samples. Moreover, in only 8% of the sequences generated in this study (4 out of 52), the similarity with sequences from GenBank was above 97%; the remaining 92% of the sequences showed sequence similarity lower than 97%. Despite such high-sequence dissimilarity levels, all Mre sequences obtained in this study clustered together with the ones previously retrieved from Glomeromycota spore collection and liverworts-associated AMF. It is additionally possible that the stringent chimera removal excluded some non-chimeric sequences. However, this allowed us to enhance our phylogenetic resolution beyond what was presented in previous studies (Naumann *et al.*, 2010; Desirò *et al.*, 2013). As a result, we could recognize at least two distinct well-supported Mre clades, here identified as Mre group A and Mre group B. However, due to high level of sequence divergence between Mre sequences clustering in the same Mre group, we hypothesize that these newly described groups can mask other still hidden clades.

Genetic and lifestyle features of endobacteria are not affected by their co-occurrence

Our present study is the first one to describe in a single fungal host the coexistence of two distinct bacterial endosymbionts. Until now, these two symbionts have been studied in isolation from each other. We found that the morphological characteristics of the two co-existing bacterial endosymbionts did not differ from those described previously in the samples where only one bacterial symbiont was present. For example, even when sharing the same cell volume, *Ca*Gg remained enclosed in a vacuole-like structure, whereas Mre were embedded directly in the cytoplasm.

Interestingly, the spore samples that we investigated showed different patterns of intersymbiont dynamics. For example, in the sample CM50 a single Mre phylotype leading to a single OTU was detected together with the homogenous *Ca*Gg population. In contrast, in the remaining samples, Mre showed high levels of nucleotide diversity and sequence divergence. It would be useful to explore which of these two scenarios is more recent and which is more evolutionarily stable.

Irrespective of the dynamic levels of Mre sequence similarity in different samples, FISH and molecular quantitative analysis revealed that Mre were unambiguously more abundant than *Ca*Gg. The stronger presence of the Mre, together with their high variability, may indicate that they are stronger colonizers of AMF. On the basis of their 16S rDNA phylogeny, Mre have been described as related to Mollicutes (Naumann *et al.*, 2010), a bacterial group that clusters with microbes (that is, *Mycoplasma*) thriving inside many eukaryotic hosts and manipulating host development, thanks to the release of effector proteins (Sugio *et al.*, 2011). Due to their capacity to interact with many AMF host genotypes, we hypothesize that Mre have been one of the factors shaping AMF evolution and/or their ecological success.

Similarities between endosymbionts of insects and AMF

The wealth of natural history and molecular evolution data available for heritable endosymbionts of insects make them into an excellent model for understanding symbiotic associations that involve vertically transmitted endobacteria. In addition to essential endosymbionts, insects can support complex communities of bacteria that include non-essential endosymbionts as well as reproductive manipulators (Moran *et al.*, 2008). Essential endosymbionts show strict vertical transmission and functional complementation with their hosts

resulting from millions of years of reciprocal selection (McCutcheon and Moran, 2010). The genomes of essential endosymbionts are usually highly reduced (McCutcheon and Moran, 2010; McFall-Ngai *et al.*, 2013). In this context, *Buchnera aphidicola* is a paradigm for primary endosymbionts. *Buchnera*'s association with aphids is ancient, being approximately 200 million years old and revolves around the endosymbiont's capacity to synthesize essential amino acids for its host (van Ham *et al.*, 2003). Due to their pleiotropic effects on their hosts, the situation is not so clear-cut for the non-essential (secondary) endosymbionts, as their transmission may be both vertical and horizontal and the ratio between cost and benefits strictly depends on environmental conditions (Ferrari and Vavre, 2011).

Given our observations that a single cell (a spore) of a fungus can host endosymbionts with distinct characteristics, it is worth considering whether the biological features of these fungal endobacteria are comparable to those of endosymbionts of insects.

In the case of *Ca*Gg, one of its hosts, *Gigaspora margarita*, can survive and multiply in the absence of the endobacterium (Lumini *et al.*, 2007), and there are natural *Ca*Gg-free isolates of Gigasporaceae (Mondo *et al.*, 2012), demonstrating that this symbiosis is facultative for the host. However, the fungal fitness can be strongly reduced by removal of the endobacteria (Lumini *et al.*, 2007; P Bonfante and M Novero, personal communication, 2013). In addition, by using codiverging partner pairs, Mondo *et al.* (2012) demonstrated that this fungal/bacterial association is ancient (at least 400 million years old) and evolutionarily stable. Analysis of the 1.72 Mb *Ca*Gg genome (Ghignone *et al.*, 2012) revealed that it is reduced when compared with the free-living related *Burkholderia* species, and that the metabolic profile of *Ca*Gg unambiguously clusters with insect endobacteria, including essential endosymbionts like *Buchnera* and *Wigglesworthia* (Moran *et al.*, 2008). These data suggest that *Ca*Gg has undergone functional convergent evolution with phylogenetically distant endobacteria. However, genome annotation also shows functional similarities with the secondary non-essential symbionts (for example *H. defensa*). On the basis of these considerations, we concluded that *Ca*Gg is an obligate intracellular symbiont, characterized by a genetic mosaic where determinants for different nutritional strategies are integrated in a reduced genome (Ghignone *et al.*, 2012). Collectively, its life history features (that is, a strict vertical transmission) as well as molecular evolution and genomic features seem to share patterns from both essential and non-essential endosymbionts of insects.

While the knowledge of the Mre biology is too limited to advance any hypothesis concerning their impact on the host biology, Mre relatedness to *Mycoplasma* and *Phytoplasma*, which are widespread parasites of animals and plants, might explain the colonization capacities of Mre, irrespectively of their role in the fungal hosts. On the other hand, it cannot be excluded that they are beneficial associates of fungi, akin to *Spiroplasma* endosymbionts that protect their insect hosts from the parasitoid pressure (Xie *et al.*, 2010). Consequently, taking into consideration the limited available empirical evidence, we conclude that classifying Mre into categories established for bacterial associates of insects is not yet possible.

Are endobacteria favoured by coenocytic hyphae?

In the rapidly evolving taxonomic classification of Glomeromycota (Redecker *et al.*, 2013), the taxon named Gigasporaceae identifies a group of AMF with distinct features of spore morphology (size, wall layering, bulbous base, germination shield) and host root colonization patterns (lack of intraradical vesicles and formation of auxiliary cells). In addition, this lineage of Glomeromycota turns out to be a preferential niche for endobacteria. Our present results confirm previous analyses (Bianciotto *et al.*, 1996, 2000, 2003; Mondo *et al.*, 2012) that demonstrated a strict association of *Ca*Gg with the Gigasporaceae. In contrast, Mre are widespread; they have been found in both basal and more recently evolved Glomeromycota taxa (Naumann *et al.*, 2010). This differential distribution pattern is one of the key distinctions between the two groups of endosymbionts.

Our present results clearly demonstrate that *Gigaspora margarita* can harbor both endosymbionts, *Ca*Gg and Mre, and this is probably true also for other Gigasporaceae taxa (A Desirò and GA da Silva, personal communication, 2013). The underlying mechanisms responsible for the propensity of Gigasporaceae to host endobacteria are unknown. However, the genome sequence of the *Ca*Gg (Ghignone *et al.*, 2012) shows that this bacterium is metabolically dependent on its fungal host. Perhaps only Gigasporaceae with their relatively large spores, which are rich in reserves of glycogen, fats and proteins (Bonfante *et al.*, 1994), can support the energetic cost of complex bacterial communities, which thrive inside a protected niche.

There is, however, increasing evidence that *Mortierella* species (Mucoromycotina) host endobacteria that are related to *Ca*Gg (Sato *et al.*, 2010; Kai *et al.*, 2012; Bonito *et al.*, 2013). These data open a novel interesting scenario: fungal endobacteria might prefer coenocytic hyphae. The absence of transverse septa may facilitate bacterial movement across the fungal mycelium, as observed in *Rhizopus microsporus* (Mucoromycotina) (Partida-Martinez and Hertweck, 2005). Recently, mitochondrial (Lee and Young, 2009; Pelin *et al.*, 2012) and nuclear (Martin, 2012) genome analyses suggested that Mucoromycotina, instead of Dikarya, is the sister group of Glomeromycota. In this context, our data

provide an additional evidence of the relationship between the two fungal lineages. The pattern of endosymbiont distribution across lineages of closely related fungal hosts raises questions about the role of symbiosis in the evolution and diversification of these fungal taxa and their associated endobacteria.

Conclusion

Our investigation has revealed for the first time that a single spore of an AMF can harbor multiple bacterial endosymbionts that represent phylogenetically diverse groups and show distinct patterns of sequence evolution. Both endosymbionts seem to retain their genetic and lifestyle peculiarities regardless of whether they colonize the host alone or together. Mre population consistently appears to be more abundant, variable and prone to recombination events than the *Ca*Gg one, suggesting that the same niche (the fungal spore) exerts a different selection pressure on its dwellers.

Our findings showing that a single fungal cell can harbor an intracellular bacterial microbiome, raise novel questions concerning molecular, cellular and metabolic interactions resulting from such complex inter-domain relationships.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank Andrea Genre (DBIOS, UNITO) for his invaluable assistance in confocal microscopy and image acquisition; Gladstone Alves da Silva (Mycology Department, Pernanbuco University) for the morphological identification of the spores; Yukari Kuga (Agriculture Faculty, Shinshu University) for her suggestions in the endobacterial ultrastructure; Stefano Ghignone and Olivier Friard (IPP-CNR and DBIOS, UNITO) for their assistance in bioinformatics; the Genebank project of NIAS (Japan) for providing the MAFF520054 isolate. Research was funded by the Ateneo Project (ex 60%) to PB.

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