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Detection of a novel intracellular microbiome hosted in arbuscular mycorrhizal fungi

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1 **Detection of a novel intracellular microbiome hosted in arbuscular mycorrhizal**
2 **fungi**

3

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22 ***Abstract***

23 Arbuscular mycorrhizal fungi (AMF) are important members of the plant microbiome.

24 They are obligate biotrophs that colonize the roots of most land plants and enhance

25 host nutrient acquisition. Many AMF themselves harbor endobacteria in their hyphae

26 and spores. Two types of endobacteria are known in Glomeromycota: rod-shaped

1 Gram-negative *Candidatus* Glomeribacter gigasporarum, *CaGg*, limited in distribution
2 to members of the order Gigasporales, and coccoid *Mollicutes*-related endobacteria,
3 *Mre*, widely distributed across different lineages of AMF. The goal of the present
4 study is to investigate the patterns of distribution and coexistence of the two
5 endosymbionts, *CaGg* and *Mre*, in spore samples of one host species, *Gigaspora*
6 *margarita*. Based on previous observations, we hypothesized that some AMF could
7 host populations of both endobacteria. To test this hypothesis we performed an
8 extensive investigation of both endosymbionts in *G. margarita* spores sampled from
9 Cameroonian soils as well as in the Japanese *G. margarita* MAFF520054 isolate
10 using different approaches (molecular phylotyping, electron microscopy, fluorescence
11 *in situ* hybridization, and quantitative real-time PCR). We found that a single AMF
12 host can harbour both types of endobacteria, with *Mre* population being more
13 abundant, variable and prone to recombination than the *CaGg* one. Both
14 endosymbionts seem to retain their genetic and lifestyle peculiarities regardless of
15 whether they colonize the host alone or together. These findings show for the first
16 time that fungi, as other eukaryotic hosts, support complex intracellular bacterial
17 microbiomes, in which distinct types of endobacteria coexist in a single cell.

18

19 **Subject Category:** Microbial population and community ecology

20

21 **Keywords:** arbuscular mycorrhizal fungi; bacterial recombination, fungal microbiome;
22 endobacteria; fluorescent *in situ* hybridization; phylogenetic analysis; quantitative
23 real-time PCR

24

25

1 **Introduction**

2 The discovery that the human body can be described as a complex ecosystem where
3 human cells interact with trillions of bacteria and other microbes has represented a
4 scientific revolution. The human microbiome, *i.e.* the microbial communities and the
5 genetic information they contain, cooperate with the human genome to regulate
6 crucial physiological processes ranging from digestion to obesity and immunity
7 (Methé *et al.*, 2012). Similarly, plants rely on microorganisms living both in their
8 tissues and in the rhizosphere (Porrás-Alfaro & Bayman, 2011; Berendsen *et al.*,
9 2012, Lundberg *et al.*, 2012; Bulgarelli *et al.*, 2012). Due to their interdependence
10 and mutual impact on each other's biology, plants and their microbiomes can be
11 viewed as “super-organisms”. To date, most of the work on plant-associated
12 microbes focused almost exclusively on bacteria (Lundberg *et al.*, 2012; Bulgarelli *et*
13 *al.*, 2012), even though eukaryotes such as fungi are also crucial components of the
14 plant microbiome. They not only thrive in the rhizosphere, but also colonize plant
15 tissues, exhibiting a range of lifestyles, including mutualism, parasitism and
16 commensalism (Porrás-Alfaro & Bayman, 2011).

17 Among plant-associated microbiota, arbuscular mycorrhizal fungi (AMF) are
18 the most widespread: they belong to an ancient monophyletic phylum, the
19 Glomeromycota (Schüßler *et al.*, 2001), and play a key role in nutrient cycling and
20 plant health due to their capacity for improving the mineral nutrition of plants (Smith &
21 Read, 2008). AMF display many unusual biological features. In addition to their
22 obligate biotrophy (Bonfante & Genre, 2010), many of them harbor endobacteria in
23 their cytoplasm (Bonfante & Anca, 2009). Bacterial endosymbionts are widespread
24 among animals (Wernegreen *et al.*, 2012; McFall-Ngai *et al.*, 2013), and in particular
25 the ones living in insect tissues have been investigated in depth (Ferrari & Vavre,

1 2011). In contrast, examples of endobacteria living inside fungal cells are much more
2 limited (Bianciotto *et al.*, 2003; Partida-Martinez & Hertweck, 2005; Lackner *et al.*,
3 2009; Naumann *et al.*, 2010; Kai *et al.*, 2012).

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

14 The *CaGg* genome sequence (Ghignone *et al.*, 2012) revealed that
15 Glomeribacter endobacteria are nutritionally dependent on the fungal host and have
16 a possible role in providing the fungus with essential factors like vitamin B12
17 (Ghignone *et al.*, 2012). Phenotypic consequences of *CaGg* removal from the host
18 include important morphological changes as well as reduced proliferation of host
19 presymbiotic hyphae. Yet, the host is not obligately dependent on the bacteria
20 (Lumini *et al.*, 2007; Mondo *et al.*, 2012). These features suggest that Glomeribacter
21 endobacteria are mutualistic associates of AMF (Lumini *et al.*, 2007). Comparisons of
22 host and symbiont phylogenies indicate that, while *CaGg* is a heritable endosymbiont
23 (Bianciotto *et al.*, 2004), it also engages in recombination and host switching, which
24 play an important role in stabilizing this 400-million-year-old association (Mondo *et*
25 *al.*, 2012). In contrast, information on the coccoid *Mre* is much more limited. Based

1 on the 16S rRNA gene sequences, this novel lineage is sister to a clade
2 encompassing the Mycoplasmatales and Entomoplasmatales (Naumann *et al.*,
3 2010). The *Mre* have been detected in 17 out of 28 investigated AMF samples from
4 culture collections, including members of Archaeosporales, Diversisporales,
5 Gigasporales and Glomerales (Naumann *et al.*, 2010), as well as in mycorrhizal thalli
6 of liverworts (Desirò *et al.*, 2013). In most of the AMF hosts and irrespectively of the
7 AMF identity, these endobacteria displayed a conspicuous variability in their 16S
8 rRNA gene sequence. Collectively, these observations indicate that *CaGg* is a stable
9 associate of Gigasporales, while the lifestyle of the *Mre* and the nature of their
10 association with Glomeromycota are uncertain. Furthermore, the interaction between
11 the two endosymbionts remains unclear, *i.e.* it is not known whether the presence of
12 one endosymbiont in the host leads to the exclusion of the other one.

13 The goal of the present study is to investigate the patterns of distribution and
14 coexistence of the two endosymbionts, *CaGg* and *Mre*, in isolates of one host
15 species, *Gigaspora margarita* W.N. Becker & I.R. Hall. Previous electron microscopy
16 observations revealed that the strain of *G. margarita* MAFF520054 harboured a
17 Gram-positive-like endobacterium (Kuga *et al.*, 2008), while molecular analysis
18 indicated the presence of *CaGg* (E. Lumini, personal communication, ref. seq.
19 AM886455). Based on these observations, we hypothesize that some AMF could
20 host populations of both endobacteria. To test this hypothesis we performed an
21 extensive investigation of both endosymbionts in *G. margarita* spores sampled from
22 Cameroonian soils as well as in *G. margarita* MAFF520054 from Japan using
23 different approaches. We found that a single AMF host can harbour both types of
24 endobacteria, with *Mre* populations being more abundant, variable and prone to
25 recombination than the *CaGg* ones. These findings show for the first time that fungi,

1 as other eukaryotic hosts, support complex microbiomes, in which distinct types of
2 endobacteria coexist in a single cell.

3

4 ***Materials and methods***

5

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

8

9 ***Sampling and sample preparation***

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

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

19

20 ***DNA extraction, amplification, and clone library analysis***

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

1 The *CaGg* 16S rRNA gene was specifically amplified with the newly
2 designed primers *CaGgADf* (5'-AGATTGAACGCTGGCGGCAT-3') and
3 *CaGgADr* (5'-ATGCGTCCTACCGTGGCCATC-3'), while the *Mre* 16S rRNA
4 gene was amplified as described in Desirò *et al.* (2013).

5 Fungal and bacterial PCR amplicons were then cloned and transformed.

6

7 *Bioinformatic analyses*

8 Sequences were assembled and curated in Mega (Tamura *et al.*, 2011),
9 aligned with MAFFT (Katoh *et al.*, 2002) or MUSCLE (Edgar, 2004), and then
10 examined for chimerism. Sequence similarity/divergence was evaluated using
11 MOTHUR (Schloss *et al.*, 2009). Nucleotide diversity (π) was calculated in
12 DNAsp v. 5.10.01 (Librado & Rozas, 2009). The *CaGg* and *Mre* 16S rRNA
13 gene sequences were grouped into operational taxonomic units (OTUs) at the
14 cutoff of 0.03 genetic distance value using MOTHUR. Phylogenetic analyses
15 were conducted using one representative sequence for each OTU. The
16 Genetic Algorithm for Recombination Detection, GARD (Kosakovsky Pond *et al.*
17 *et al.*, 2006), was used to identify recombination breakpoints in 16S rRNA genes
18 of *CaGg* and *Mre*. Alignments and trees are available in TreeBASE
19 (submissions XXXX) (Piel *et al.*, 2002). Representative DNA sequences are in
20 GenBank (XXXX).

21

22 *Ultrastructural analyses*

23 Single *G. margarita* spores from CM23 and CM 47 samples were processed
24 by using high-pressure-freezing followed by freeze-substitution. Single spores
25 floating in water were transferred in the cavity of an aluminium carrier with a

1 pipette. Excess of water was drawn off with filter paper and the space was
2 filled with 1-Hexadecene. The sandwich was completed with a flat specimen
3 carrier and frozen in a HPM 100 high-pressure freezing machine (Leica
4 Microsystems, Wetzlar, Germany) (McDonald *et al.*, 2010). Samples were
5 then freeze-substituted, resin embedded, and processed for transmission
6 electron microscopy.

7

8 *FISH experiments and Confocal Microscopy*

9 Sterilized spores of the samples CM23, CM47, CM50, CM52 and *G. margarita*
10 BEG34 were fixed as described in Naumann *et al.*, (2010). The *Mre* specific
11 probe BLOsADf2 (Desirò *et al.*, 2013), together with a newly designed specific
12 *CaGg* 16S rRNA probe (*CaGgADf1* 5'-CTATCCCCCTCTACAGGAYAC-3'),
13 were used to label the endobacteria. In addition, the eubacterial probe
14 EUB338 (Amann *et al.*, 1990) and the *Buchnera*-specific probe ApisP2a
15 (Koga *et al.*, 2003) were used. Spores were observed using a Leica TCS-SP2
16 confocal microscope (Leica Microsystems).

17

18 *Quantification of the bacterial populations*

19 The sample CM23 (containing both *Mre* and *CaGg*) was selected for the
20 relative quantification of the two bacterial populations by real-time qPCR.
21 Briefly, the 16S rDNA gene sequences obtained for both *CaGg* and *Mre* were
22 used to design two distinct qPCR primer pairs. Template plasmids containing
23 the target DNA sequences were constructed to generate a standard curve as
24 an external standard. The number of target DNA sequences present in each

1 PCR mixture was calculated by comparing the crossing points of the samples
2 with those of the standards.

3

4 **Results**

5

6 *Identity of AMF*

7 To confirm the morphological identification of AMF originating from Cameroon and
8 Japan as *Gigaspora margarita*, we analysed their 18S, 28S and ITS rRNA gene
9 regions. These analyses revealed that all the fungi could be identified as *G. margarita*
10 (Figures 1 and S3). As expected, the 18S rRNA gene analysis led to an unresolved,
11 polytomic phylogeny (not shown), while a better resolution was provided by the 28S
12 rRNA gene (Figure 1) and the ITS region (Figure S3).

13

14 *Identity of endobacteria*

15 Bacterial 16S rRNA gene sequences were PCR-amplified from single AMF
16 spores using primers specific for *CaGg* and *Mre* (Naumann *et al.*, 2010) to
17 detect endosymbiont presence. Most samples harboured both types of
18 endobacteria with the exception of the *G. margarita* samples CM3 and CM52,
19 which contained only *Mre* (Table 1). The absence of *CaGg* in the samples
20 CM3 and CM52 was confirmed by real-time qPCR (data not shown), which
21 can detect up to ten bacterial cells (Salvioli *et al.*, 2008).

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

1 The RFLP analyses of *CaGg* 16S rRNA gene sequences revealed a
2 single RFLP profile for each 10-spores sample, suggesting a limited
3 intrasample variability, which was further confirmed by sequence analyses.
4 The obtained sequences were grouped into OTUs at 97% of sequence
5 similarity and, as expected, a single OTU for each sample was obtained
6 (Table 2). Phylogenetic analyses of *CaGg* sequences retrieved from spore
7 samples showed that they clustered with other *CaGg* sequences available in
8 GenBank (Figure 2).

9 Sequencing of the *Mre* 16S rRNA gene clones generated a total of 118
10 sequences (Table 3). To eliminate potential PCR artefacts expected in
11 amplifications from complex templates such as *Mre* populations (Naumann *et*
12 *al.*, 2010), the obtained sequences were submitted to a rigorous chimera
13 screen, which reduced the total amount to 52 sequences (Table 3). They were
14 grouped into OTUs at 97% sequence similarity (Table 3). Most of the
15 sequences (48 out of 52) showed sequence similarity values lower than 97%
16 when compared to the *Mre* sequences obtained from GenBank, suggesting
17 the presence of novel phylotypes (Table 3).

18 Despite the high variability, all retrieved *Mre* sequences clustered
19 together with those obtained in previous studies (Naumann *et al.*, 2010;
20 Desirò *et al.*, 2013) (Figure 3). Moreover, because the resulting phylogenies
21 presented here are better supported and resolved than those constructed in
22 previous works (Naumann *et al.*, 2010; Desirò *et al.*, 2013), we conclude that
23 there are at least two distinct and well supported *Mre* clades, identified as *Mre*
24 group A and *Mre* group B (Figure 3), and that the level of sequence
25 divergence among sequences clustering in the same *Mre* group reached up to

1 15 and 16% in *Mre* group A and B, respectively. Overall, in all the samples,
2 with the only exception of CM50, *CaGg* showed a high level of intra-host
3 sequence similarity, whereas *Mre* revealed high levels of intra-host sequence
4 diversity.

5

6 *Recombination detection*

7 To explore the underlying causes of differences in sequence evolution
8 patterns between *CaGg* and *Mre*, we used GARD (Kosakovsky Pond *et al.*,
9 2006) to look for evidence of recombination in 16S rRNA genes of the two
10 endosymbionts associated with AMF from Cameroon and Japan. No evidence
11 of recombination was detected in the *CaGg* sequences. In contrast, in the *Mre*
12 dataset, we found that the AIC_C score of 8529.9 for the best-fitting model
13 allowing for different topologies of the alignment segments defined by
14 recombination breakpoints was lower than the AIC_C score of 8819.4 for the
15 model that assumed the same topology for all segments, indicating that a
16 multiple tree model is preferable over a single tree model. Using the KH test,
17 one breakpoint at the alignment position 479 was identified as resulting in
18 significant topological incongruence between segments ($P < 0.001$, Figure
19 S4).

20

21 *Localization of the two bacterial morphotypes in AMF cells: high* 22 *pressure/freeze-substitution and transmission electron microscopy*

23 We used electron microscopy to confirm the cytoplasmic location of both
24 types of endobacteria. To ensure proper preservation of endosymbiont cells
25 and fungal organelles, which could be jeopardized by the very thick fungal cell

1 wall (12-16 μm , Lumini *et al.*, 2007), we used high pressure and freeze-
2 substitution specimen preparation. On the basis of the previous molecular
3 analysis, two isolates of *G. margarita* (CM23 and CM47) were selected for this
4 experiment. When inspected under the electron microscope, CM23 and CM47
5 presented both the rod-shaped and coccoid bacteria in the same area of their
6 cytoplasm (Figure 4). The rod-shaped *CaGg* were 330-550 x 960-1050 nm in
7 size, with a layered, Gram-negative type cell wall (Figure 4A, B) and were
8 located inside a vacuole-like organelle (Figure 4A), consistent with reports
9 from earlier studies (Bianciotto *et al.*, 1996, 2003). The vacuole revealed an
10 electron dense matrix, which was identified as of protein origin (Bonfante *et*
11 *al.*, 1994) (Figure 4A). In other cases, the matrix was reduced in size and the
12 bacterium was more closely surrounded by the membrane of fungal origin
13 (Figure 4B). In contrast, the coccoid *Mre*s were directly embedded in the
14 fungal cytoplasm (Figure 4A, C). They were consistently smaller, 300-600 nm
15 in size, with a homogeneous, Gram-positive-like cell wall (Figure 4C).

16

17 *Localization of the two endosymbionts in AMF spores: FISH*

18 To further validate our molecular and morphological observations of the *CaGg*
19 and *Mre* coexistence in *G. margarita*, we performed fluorescence *in situ*
20 hybridization (FISH) experiments in samples CM23, CM47, CM50, and CM52.
21 *G. margarita* BEG34 was used as negative control, since *Mre* have never
22 been found in this isolate (Naumann *et al.*, 2010). We used two probes:
23 *CaGgADf1*, which was designed to specifically detect *CaGg*, and *BLOsADf2*
24 (Desirò *et al.*, 2013), which targeted entire *Mre* variability contained in our
25 spore samples. In agreement with PCR results, we did not observe any *CaGg*

1 signal in CM52, where *CaGg* have never been detected by PCR-amplification
2 of 16S rRNA gene. Similarly, we did not observe any *Mre* signals in BEG34.
3 On the contrary, the two specific probes produced simultaneous FISH signals
4 in the spores where the presence of both bacterial types was expected
5 (Figures 5 and 6). The number of fluorescent signals suggested a more
6 abundant presence of *Mre* than *CaGg* in the spores with both types of
7 bacteria. The fluorescent signals were located in the fungal cytoplasm and
8 never on the spore surface. Importantly, the fluorescent signal of the probes
9 BLOsADf2 (Desirò *et al.*, 2013) and *CaGgADf1* were always co-localized with
10 the fluorescence given by the general bacterial probe EUB338 (Amann *et al.*,
11 1990) (Fig. 5). No fluorescent signal was detected with the negative control
12 probe ApisP2a (Koga *et al.*, 2005) (Fig. 6E). Pre-treatment with RNase, as
13 well as control hybridization with nonsense probes, did not provide any FISH
14 signal. A weak autofluorescence of the fungal cytoplasm, probably deriving to
15 the use of aldehydic fixatives, was visible in all spore samples. Hence, FISH
16 experiments, validating the PCR results, confirmed the simultaneous
17 presence of *Mre* and *CaGg* in some *G. margarita* samples.

18

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

20 To further examine differences in *Mre* and *CaGg* abundance suggested by
21 FISH experiments, we used real-time quantitative PCR (qPCR) to quantify the
22 bacterial populations present in the *G. margarita* sample CM23 that was
23 previously shown to contain both *Mre* and *CaGg* endobacteria. The 16S rRNA
24 gene was used as a target gene, but while in the *CaGg* genome the 16S
25 rRNA gene is present in a single copy (Ghignone *et al.*, 2012), in *Mre* one or

1 at most two rRNA gene copies are expected based on the comparison with
2 the closest microbes already sequenced (Fraser *et al.*, 1995; Glass *et al.*,
3 2000; Jaffe *et al.*, 2004; Minion *et al.*, 2004; Vasconcelos *et al.*, 2005; Bai *et*
4 *al.*, 2006).

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

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

19

20 **Discussion**

21 A combination of morphological, molecular, and phylogenetic analyses
22 demonstrates that *Gigaspora margarita* spores host a complex microbiome
23 consisting of rod-shaped and coccoid bacteria. The two bacterial groups are
24 very distinct not only in their phylogenetic placement, *i.e.* *Candidatus*
25 *Glomeribacter gigasporarum* is closely related to Burkholderiaceae, while the

1 coccoid endobacteria are related to the Gram-positive *Mollicutes*, but also in
2 their genetic features.

3

4 *Sharing the same host and revealing intra-host diversity*

5 Notwithstanding the endobacteria share the same fungal host, a relevant
6 difference in genetic diversity patterns between them was revealed. While
7 *CaGg* shows a high level of intra-host sequence similarity, the *Mre* are
8 characterized by high levels of intra-host sequence diversity. One of the
9 underlying causes of differences in sequence evolution patterns between
10 *CaGg* and *Mre* may be differences in their lifestyle. For example, in *Mre*, we
11 found evidence of recombination, which was not apparent in *CaGg*. This
12 finding was supported by some genomic features of *CaGg* genome:
13 notwithstanding its high repetitive DNA (15%), *CaGg* contains a low number of
14 active insertion sequences, which are considered important determinants for
15 recombination (Ghignone *et al.*, 2012). Indeed, a recent study of *CaGg*, using
16 a set of four marker genes, revealed that recombination is not entirely absent
17 from the *CaGg* evolutionary history and, together with host switching, may
18 play an important role in evolutionary stability of *CaGg* association with
19 Glomeromycota (Mondo *et al.*, 2012). Detecting evidence of recombination in
20 a single gene of *Mre* sampled in the present study may suggest that *Mre*
21 engage in more frequent recombination than *CaGg*. Interestingly, cryptic
22 prophage remnants have been detected in the genome of the *Mre*-related
23 phytoplasma, leading to the suggestions that these genetic elements may
24 have played important roles in generating phytoplasma genetic diversity (Wei
25 *et al.*, 2008).

1

2 *Phylogenetic divergence patterns of the co-existing endobacteria*

3 The extensive phylogenetic analysis performed on the endobacteria thriving in
4 the cytoplasm of five spore samples and their comparison with data from
5 previous investigations (Bianciotto *et al.*, 1996, 2000, 2003; Mondo *et al.*,
6 2012) confirmed that the 16S rRNA gene sequences of CaGg were relatively
7 conserved, irrespectively of the geographic origin of the fungal host. However,
8 our careful analyses showed that the sequence similarity between CaGg from
9 *G. margarita* MAFF520054 isolate and the already sequenced CaGg from *G.*
10 *margarita* BEG34 was below the critical level of 97%. In fact, although this
11 distinction is controversial (Rossello-Mora, 2003), it is generally accepted that
12 sequences with similarity greater than 97% are typically assigned to the same
13 species and those with similarity greater than 95% to the same genus
14 (Stackebrandt & Goebel, 1994; Everett *et al.*, 1999; Gevers *et al.*, 2005).
15 Consequently, further work is needed to resolve whether CaGg from *G.*
16 *margarita* MAFF520054 and *G. margarita* BEG34, which show sequence
17 similarity lower than 97% and a different location inside the CaGg
18 phylogenetic tree, represent distinct taxa.

19 In contrast to CaGg and despite the stringent removal of chimeric
20 sequences, the 16S rRNA gene sequences of *Mollicutes*-related endobacteria
21 turned out to be highly variable inside at least four out of five spore samples.
22 Moreover, in only 8% of the sequences generated in this study (4 out of 52),
23 the similarity with sequences from GenBank was above 97%; the remaining
24 92% of the sequences showed sequence similarity lower than 97%. Despite
25 such high sequence dissimilarity levels, all *Mre* sequences obtained in this

1 study clustered together with the ones previously retrieved from
2 Glomeromycota spore collection and liverworts-associated AMF. It is
3 additionally possible that the stringent chimera removal excluded some non-
4 chimeric sequences. However, this allowed us to enhance our phylogenetic
5 resolution beyond what was presented in previous studies (Naumann *et al.*,
6 2012; Desirò *et al.*, 2013). As a result, we could recognize at least two distinct
7 well supported *Mre* clades, here identified as *Mre* group A and *Mre* group B.
8 However, due to high level of sequence divergence between *Mre* sequences
9 clustering in the same *Mre* group, we hypothesize that these newly described
10 groups can mask other still hidden clades.

11

12 *Morphological aspects of endobacteria are not affected by their co-*
13 *occurrence*

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

22 Interestingly, the spore samples that we investigated showed different
23 patterns of intersymbiont dynamics. For example, in the sample CM50, only
24 one *Mre* phylotype revealed high values of sequence similarity and
25 consequently a limited number of *Mre* single OTUs was detected together with

1 the homogenous CaGg population. In contrast, in the remaining samples, *Mre*
2 showed higher levels of nucleotide diversity and sequence divergence. It
3 would be useful to explore which of these two scenarios is more recent and
4 which is more evolutionarily stable.

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

16

17 *Similarities between endosymbionts of insects and AMF*

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

1 selection (McCutcheon & Moran, 2010). The genomes of essential
2 endosymbionts are usually highly reduced (McCutcheon & Moran, 2010;
3 McFall-Ngai *et al.*, 2013). In this context, *Buchnera aphidicola* is a paradigm
4 for primary endosymbionts. *Buchnera*'s association with aphids is ancient,
5 being approximately 200 million years old and revolves around the
6 endosymbiont's capacity to synthesize essential amino acids for its host (van
7 Ham *et al.*, 2003). Due to their pleiotropic effects on their hosts, the situation
8 is not so clear-cut for the non-essential (secondary) endosymbionts, since
9 their transmission may be both vertical and horizontal and the ratio between
10 cost and benefits strictly depends on environmental conditions (Ferrari &
11 Vavre, 2011). The effects of secondary symbionts on their host are pleiotropic,
12 but one of the best understood is their action in the so-called tritrophic
13 interactions. For example, the secondary symbiont *Hamiltonella defensa*
14 confers on its aphid host an increased level of resistance towards the
15 parasitoid wasp when compared to the uninfected aphids of the same
16 genotype (Oliver *et al.*, 2003).

17 Reproductive parasites manipulate their insect host reproduction in
18 favour of their own transmission (Engelstädter & Hurst, 2009). Examples of
19 reproductive manipulators include *Wolbachia* and *Rickettsia* (Engelstädter &
20 Hurst, 2009) as well as *Spiroplasma* (Anbutsu & Fukatsu, 2011). Their
21 lifestyles can be highly dynamic showing fast transitions between parasitism
22 and mutualism (Weeks *et al.*, 2007).

23 Given our observations that a single cell (a spore) of a fungus can host
24 endosymbionts with distinct characteristics, it is worth considering whether the

1 biological features of these fungal endobacteria are comparable to those of
2 endosymbionts of insects.

3 In the case of CaGg, one of its hosts, *Gigaspora margarita*, can survive
4 and multiply in the absence of the endobacterium (Lumini *et al.*, 2007), and
5 there are natural CaGg-free isolates of Gigasporales (Mondo *et al.*, 2012),
6 demonstrating that this symbiosis is facultative for the host. However, the
7 fungal fitness can be strongly reduced by removal of the endobacteria (Lumini
8 *et al.*, 2007; P. Bonfante and M. Novero 2013, unpublished data). In addition,
9 by using codiverging partner pairs, Mondo *et al.* (2012) demonstrated that this
10 fungal/bacterial association is ancient (at least 400 million years old) and
11 evolutionarily stable. Analysis of the 1.72 Mb CaGg genome (Ghignone *et al.*,
12 2012) revealed that it is reduced when compared with the free-living related
13 *Burkholderia* species, and that the metabolic profile of CaGg unambiguously
14 clusters with insect endobacteria, including essential endosymbionts like
15 *Buchnera* and *Wigglesworthia* (Moran *et al.*, 2008). These data suggest that
16 CaGg has undergone functional convergent evolution with phylogenetically
17 distant endobacteria. However, genome annotation also shows functional
18 similarities with the secondary non-essential symbionts (for example *H.*
19 *defensa*). On the basis of these considerations, we concluded that CaGg is an
20 obligate intracellular symbiont, characterized by a genetic mosaic where
21 determinants for different nutritional strategies are integrated in a reduced
22 genome (Ghignone *et al.*, 2012). Collectively, its life history features (*i.e.* a
23 strict vertical transmission) as well as molecular evolution and genomic
24 features seem to share patterns from both essential and non-essential
25 endosymbionts of insects.

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

11

12 *Are endobacteria favoured by coenocytic hyphae?*

13 In the rapidly evolving taxonomic classification of Glomeromycota (Redecker
14 *et al.*, 2013), the taxon named Gigasporales (Oehl *et al.*, 2011; da Silva *et al.*,
15 2013) identifies a group of AMF with distinct features of spore morphology
16 (size, wall layering, bulbous base, germination shield) and host root
17 colonization patterns (lack of intraradical vesicles and formation of auxiliary
18 cells). In addition, this lineage of Glomeromycota turns out to be a preferential
19 niche for endobacteria. Our present results confirm previous analyses
20 (Bianciotto *et al.*, 1996, 2000, 2003; Mondo *et al.*, 2012) that demonstrated a
21 strict association of *CaGg* with the Gigasporales. In contrast, the *Mre* are
22 widespread; they have been found in both basal and more recently evolved
23 Glomeromycota taxa (Naumann *et al.*, 2010). This differential distribution
24 pattern is one of the key distinctions between the two groups of
25 endosymbionts.

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

11 There is, however, increasing evidence that *Mortierella* species
12 (Mucoromycotina) host endobacteria that are related to *CaGg* (Sato *et al.*,
13 2010; Kai *et al.*, 2012; Bonito *et al.*, 2013). These data open a novel
14 interesting scenario: fungal endobacteria might prefer coenocytic hyphae. The
15 absence of transverse septa may facilitate bacterial movement across the
16 fungal mycelium, as observed in the *Burkholderia rhizoxinica* endosymbiont of
17 *Rhizopus microsporus* (Partida-Martinez & Hertweck, 2005). In addition, these
18 data support a link between Glomeromycota and Mucoromycotina, which both
19 belong to the group of the basal fungi: this has already been suggested by the
20 similarities in their mitochondrial genomes (Lee & Young, 2009; Pelin *et al.*,
21 2012) and by the assembled genome of *Rhizophagus irregularis* (M.Martin,
22 Ton Bisseling..personal communication). This pattern of endosymbiont
23 distribution across lineages of closely related fungal hosts raises questions
24 about the role of symbiosis in the evolution and diversification of these fungal
25 taxa and their associated endobacteria.

1

2 **Conclusion**

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

11 Our findings showing that a single fungal cell can harbour a complex
12 microbiome, raise novel questions concerning molecular, cellular and
13 metabolic interactions resulting from such complex inter-domain relationships.

14

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25

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2

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15
16 **Figure 1** Phylogenetic placement of Cameroonian and Japanese spore
17 samples inside the Gigasporales tree. The fungal phylogeny was
18 reconstructed using partial 28S rRNA gene sequences. The DNA sequences
19 retrieved in this work are in bold. All the thirteen spore samples are located
20 inside the Gigasporaceae clade, close to *Gigaspora margarita*. Supported
21 values are from Bayesian/maximum likelihood/maximum parsimony analyses.
22 The Bayesian and maximum likelihood analyses were performed with GTR+G
23 nucleotide substitution model. Dashes instead numbers imply that the
24 topology was not supported in the respective analysis.

25
26 **Figure 2** Phylogenetic placement of representative *Candidatus*
27 *Glomeribacter gigasporarum* partial 16S rRNA gene sequences retrieved from
28 spores of AMF. The DNA sequences retrieved in this work are in bold. The
29 tree encompasses several CaGg groups. Sequences from *G. margarita*

1 sample CM47 and CM50 cluster in a group sister to the one (with thickened
2 branches) including *CaGg* from *G. margarita* BEG34 isolate (highlighted in
3 gray) and from the Cameroonian CM21 and CM23 samples. The 16S rRNA
4 gene sequences from the Japanese sample MAFF520054 are located in a
5 different and more basal position inside the tree, together with other *CaGg*
6 sequences retrieved from worldwide *G. margarita* isolates. The number of
7 sequences included in each OTU is in brackets. Cameroonian isolates
8 showed 97-100% sequence similarity with Gigasporales isolates (*i.e.*
9 *Gigaspora decipiens*, *G. gigantea*, *G. margarita*, including the isolate BEG34,
10 *G. rosea*, *Racocetra castanea* and *R. verrucosa*) which are located in the
11 upper part of the tree. By contrast, *CaGg* sequence similarity, in particular of
12 the samples CM47 and CM50, decreased to 96% relative to *CaGg* sequences
13 retrieved from other worldwide isolates of *Cetraspora pellucida* and *G.*
14 *margarita*, including the *G. margarita* isolate MAFF520054. Supported values
15 are from maximum likelihood/Bayesian/maximum parsimony analyses. The
16 maximum likelihood and Bayesian analyses were performed with GTR+G and
17 TIM3+G nucleotide substitution models, respectively. Dashes instead
18 numbers imply that the topology was not supported in the respective analysis.

19

20 **Figure 3** Phylogenetic placement of representative *Mollicutes*-related
21 endobacteria partial 16S rRNA gene sequences retrieved from AM spores
22 within the *Mollicutes* clade. The DNA sequences retrieved in this work are in
23 bold. The tree encompasses at least two main and well supported groups
24 (*Mre* group A and B) which also include sequences retrieved in previous
25 experiments from AM spore collection (Naumann *et al.*, 2010) and AMF

1 liverworts-associated (Desirò *et al.*, 2013). The number of sequences included
2 in each OTU is in brackets. Supported values are from Bayesian/maximum
3 likelihood/maximum parsimony analyses. The Bayesian and maximum
4 analyses were performed with GTR+G nucleotide substitution model. Dashes
5 instead numbers imply that the topology was not supported in the respective
6 analysis.

7

8 **Figure 4** Electron microscopy of *Gigaspora margarita* sample CM23. (A) The
9 two bacterial types, *CaGg* (arrow) and *Mre* (arrowhead) are present in the
10 same district of the sporal fungal cytoplasm (fc). The rod-shaped type is
11 constantly located inside a vacuole-like organelle (v). The vacuole reveals an
12 electron dense matrix (m), identified as of protein origin. (B) Sometimes *CaGg*
13 (here cut in a transversal section) is more closely surrounded by the
14 membrane of fungal origin (arrow). (C) The *Mre* is directly embedded in the
15 fungal cytoplasm. Scale bars: (A) 1,5 μm ; (B) 0,26 μm ; (C) 0,17 μm .

16

17 **Figure 5** FISH on a crushed spore of *Gigaspora margarita* sample CM21. (A)
18 Bright-field image of the fungal cytoplasm (fc) trapped in a drop of agarose is
19 shown. (B) Triple labelling of the endobacteria with the *Mre*-specific probe
20 BLOsADf2 (red), the *CaGg*-specific probe *CaGcADf1* (blue) and the
21 eubacterial-probe EUB338 (green); bacteria are seen as coccoid or rod-
22 shaped fluorescent spots (arrowheads); in this image, where red and green or
23 blue and green channels are overlaid, bacteria are visualized as fluorescent
24 orange or light blue spots inside the brown cytoplasm. The corresponding red,
25 blue and green channels are shown in C, D and E. The insets show the

1 magnification of some *Mre* and *CaGg* cells surrounded by the fungal
2 cytoplasm. Scale bars: 12 μm , 3 μm in the insets.

3

4 **Figure 6** FISH on a crushed spore of *Gigaspora margarita* sample CM23. (A)
5 Bright-field image of the fungal cytoplasm (fc) trapped in a drop of agarose is
6 shown. (B) Triple labelling of the endobacteria with the *Mre*-specific probe
7 BLOsADf2 (red), the *CaGg*-specific probe CaGcADf1 (blue) and the
8 *Buchnera*-specific probe ApisP2a (green) used as negative control; bacteria
9 are seen as coccoid or rod-shaped fluorescent spots (arrowheads). The
10 corresponding red and blue channels are shown in C and D. (E) No presence
11 of non-specific fluorescent signal is detected. The insets show the
12 magnification of some *Mre* and *CaGg* cells surrounded by the fungal
13 cytoplasm. Scale bars: 8 μm , 3 μm in the insets.

14

15 **Figure S1** Serial dilutions of the standard plasmids were used in individual
16 real-time qPCR to generate standard curves for the *CaGg* and *Mre* 16S rRNA
17 genes. The R^2 values, efficiencies and slopes are shown for each reaction.

18

19 **Figure S2** Melting curve analysis of the fragments obtained by real-time q-
20 PCR in the following conditions: (A) *Gigaspora margarita* CM23 spore DNA
21 amplified with the *CaGgAD* primer pair; (B) Individual plasmids carrying the
22 three variants of the *Mre* 16S rDNA amplified with the CMsAD primer pair; (C)
23 *G. margarita* CM23 spore DNA amplified with the *Mre* specific primer pair. The
24 amplification of the sporal DNA with *CaGg* primers originated a unique
25 specific melting peak. As far as the *Mollicutes*-specific amplification is

1 concerned, the melting analysis showed that all the three variants of the *Mre*
2 16S rDNA could be amplified with a single primer pair, and that such different
3 amplicons can be simultaneously obtained and discriminated when the spore
4 DNA is used as a template.

5

6 **Figure S3** Phylogenetic placement of Cameroonian and Japanese spore
7 samples inside the Gigasporales tree. The fungal phylogeny was
8 reconstructed using partial ITS sequences. The DNA sequences retrieved in
9 this work are in bold. All the thirteen spore samples are located inside the
10 Gigasporaceae clade, close to *Gigaspora margarita*. Supported values are
11 from Bayesian/maximum likelihood/maximum parsimony analyses. The
12 partitioned Bayesian analysis was performed with TVM+G, K80+G, and
13 TPM2uf+G nucleotide substitution models for ITS1, 5.8S and ITS2 regions,
14 respectively. The maximum likelihood analysis was performed with GTR+G
15 nucleotide substitution model. Dashes instead numbers imply that the
16 topology was not supported in the respective analysis.

17

18 **Figure S4** Segment-specific ML topologies reconstructed for two incongruent
19 segments of the *Mre* 16S rRNA gene sequence alignment. The breakpoint at
20 position 479 was identified by the GARD method and is supported by the
21 Kishino-Hasegawa test ($P < 0.001$). The trees were mid-point rooted. Values
22 above branches represent ML bootstrap support over 70% (1,000 replicates).
23 (A) Topology for the segment of nucleotide positions 1-479. (B) Topology for
24 the segment of nucleotide positions 480-1109.