Diverse bacterial communities are recruited on spores of different arbuscular mycorrhizal fungal isolates

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RUNNING HEAD: Diverse bacterial communities recruited on AMF spores

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Abstract

Arbuscular mycorrhizal fungi (AMF) establish mutualistic symbioses with the roots of most food crops, playing a key role in soil fertility and plant nutrition and health. The beneficial activity of AMF may be positively affected by bacterial communities living associated with mycorrhizal roots, spores and extraradical hyphae. Here we investigated the diversity of bacterial communities associated with the spores of six AMF isolates, belonging to different genera and species and maintained for several generations in pot-cultures with the same host plant, under the same environmental conditions and with the same soil. The occurrence of large bacterial communities intimately associated with spores of the AMF isolates was revealed by PCR-DGGE analysis and sequencing of

DGGE bands. Cluster and canonical correspondence analysis showed that the six AMF isolates displayed diverse bacterial community profiles unrelated with their taxonomic position, suggesting that each AMF isolate recruits on its spores a different microbiota. The 48 sequenced fragments were affiliated with *Actinomycetales*, *Bacillales*, *Pseudomonadales*, *Burkholderiales*, *Rhizobiales* and with *Mollicutes* related endobacteria (Mre). For the first time, we report the occurrence of Mre in *F. coronatum* and *R. intraradices* and sequences related to endobacteria of *Mortierella elongata* in *F. coronatum* and *F. mosseae*. The bacterial species identified are known to possess diverse and specific physiological characteristics and may play multifunctional roles affecting the differential performance of AMF isolates, in terms of infectivity and efficiency.

Keywords: mycorrhizosphere; AMF spores; spore-associated bacteria; PCR-DGGE; bacterial diversity

Introduction

Soil rhizosphere, the area surrounding the plant root system, is a complex and dynamic environment colonized by a wide range of microorganisms, *i.e.* bacteria, fungi, virus and archaea, which coexist and interact among themselves and with plant roots (Philippot et al. 2013). An important group of rhizosphere beneficial microorganisms is represented by arbuscular mycorrhizal fungi (AMF, *Glomeromycota*), which establish mutualistic symbioses with roots of most food crops and play a key role in soil fertility and plant nutrition and health (Smith and Read 2008). AMF provide essential agro-

ecosystem services and are considered as fundamental biofertilisers and bioenhancers in sustainable and organic agriculture (Gianinazzi et al. 2010). The development and performance of the mycorrhizal symbiosis may be affected by different factors, including agronomic management (Njeru et al. 2014) and bacteria living in the mycorrhizosphere, *i.e.* those associated with mycorrhizal roots, spores, sporocarps and extraradical hyphae (Rambelli 1973). Mycorrhizospheric bacteria have different functional abilities, such as the promotion of spore germination and mycorrhizal activity (Mayo et al. 1986; Xavier and Germida 2003; Giovannetti et al. 2010), the production of antibiotics providing protection against different species of fungal plant pathogens (Citernesi et al. 1996; Budi et al. 1999; Li et al. 2007; Bharadwaj et al. 2008b) and the supply of nutrients and growth factors (Barea et al. 2002; Bharadwaj et al. 2008b). In particular, isolates of *Streptomyces* spp. were able to increase the germination of *Gigaspora margarita* spores by releasing volatile compounds in axenic culture (Carpenter-Boggs et al. 1995), while *Paenibacillus validus* stimulated mycelial growth of *Glomus intraradices* Sy167 up to the formation of fertile spores even in the absence of plant roots (Hildebrandt et al. 2006). Phosphate solubilizing bacteria living in the hyphosphere of *Rhizophagus irregularis* positively interacted with the AM fungus, promoting the mineralization of soil phytate and plant phosphorus uptake (Zang et al. 2014). Moreover, AMF-associated bacteria displayed multifunctional activities, from soil aggregation (Rillig et al. 2005) to mineralization of organic nutrients and production of bioactive compounds (Jansa et al. 2013).

Several ultrastructural studies showed that bacteria associated with AMF spores and sporocarps were embedded either in the spore wall layers or in the microniches formed by the peridial hyphae interwoven around the spores (Walley and Germida 1996; Filippi

et al. 1998) or inside the cytoplasm as intracellular entities (Mosse 1970; MacDonald and Chandler 1981; MacDonald et al. 1982; Bianciotto et al. 1996). Other authors, using culturing techniques, isolated different bacterial communities from the spore walls of *Glomus versiforme* and *Rhizophagus clarum* NT4 (Mayo et al. 1986; Xavier and Germida, 2003), from the protoplasts of *Gigaspora margarita* spores (Cruz et al. 2008) and from *Rhizophagus intraradices* and *Funneliformis mosseae* spores (Bharadwaj et al. 2008a). Afterward, direct molecular approaches, without any cultivation step, were applied to obtain a more exhaustive description of bacterial diversity associated with AMF spores. In particular, PCR-denaturing gradient gel electrophoresis (DGGE) analysis was used to detect bacteria associated with *Funneliformis geosporum*, *Septoglomus constrictum* and *G. margarita* spores (Roesti et al. 2005; Long et al. 2008). Other molecular approaches detected *Mollicutes*-related endobacteria (Mre) and unculturable endosymbionts across different lineages of AMF (Naumann et al. 2010; Desirò et al. 2014).

Since AMF are obligate symbionts which grow and sporulate in the soil surrounding the roots, the composition of the bacterial populations living strictly associated with their spores may vary depending on environmental variables, such as soil types and host plants (Roesti et al. 2005; Long et al. 2008; Bharadwaj et al. 2008a).

So far, the few data available were obtained through investigations carried out only on a small number of AMF species. Moreover, the question as to whether specific bacterial populations are associated to specific AMF taxa remains to be answered.

Here, we approached these issues by assessing whether diverse bacterial communities are associated with AMF spores belonging to different genera, species and isolates, maintained for several generations in pot-cultures with the same host plant, under the

same environmental conditions and in the same soil. We analysed six different AMF originating from diverse geographical areas and grown in our collection for at least 15 years: three isolates of the species *F. mosseae* originating from U.S.A and France, two *R. intraradices* isolates from France and Italy and one *Funneliformis coronatum* isolate from Italy. Such isolates showed a high level of intraspecific and interspecific diversity, as revealed by genetic and phenotypic characterization (Giovannetti et al. 2003; Avio et al. 2009).

In order to overcome underestimates due to the constraints of culture media and cultivation conditions, as well as to the presence of bacteria in viable but non-culturable state, we utilized a culture-independent approach, such as PCR-DGGE analysis of the 16S rRNA gene, which allows fingerprinting of the bacterial species occurring in the relevant biological sample. Our study was aimed at (i) identifying the bacterial species loosely and strictly associated with the spores of different AMF isolates and (ii) determining whether specific bacterial communities are recruited on spores of different AMF taxa.

Materials and methods

Fungal material

The AMF used in this work were the following: three isolates belonging to the species *F. mosseae* (T.H. Nicolson & Gerd.) C. Walker & Schuessler (IMA1, IN101C, AZ225C), one isolate of *F. coronatum* (Giovann.) C. Walker & Schüßler (IMA3) and two isolates of *R. intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & Schüßler (IMA5 and IMA6) (Table 1). Spores and sporocarps of AMF were obtained from pot-cultures maintained in the collection of the Microbiology Labs of the Department of

Agricultural, Food and Environment, University of Pisa, Italy (International Microbial Archives, IMA). Each isolate was maintained for at least 15 generations under the same growth conditions, in sterilized calcinated clay (OILDRI, Chicago, IL, USA) field soil (1:1, v/v) pot cultures using a mixture of *Trifolium alexandrinum* L. and *Medicago sativa* L. as host plants.

Spore collection

Spores and sporocarps were extracted from soil of three pot-cultures for each AMF isolate, using the wet sieving and decanting technique, down to a mesh size of 50 µm (Gerdemann and Nicolson 1963). Spores retained on sieves, or extracted from sporocarps using needles under a dissecting microscope, were flushed into Petri dishes and, for each isolate, 100 spores were manually collected with a capillary pipette under a dissecting microscope (Leica MS5, Milano, Italy). Only intact and healthy spores were selected. Bacterial communities loosely and strictly associated to AMF spores were analysed by suspending spores in 1 mL sterile water in a 1.5-mL Eppendorf tube and vigorously washing using a vortex mixer at 1,500 rpm for 1 min. Spores were washed 15 times in succession in sterile water and then homogenized in 100 µL water. The rinsing water of the first and the 15th washings was preserved, along with spore homogenates, in order to be used for DNA extraction. Spores were not washed further, as a preliminary experiment showed that no bacterial colonies developed on tryptic soy agar after the 15th washing.

The above described procedure, from spore collection and selection to DNA extraction, was replicated three times for each AMF isolate.

DNA extraction and PCR amplification

DNA extracts were prepared for each isolate from the rinsing water of the first and the 15th washing of spores, centrifuged for 20 min at 11,699g, as well as from spores washed 15 times and crushed in 100 µL sterile physiological water using "MasterPureTM Yeast DNA Purification" kit (Epicentre), according to the manufacturer's protocol. For the analysis of bacterial communities, the amplification of the variable region V3-V5 of 16S rDNA was carried out using the primers 341F (CCTACGGGAGGCAGCAG) and 907R (CCGTCAATTCCTTTRAGTTT) (Yu and Morrison 2004). At its 5' end, the primer 341F had an additional 40-nucleotide GC-rich tail (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3'). Amplification reaction was prepared in a final volume of 50 µL, using 10-20 ng of DNA, 5 µL of 10X Gold Buffer $MgCl_2$ free (Applied Biosystem), 2 mM of $MgCl_2$ (Applied Biosystem), 1.25 U of AmpliTaq Gold (Applied Biosystem), 0.2 mM of each dNTP (GeneAmp dNTP Mix, Applied Biosystem) and 0.5 µM of each primer (Primm). The fragment obtained was about 560 bp long. The reaction was carried out using an iCycler-iQ Multicolor Real-Time PCR Detection System (Biorad) with the following denaturation, amplification and extension procedure: at 95°C for 10 min; at 94°C for 30 sec, at 55°C for 30 sec, at 72°C for 60 sec (for 35 cycles); at 72°C for 10 min. The presence of amplicons was confirmed by electrophoresis in 1.5% (w/v) Agarose I (Euroclone) in TBE 1 x buffer (Euroclone) gels stained with ethidium bromide (0.5 µg mL^{-1}). All gels were visualized and captured as TIFF format files by the Liscap program for Image Master VDS system (Pharmacia Biotech).

164

165 **DGGE and profile analyses**

166 The amplicons were analyzed using the DCodeTM Universal Mutation Detection
167 System Biorad. Twenty µL of the PCR products plus 20 µL of buffer 2 x made with
168 70% glycerol, 0.05% xylene cyanol and 0.05% bromophenol bleu were loaded on a 8%
169 polyacrylamide-bisacrilamide (37.5:1) gel with a urea-formamide denaturing gradient
170 ranging from 30% to 65%. A composite mix of bacterial 16S rRNA gene fragments
171 from *Bacillus megaterium* DSMZ 32, *Bacillus subtilis* DSMZ 1970, *Pseudomonas*
172 *fluorescens* 19/5, *Rhizobium leguminosarum* RL 11, *Serratia marcescens* B2,
173 *Sinorhizobium meliloti* SM 8, *Streptomyces* sp. ATB 42 were added as reference DGGE
174 marker (M). Gels were run at 80 V and 60 °C for16 hours and stained for 30' in 500 mL
175 of TAE 1 x buffer containing 50 µL of Sybr Gold Nucleic Acid Gel Stain (Molecular
176 Probes, Invitrogen). The profiles were visualized under UV illumination and captured as
177 TIFF format file by Liscap program for Image Master VDS System (Pharmacia
178 Biotech). Band patterns in different DGGE lanes were compared with the ImageMaster
179 1D Elite v3.00 software (Pharmacia Biotech). The lanes were normalized to contain the
180 same amount of total signal after background subtraction and the gel images were
181 straightened and aligned to give a densitometric curve. Bands were assigned and
182 matched automatically and then checked manually. Band positions were converted to Rf
183 values between 0 and 1, and profile similarity was calculated by determining Dice's
184 similarity coefficients for the total number of lane patterns from the DGGE gel. The
185 similarity coefficients calculated were then used to generate the dendrograms utilizing
186 the clustering method UPGMA (Unweighted Pair Group Method Using Arithmetic
187 Average). Community profiles were analysed by canonical correspondence analysis

(CCA) performed from a data matrix constructed based on relative band intensity and position. The significance of data was assessed by the Monte Carlo permutation test (Canoco 5 software). DGGE banding data were used to estimate four different indices treating each band as an individual operational taxonomic unit (OTU). Richness (S) indicates the number of OTUs present in a sample and was determined by the number of fragments. The overall diversity index of Shannon-Weaver (H) and the dominance index of Simpson (D) were calculated using the equations $H = -\sum(P_i \times \ln P_i)$ and $D = \sum P_i^2$ respectively, where the relative importance of each OTU is $P_i = n_i/N$ and n_i is the peak intensity of a band and N is the sum of all peak intensities in a lane. Evenness index (E), which allows the identification of dominant OTUs, was calculated as $E = H/\ln S$. 2.4. One-way ANOVA was applied to diversity indices with isolate as a variability factor, after checking the normal distribution of data. The means were compared by the Tukey's test ($P < 0.05$). Analyses were carried out with the SPSS version 20 software (IBM Corp., Armon, NY Inc, USA).

DGGE bands Sequencing

The bands of interest in the DGGE profiles were cut out from the gels for sequencing. DNA was extracted by eluting for 3 days in 50 µL 10 mM TE at 4°C. One µL of the supernatant diluted 1:100 was used to re-amplify the V3-V5 region of the DNA according to the PCR protocol described above, except that a 341F primer without the GC-rich tail and a 25 clycles amplification protocol were used. The amplification product were then purified with the EuroGold Cycle Pure Kit (Euro Clone) according to the manufacturer's protocol, quantified and 5' sequenced at the BMR Genomics

211 (Padova, Italy). Sequences were analyzed using BLAST on the NCBI web
212 (http://www.NCBI.nim.nih.gov/). The related sequences were collected and aligned
213 using MUSCLE (Edgar 2004a; 2004b), and phylogenetic trees were constructed using
214 the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura
215 1980) in Mega 5.2 software (http:// www.megasoftware.net/) with 1,000 bootstraps
216 replicates.
217 The DGGE band sequences were submitted to the European Nucleotide Archive under
218 the accession numbers from LN554945 to LN554985.
219

220 **Results**

221 **Bacterial communities loosely and strictly associated with AMF spores**
222 In this experiment DNA was extracted from the rinsing water of the first and the 15th
223 washing of spores of the different AMF isolates and directly from spore homogenates
224 after the 15th washing, and then analysed by PCR-DGGE to assess the diversity of
225 bacterial communities strictly and loosely associated with AMF spores. A DNA
226 fragment of approximately 600 bp, corresponding to the V3-V5 region of the 16S
227 rDNA, was successfully amplified from all samples. DGGE analyses of PCR products
228 showed the highest number of fragments in the profiles obtained from spore
229 homogenate samples, while only a few bands occurred in the profile of the 15th
230 washing. Most fragments found in the first washing were shared with those obtained
231 from spores. Such results were consistent in the six AMF isolates. DGGE profiles
232 obtained from the isolate *R. intraradices* IMA6 are reported in Fig. 1. The relevant
233 dendrogram (Fig. 2) detected a low similarity (15%) between the banding patterns of
234 the 15th washing and those of the first washing and spore homogenates, which, anyway,

grouped in two separate sub-clusters, showing a similarity index of 65%. Consistent results were found for the other five AMF isolates. In particular, *R. intraradices* IMA5 and *F. coronatum* IMA3 dendrograms showed a low level of similarity (13% and 14%, respectively) between the banding patterns of the 15th washing and those of the first washing and spore homogenates, which grouped in a single cluster, with a similarity of 54% and 58%, respectively (Fig. S1 A and B, supplementary electronic material). DGGE profiles and dendrograms obtained from the three *F. mosseae* isolates, AZ225C, IMA1 and IN101C, showed analogous results (Fig. S1 C-E, supplementary electronic material).

The bands of interest from the DGGE profiles of the first washing, the 15th washing and spore homogenates were excised and sequenced to determine their affiliation. Fig. 3 shows the relative percentage of the most abundant bacterial genera detected in the different samples of each AMF isolate. Among the microorganisms identified, *Actinobacteria* were always detected in all AMF isolates. In particular, for the isolates *F. coronatum* IMA3, *F. mosseae* AZ225C and *R. intraradices* IMA6, the sequences were affiliated with the genus *Arthrobacter*, for the isolate *F. mosseae* IN101C with the genus *Propionibacterium*, for the isolate *F. mosseae* IMA1 with the genus *Amycolatopsis* and for the isolate *R. intraradices* IMA5 with the genus *Streptomyces.* Sequences of *Bacillus*, *Pseudomonas* and different genera belonging to the *Rhizobiales* were also recovered. Among the bacterial genera strictly associated with AMF spores, we identified sequences affiliated with *Mollicutes* related endobacteria (Mre).

Diversity of the bacterial communities strictly associated with spores of different AMF isolates

The diversity of the bacterial communities strictly associated with spores of the different AMF isolates was assessed by cluster analysis of DGGE profiles obtained from spore homogenates (Figs. 4 and 5). In particular, the relevant dendrogram showed a low similarity level among the 6 AMF isolates. A main cluster formed by two subclusters with a similarity of 37% was identified. The first subcluster included the isolates *F. mosseae* AZ225C and *R. intraradices* IMA6, with a similarity of 45%, while the second included the isolates *F. coronatum* IMA3 and *F. mosseae* IMA1, with a similarity of 42%. The other two AMF isolates, *F. mosseae* IN101C and *R. intraradices* IMA5 grouped separately in two different clusters showing a level of similarity of 30 and 35% respectively, compared with the other ones. These findings were also confirmed by the CCA of the DGGE bacterial community profiles performed on a data matrix constructed on relative band intensity and position (Fig. 6). The different AMF taxa accounted for 49.3% of total variation, while the relationship among the response and explanatory variables was statistically significant at $p = 0.002$, as revealed by the permutation test on the first axis and all axes. The distances among the isolates of the different AMF species were shorter than those among the isolates belonging to the same AMF species, indicating that each AMF isolate displayed a bacterial community specific profile unrelated with their taxonomic position.

In this study DGGE profiles obtained from AMF spore homogenates were also used to estimate Richness (S), Shannon-Weaver (H), Simpson (D) and Eveness (E) biodiversity indices (Table 2). No statistically significant differences were found among AMF isolates for S, H and D biodiversity indices, while for the E index the isolates *R. intraradices* IMA5 and IMA6 were statistically different from *F. mosseae* IN101C.

Nevertheless, all isolates showed high E values (more than 0.95), which implies that their spores were conducive to the establishment and maintenance of large bacterial communities.

In order to identify the bacterial species occurring in spore homogenates of the different AMF, the DGGE bands of interest were excised, sequenced and affiliated to bacterial species by using BLAST and phylogenetic trees analyses. Fig. 4 shows the 48 sequenced fragments marked with a progressive number as well as with their correspondent affiliation to the bacterial Order, while Fig. 7 shows the related phylogenetic tree with the affiliation of sequences to bacterial species. Sequences resulted to be affiliated with *Actinomycetales* (e.g. *Arthrobacter, Streptomyces, Amycolatopsis* and *Propionibacterium*), *Bacillales* (e.g. *Paenibacillus, Bacillus*) *Pseudomonadales* (*Pseudomonas*), *Burkholderiales* (e.g. *Methylibium, Ideonella, Herbaspirillum, Duganella, Massilia*), *Rhizobiales* (e.g. *Rhizobium, Sinorhizobium, Agrobacterium*) and to *Mollicutes* related endobacteria.

Bands whose sequences were affiliated to the order *Actinomycetales* were found in all AMF isolates. In particular, bands 1, 9, 14, 24 and 43 were related to *Arthrobacter phenanthrenivorans*, and bands 18 and 44 to *A. aurescens*, with high sequence similarity (from 98% to 100%). Bands 15 and 45 were affiliated to *Streptomyces flavogriseus* and band 39 to *S. phaeochromogenes*, with a similarity of 99%. Finally, band 46, specific of *F. mosseae* IMA1, and band 48, specific of the isolate *F. mosseae* IN101C, were related to the *Amycolatopsis* and *Propionibacterium*, respectively.

We found also *Burkholderiales* in 4 AMF isolates out of 6: band 47 from isolate *F. mosseae* IN101C was affiliated to the genus *Methylibium*, bands 13 and 19 from isolate

305 *F. mosseae* AZ225C were related to *Ideonella* sp. and to *Duganella nigrescens*,
306 respectively, and band 40 was affiliated to *Massilia aurea* and *Janthinobacterium* sp.
307 With regard to the *Rhizobiales* order, sequences related to *Sinorhizobium meliloti*
308 occurred in three AMF isolates (bands 12, 23, 7 and 26) while *Agrobacterium*
309 *radiobacter* and *Rhizobium giardinii* showed specific association, respectively, with *F.*
310 *coronatum* IMA3 (5 and 6) and *F. mosseae* AZ225C (22). Members of *Bacillales* and
311 *Pseudomonadales* were found less frequently associated with our AMF isolates. In
312 particular, two bands (34 and 35) were only present with *R. intraradices* IMA5 and
313 were affiliated with *Pseudomonas putida*, while two other bands - one related to
314 *Paenibacillus castaneae* (21) and the other one to *Bacillus firmus* (42) - were,
315 respectively, present in the profile of *F. mosseae* AZ225C and *R. intraradices* IMA6.
316 In addition, several bands related to Mre occurred in *F. mosseae* IN101C (bands 25 and
317 27), *F. mosseae* IMA1 (bands 30 and 29), *F. coronatum* IMA3 (bands 2 and 3) and *R.*
318 *intraradices* IMA5 (band 33). Most of our sequences (5 out of 7) displayed similarity
319 values from 97% to 99% with those related to Mre of *Glomeromycota* spores and
320 liverworts-associated AMF retrieved from GeneBank, and clustered all together, in a
321 well-supported clade (Fig. 7). Interestingly, some sequences showed high divergence,
322 up to 24%, even when originating from the same isolate (e.g. bands 29 and 30). On the
323 other hand, sequences from bands 27 and 33 displayed a high similarity (99%), although
324 they were obtained from two different AMF isolates.
325 Finally, sequences from band 4 (*F. coronatum* IMA3) and band 28 (*F. mosseae*
326 IN101C) showed 94% similarity with those of *Mortierella elongata* endobacteria, in the
327 family *Burkholderiaceae*.
328

Discussion

Bacterial communities loosely and strictly associated with AMF spores

The assessment of the diversity of bacterial communities strictly and loosely associated with spores of the six AMF showed that there was no specific band characterizing the profiles of the first spore washing, as all the fragments detected were in common with those obtained from spore homogenates. Therefore, there was no specific bacterial community loosely associated with AMF spores. After 15 washings only few fragments were detectable in the DGGE profiles, showing that most bacterial species were washed away. On the other hand, the higher number of DGGE fragments found in spore homogenates than in washings represents evidence of the occurrence of large bacterial communities intimately associated with AMF spores. The detection of several DGGE fragments shared by the first washing and spore homogenates can be ascribed to physiological characteristics of some bacterial populations, whose growth may be influenced by specific spore wall composition and structure or spore exudates (Roesti et al. 2005; Sbrana et al. 1995).

The phylogenetic identification confirmed the results obtained by DGGE profiles analyses, *i.e*. the occurrence of the highest bacterial diversity in spore homogenates. The sequencing of the DGGE bands of interest detected many *Actinobacteria*, belonging to the genera *Arthrobacter*, *Propionibacterium*, *Amycolatopsis* and *Streptomyces*, in all AMF isolates, both in the first washing and in spore homogenates. The persistence of such bacteria in spore homogenates may be ascribed to their particular physiological characteristics, *i.e*. chitinolytic activity. Indeed, spore wall degrading activity was found in bacteria embedded in spore walls of *F. mosseae*, where chitinolytic bacteria represented 72% of all the isolated chitinolytic microorganisms

353 (Filippi et al. 1998). Other authors reported that most sequences associated with *G.*
354 *geosporum* and *G. constrictum* spores were affiliated with bacteria able to hydrolyze
355 biopolymers (Roesti et al. 2005).
356 Among the bacterial genera strictly associated with AMF spores, *Bacillus*,
357 *Pseudomonas* and different genera belonging to the *Rhizobiales* were retrieved. Such
358 bacteria were probably not washed away as a result of their ability to form biofilms by
359 producing exopolysaccharides, which allows them to efficiently colonize roots and
360 mycorrhizal hyphae (Toljander et al. 2006). Interestingly, we identified also sequences
361 affiliated with *Mollicutes* related endobacteria (Mre), that have been reported to be
362 localized inside the cytoplasm of AMF spores (Naumann et al. 2010).
363
364 **Diversity of the bacterial communities strictly associated with spores of different**
365 **AMF isolates**

366 Cluster and canonical correspondence analyses of DGGE patterns obtained from spore
367 homogenates showed that the six AMF isolates displayed diverse bacterial community
368 profiles unrelated with their taxonomic position, suggesting that each AMF isolate
369 recruits on its spores a different microbiota. Considering that the AMF isolates analysed
370 in our study were maintained for several generations in pot-cultures with the same host
371 plant, under the same environmental conditions and in the same soil substrate, we can
372 infer that the composition of the bacterial communities associated with spores is
373 affected by the genetic diversity of each AMF isolate. Such claim is well supported by
374 our data, which were obtained by analysing six different AMF isolates belonging to two
375 genera and three species. Indeed, the isolates of *F. mosseae* AZ225C, IMA1 and
376 IN101C were previously reported to encompass a high genetic variability (Giovannetti

et al. 2003; Avio et al. 2009). Previous works showed that spores of *Funneliformis geosporum* and *S. constrictum*, harvested from pot cultures with two host plants, harbored different bacterial populations, which were more influenced by a specific spore wall composition or AMF exudates than by specific root exudates (Roesti et al. 2005). By contrast, Long et al. (2008) reported that the composition of the bacterial community associated to *G. margarita* spores was influenced by both culture substrates and host plants.

The sequencing of the DGGE bands from spore homogenates confirmed that each AMF isolate was characterized by a diverse bacterial community composition. Members of the *Actinomycetales* order were regularly retrieved from all AMF isolates, confirming their widespread occurrence in the mycorrhizosphere (Ames et al. 1989; Filippi et al. 1998) where they may contribute to AMF functionality, in terms of infectivity and efficiency (Bharadwaj et al. 2008b; Hamdali et al. 2008; Giovannetti et al. 2010). We found species of the genera *Arthrobacter*, whose members are metabolically and ecologically diverse, able to survive in environmentally harsh conditions for extended periods of time; they are also known for their ability to biodegrade a variety of environmental pollutants and chemicals (Mongodin et al. 2006). Sequences affiliated to *Streptomyces* were also retrieved. Such bacteria are abundant in the soil and capable of producing a vast array of complex and biologically active secondary metabolites and a large number of enzymes that break down insoluble organic polymers, including chitin and cellulose (Seipke et al. 2012).

Members of the *Burkholderiales*, *Rhizobiales*, *Bacillales* and *Pseudomonadales* orders were differentially retrieved from the diverse AMF isolates. The occurrence of *Methylibium*, *Ideonella*, *Duganella*, *Massilia* and *Janthinobacterium* within the

401 *Oxalobacteriaceae* family (*Burkholderiales*) is consistent with previous reports showing
402 that members of *Oxalobacteriaceae* were particularly abundant on AMF hyphae
403 (Scheublin et al. 2010). A bacterial strain belonging to *Oxalobacteriaceae* able to
404 promote spore germination, hyphal growth and root colonization of *F. mosseae* was
405 isolated from mycorrhizal roots (Pivato et al. 2009). Moreover, a bacterium isolated
406 from protoplasts of *G. margarita* spores, closely related to *Janthinobacterium lividum,*
407 showed strong antagonistic effects against pathogenic fungi and P solubilisation activity
408 (Cruz et al. 2008).
409 Sequences affiliated to *Rhizobiales*, *Bacillales* and *Pseudomonadales* orders were found
410 less frequently associated with our AMF isolates. However, their occurrence in strict
411 association with AMF spores suggest their possible role in the promotion of spore
412 germination, mycelial growth and mycorrhizal colonization (Mayo et al. 1986; Xavier
413 and Germida 2003; Bharadwaj et al. 2008b). Recent findings reported that members of
414 *Bacillales* may have a role as mycorrhiza helper bacteria, increasing mycorrhiza
415 formation and plant growth (Zhao et al. 2014).
416 Here, we report for the first time the occurrence of several sequences related to Mre in
417 *F. coronatum* and *R. intraradices*, in addition to *F. mosseae*. Moreover, from *F.*
418 *coronatum* and *F. mosseae* we obtained sequences similar to those of an endobacterium
419 of *Mortierella elongata* (Sato et al. 2010), showing that our isolates concomitantly
420 harbor two different types of endobacteria, related to *Mollicutes* and to
421 *Burkholderiaceae*.
422 In conclusion, the differences in bacterial communities associated with the different
423 AMF may represent the outcome of a selection that can be ascribed to the rich niche
424 (the sporosphere), providing nutrients for their establishment and metabolic activity, or

425 rather the result of tripartite (AMF, plant roots and bacteria) beneficial interactions
426 producing mutualistic advantages for the three partners (Jansa et al. 2013). In the latter
427 hypothesis, besides the well-known benefits of the mycorrhizal symbiosis for host
428 plants and fungal symbionts, new properties could result from the tripartite
429 multifunctional association (Rillig et al. 2005). The complexity of such a system may
430 result further increased, as spore associated bacteria could be tranferred to the hyphal
431 surface of germlings and extraradical mycelium, and to the relevant soil volume
432 (hyphosphere), possibly playing important functional roles for plant growth and
433 symbiosis establishment (Scheublin et al. 2010; Jansa et al. 2013). The quantity and
434 quality of spore and hyphal exudates and the composition of the spore wall layers of
435 each AMF isolate could differentially affect the selection of the resident bacterial
436 strains, which might play differential roles in enhancing nutrient availability to fungal
437 mycelium and plant roots (e.g. phosphate solubilising, nitrogen fixing and chitinolytic
438 bacteria), in controlling plant pathogens (e.g. siderophore and antibiotic producing
439 bacteria) and promoting plant growth (e.g. plant growth promoting bacteria).
440 Variable combinations of these effects may be at the basis of the differential
441 performance of AMF isolates, in terms of infectivity and efficiency (Giovannetti and
442 Avio 2002). The isolation of bacteria strictly associated with AMF spores and the
443 investigation of their functional significance represent further essential steps, in order to
444 select the best AMF/bacteria combinations to be used as biofertilizers and bioenhancers
445 in sustainable agroecosystems.
446
447
448 **Acknowledgments**

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452

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582

583 **Table 1** List of arbuscular mycorrhizal fungal isolates studied in the present work

Fungal species	Isolate code	Geographic origin	Biome	Collector	Original inoculum supplier [a]
F. coronatum	IMA3	Tuscany, Italy	Mediterranean sand dunes	M. Giovannetti	IMA, Pisa, ITALY
F. mosseae	AZ225C	Arizona, USA	Subtropical desert	J.C. Stutz	INVAM, Morgantown, WV, USA
F. mosseae	IMA1	Kent, UK	Unknown	B. Mosse	Rothamsted Experimental Station, UK
F. mosseae	IN101C	Indiana, USA	Temperate grassland	R. Kemery	INVAM, Morgantown, WV, USA
R. intraradices	IMA5	Liguria, Italy	Mediterranean grassland	M. Giovannetti	IMA, Pisa, ITALY
R. intraradices	IMA6	Burgundy, France	Temperate agricultural	A. Trouvelot	V. Gianinazzi-Pearson

584

585 [a] Abbreviations: INVAM, International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi. IMA, International Microbial
586 Archives
587

588 **Table 2** Richness (S), Shannon-Weaver (H), Simpson (D) and Evenness (E) indices
589 calculated from DGGE profile of the bacterial community associated to different AMF
590 isolates (mean ± standard error). Means with different letters are significantly different
591 ($p < 0.05$)

592

Isolate	S	H	D	E
F. coronatum IMA3	17.67±2.73 a	2.79±0.14 a	0.07±0.01 a	0.98±0.01 ab
F. mosseae AZ225C	13.67±0.33 a	2.53±0.03 a	0.08±0.00 a	0.97±0.01 ab
F. mosseae IN101C	14.00±1.15 a	2.53±0.08 a	0.08±0.01 a	0.96±0.00 a
F. mosseae IMA1	15.67±2.60 a	2.67±0.20 a	0.07±0.02 a	0.98±0.01 ab
R. intraradices IMA5	14.67±2.40 a	2.64±0.18 a	0.07±0.01 a	0.99±0.00 b
R. intraradices IMA6	17.00±4.16 a	2.74±0.28 a	0.07±0.02 a	0.99±0.00 b
ANOVA results				
$F_{5,12}$	0.410	0.361	0.225	4.584
p	0.833	0.865	0.944	0.014

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602 **FIGURE LEGENDS**

603 **Fig. 1** DGGE analysis of *R. intraradices* IMA6 associated bacterial communities. I RW,
604 rinsing water of the first spore washing; XV RW, rinsing water of the 15th spore
605 washing; Spores, homogenate of spores after the 15th washing; M, Marker.

606 **Fig. 2** Dendrogram obtained by UPGMA (Unweighted Pair Group Method Using
607 Arithmetic Average) based on the DGGE profiles of the bacterial community associated
608 to isolate *R. intraradices* IMA6. I RW, rinsing water of the first spore washing; XV
609 RW, rinsing water of the 15th spore washing; Spores, homogenate of spores after the
610 15th washing; M, Marker. The relationships among samples are based on similarity,
611 evaluated by the Dice coefficient. Bootstrap (1,000 replicates) values below 50 are not
612 shown.

613 **Fig. 3** Relative abundance (%) of bacterial genera detected in each sample of
614 geographically different *F. coronatum*, *F. mosseae* and *R. intraradices* isolates. I RW,
615 rinsing water of the first spore washing; XV RW, rinsing water of the 15th spore
616 washing; Spores, homogenate of spores after the 15th washing. For each sample the
617 percentage was calculated as follows: the number of sequences referred to a genus
618 divided by the total number of sequences identified. Mre: Mollicutes-related
619 endobacteria.

Fig. 4 DGGE analysis of bacterial communities associated with spores of different isolates of *F. coronatum*, *F. mosseae* and *R. intraradices*. Marker (M). Sequenced fragments are marked with progressive numbers.

Fig. 5 Dendrogram obtained by UPGMA (Unweighted Pair Group Method Using Arithmetic Average) based on the DGGE profiles of the bacterial community associated with spores of different isolates of *F. coronatum*, *F. mosseae* and *R. intraradices*. Marker (M). The relationships among samples are based on similarity, evaluated by the Dice coefficient. Bootstrap (1,000 replicates) values below 50 are not shown.

Fig. 6 Ordination plot generated by canonical correspondence analysis, representing the relationships between spore-associated bacterial communities identified by the DGGE profiles obtained for *F. coronatum*, *F. mosseae* and *R. intraradices* different isolates. ● *F. coronatum* IMA3; ▲ *F. mosseae* AZ225C; ● *F. mosseae* IN101C; ◆ *F. mosseae* IMA1; ■ *R. intraradices* IMA5; ▼ *R. intraradices* IMA6. Values on the axes indicate the percentage of the total variation explained.

Fig. 7 Affiliation of the sequences retrieved from the DGGE gel bands (marked in Fig. 4) with the existing 16S rRNA gene sequences. Phylogenetic analysis inferred by using the Maximum Likelihood method. Bootstrap values below 70 are not shown. The sequences from the database are indicated by their accession numbers. The DNA sequences retrieved in this work are indicated by their corresponding band numbers (Fig. 4). ● *F. coronatum* IMA3; ▲ *F. mosseae* AZ225C; ● *F. mosseae* IN101C; ◆ *F. mosseae* IMA1; ■ *R. intraradices* IMA5; ▼ *R. intraradices* IMA6.

Figures

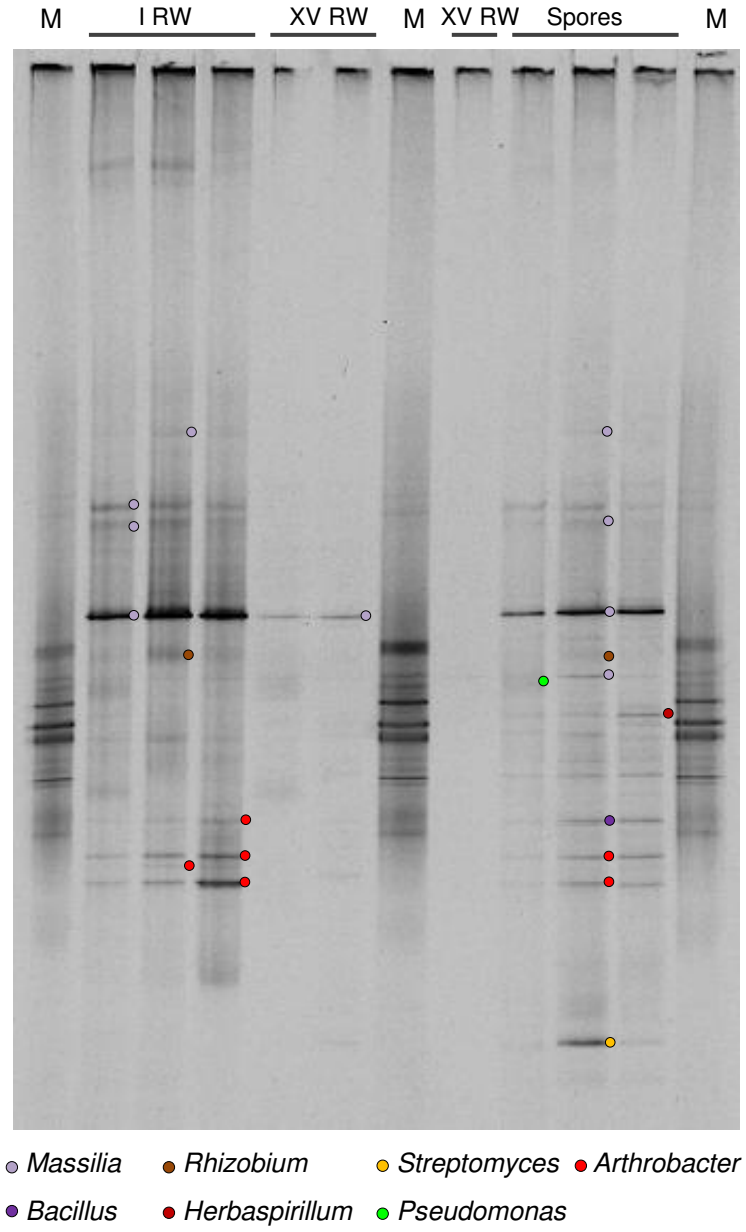
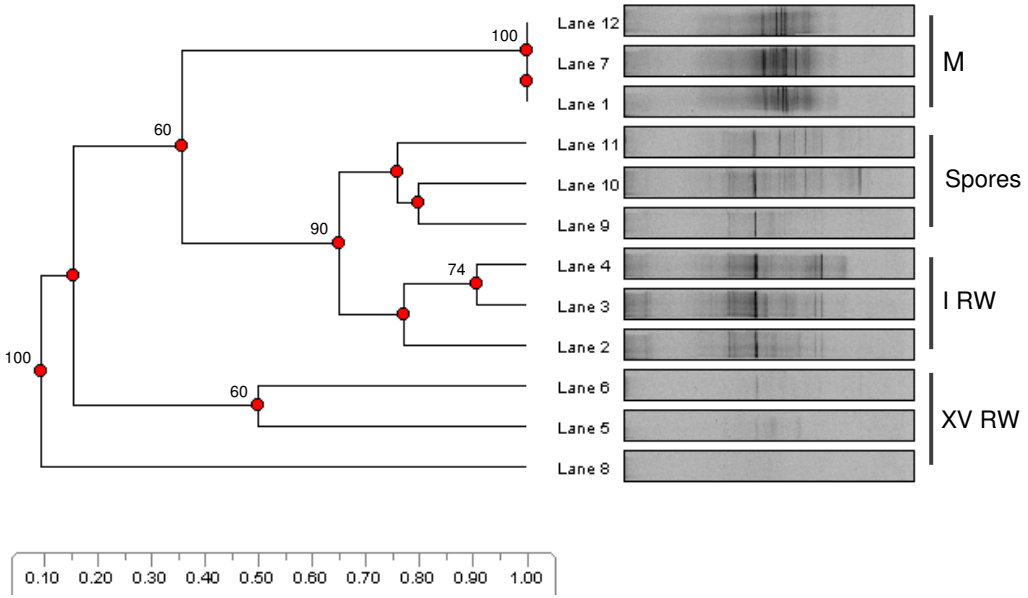


Fig. 1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74
100
60
Lane 12
Lane 7
Lane 1
Lane 11
Lane 10
Lane 9
Lane 4
Lane 3
Lane 2
Lane 6
Lane 5
Lane 8
M
Spores
I RW
XV RW
0.10 0.20 0.30 0.40 0.50 0.60 0.70 0.80 0.90 1.00

Fig. 2

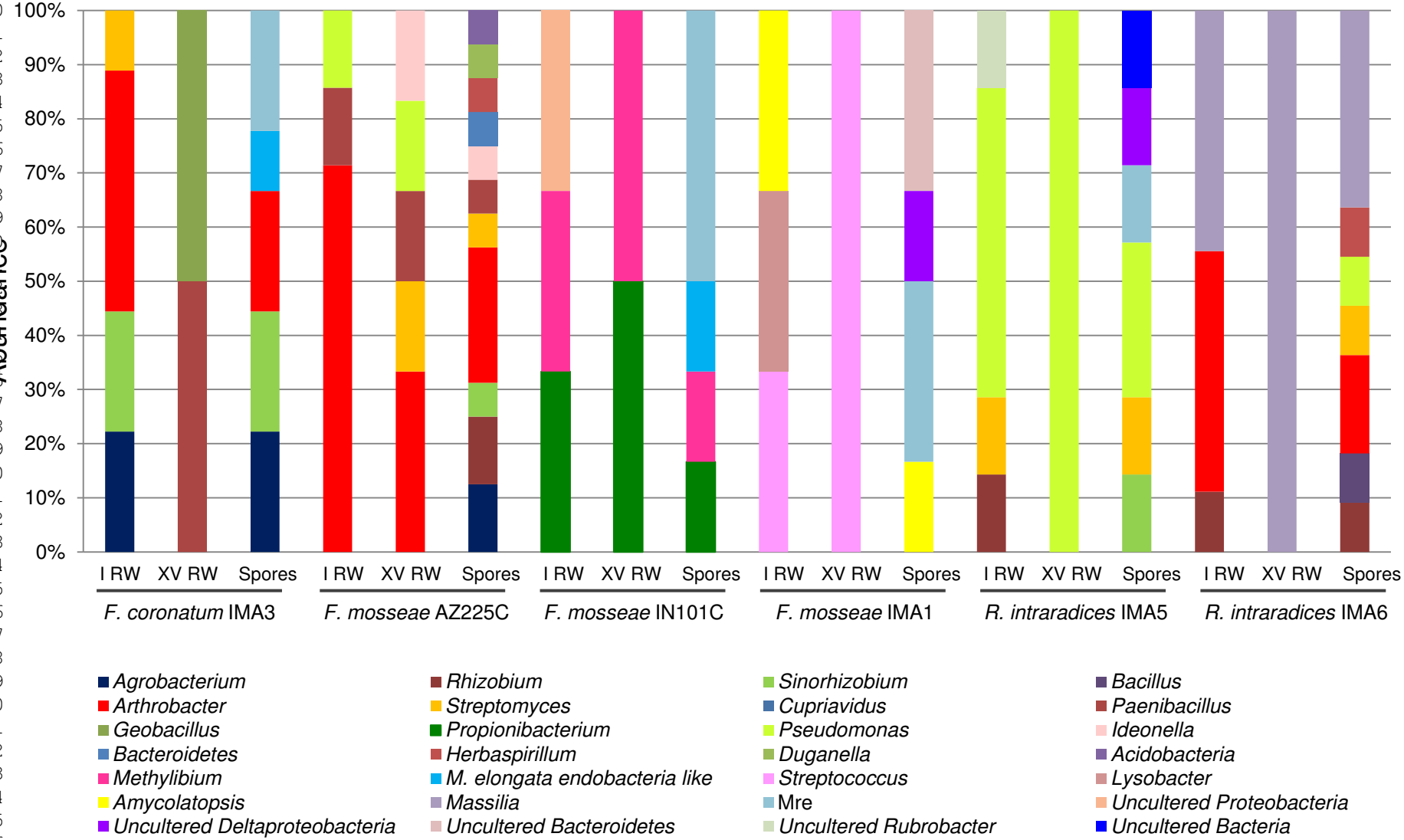
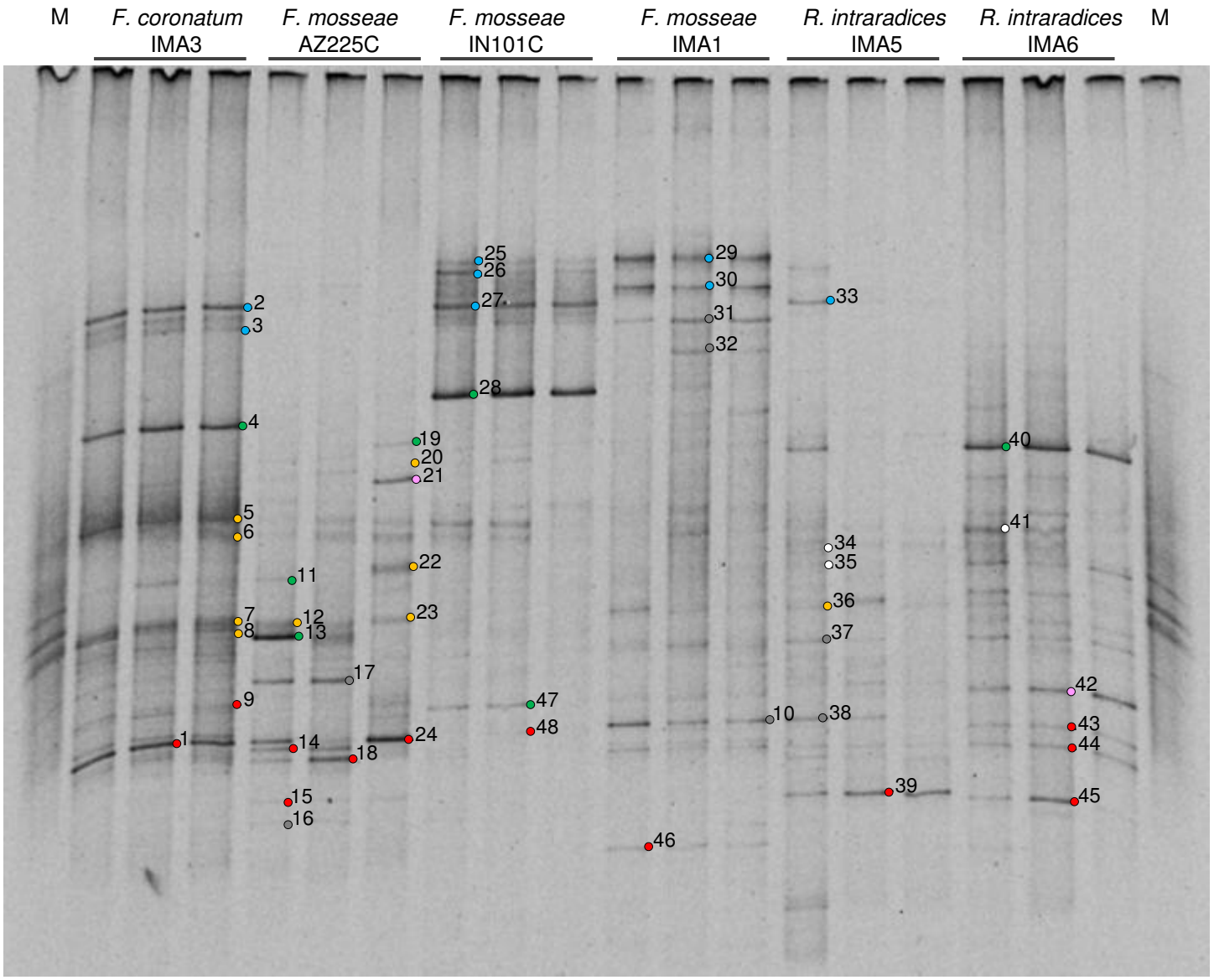

Abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
I RW
XV RW
Spores
F. coronatum IMA3
F. mosseae AZ225C
F. mosseae IN101C
F. mosseae IMA1
R. intraradices IMA5
R. intraradices IMA6
Agrobacterium
Arthrobacter
Geobacillus
Bacteroidetes
Methylibium
Amycolatopsis
Uncultered Deltaproteobacteria
Rhizobium
Streptomyces
Propionibacterium
Herbaspirillum
M. elongata endobacteria like
Massilia
Uncultered Bacteroidetes
Sinorhizobium
Cupriavidus
Pseudomonas
Duganella
Streptococcus
Mre
Uncultered Rubrobacter
Bacillus
Paenibacillus
Ideonella
Acidobacteria
Lysobacter
Uncultered Proteobacteria
Uncultered Bacteria

Fig. 3


M
F. coronatum IMA3
F. mosseae AZ225C
F. mosseae IN101C
F. mosseae IMA1
R. intraradices IMA5
R. intraradices IMA6
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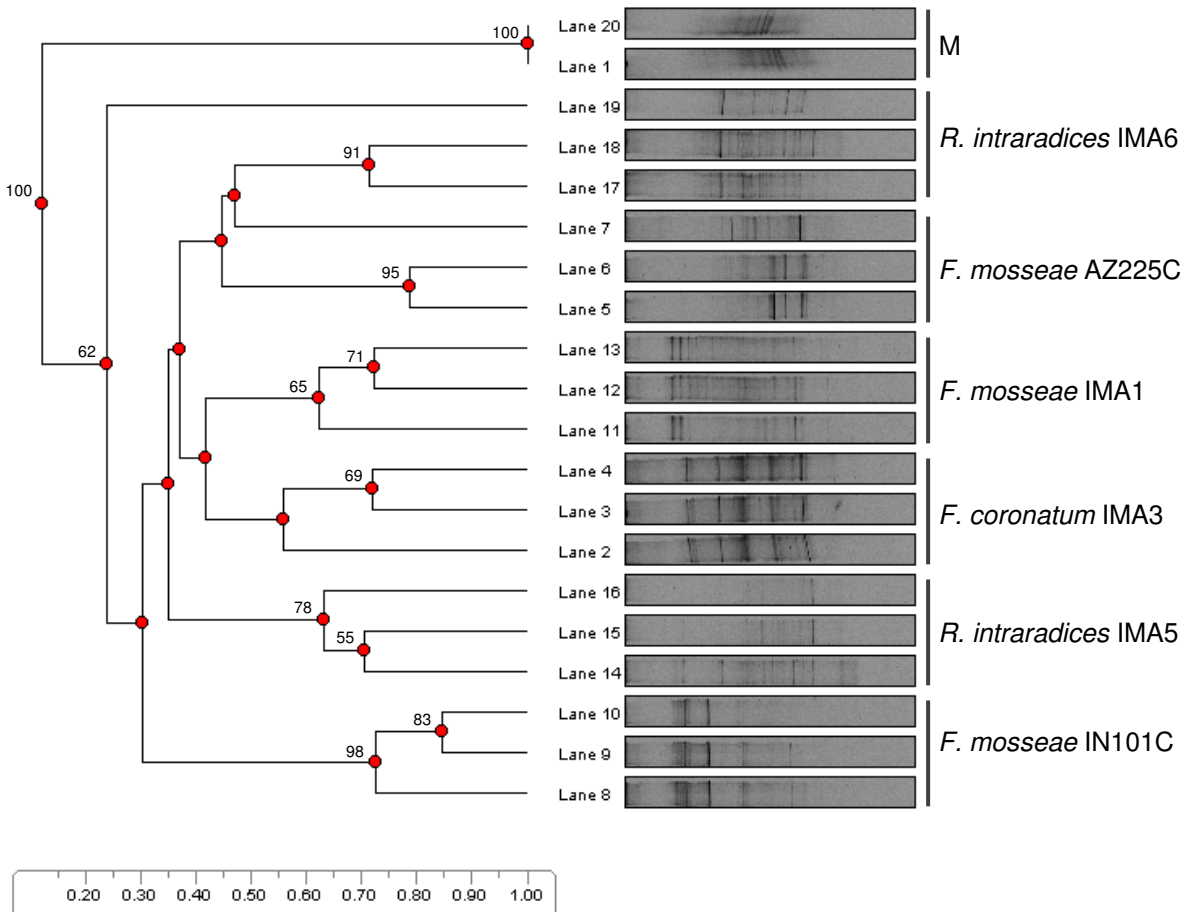
Actinomycetales
Mollicutes related endobacteria
Burkholderiales
Rhizobiales
Bacillales
Pseudomonadales
Uncultered

Fig. 4


100
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Lane 20
Lane 1
Lane 19
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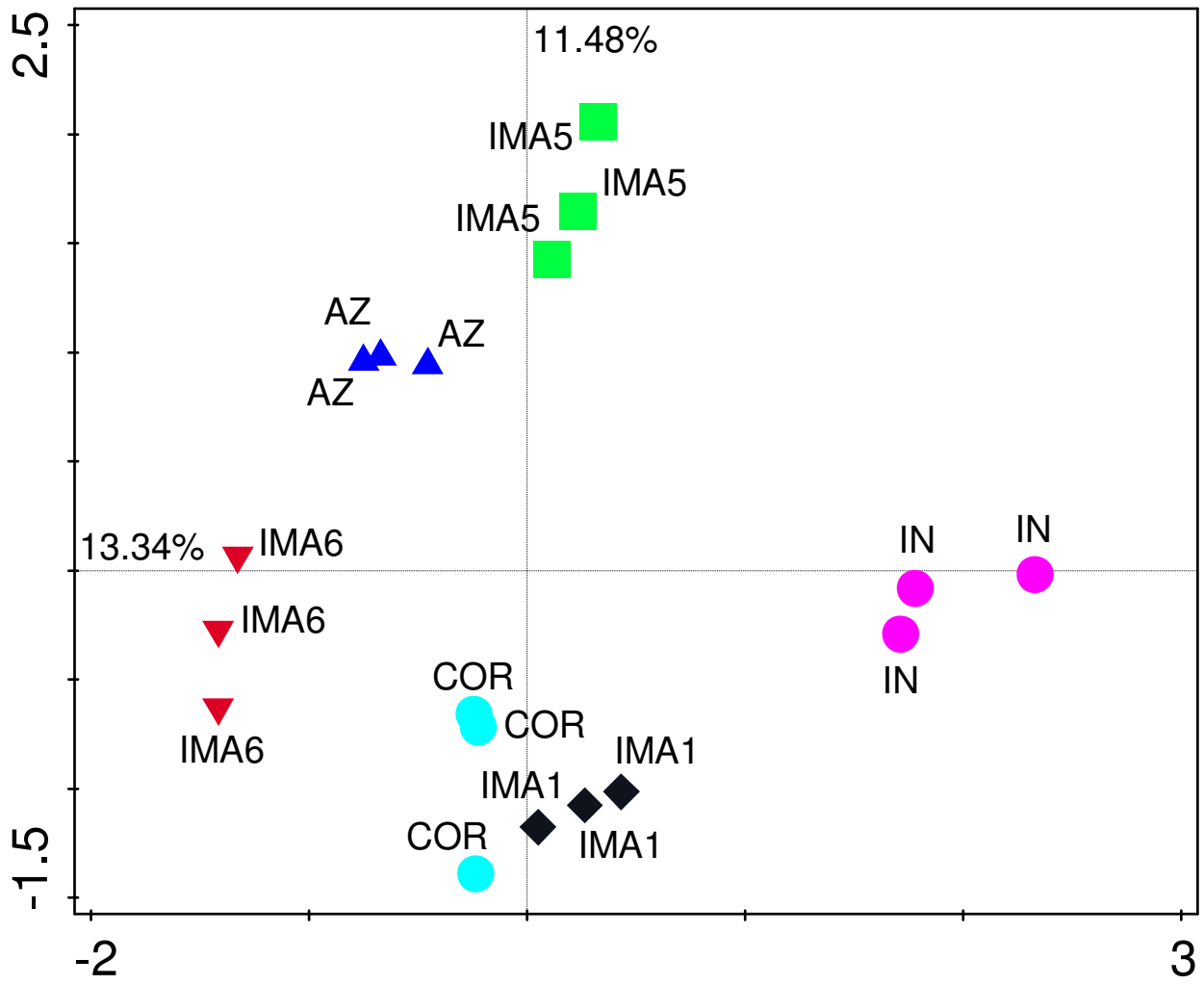
Lane 13
Lane 12
Lane 11
Lane 4
Lane 3
Lane 2
Lane 16
Lane 15
Lane 14
Lane 10
Lane 9
Lane 8
M
R. intraradices IMA6
F. mosseae AZ225C
F. mosseae IMA1
F. coronatum IMA3
R. intraradices IMA5
F. mosseae IN101C
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00

Fig. 5


2.5
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-2
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13.34%
IMA5
IMA5
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AZ
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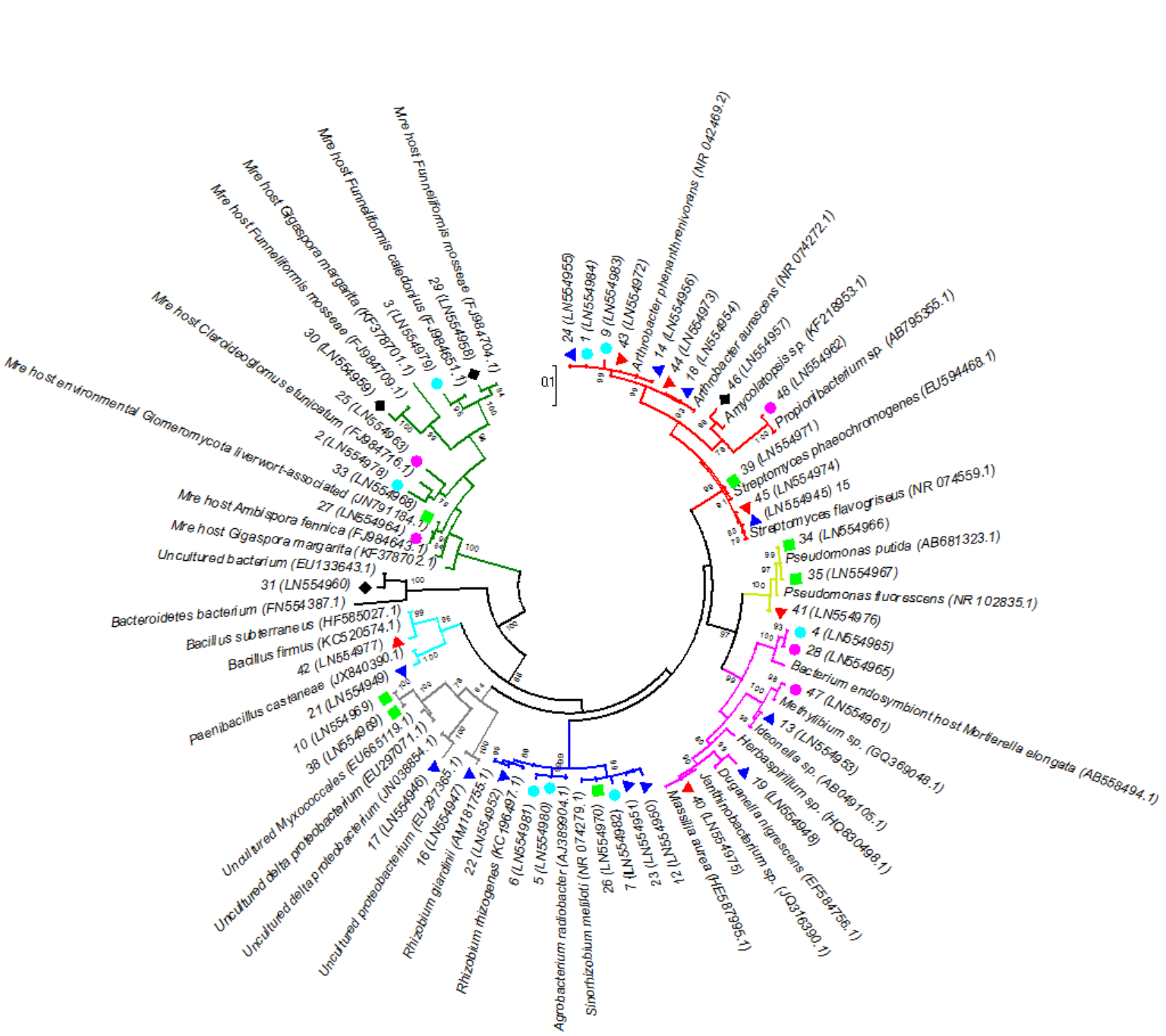
IMA1
IMA1
IMA1

Fig. 6


0.1
24 (LN554955)
1 (LN554984)
9 (LN554983)
43 (LN554972)
Arthrobacter phenanthrenivorans (NR 042469.2)
14 (LN554956)
44 (LN554973)
18 (LN554954)
Arthrobacter aurescens (NR 074272.1)
46 (LN554957)
Amycolatopsis sp. (KF218953.1)
48 (LN554962)
Propionibacterium sp. (AB795355.1)
39 (LN554971)
Streptomyces phaeochromogenes (EU594468.1)
45 (LN554974)
45 (LN554945) 15
Streptomyces flavogriseus (NR 074559.1)
34 (LN554966)
Pseudomonas putida (AB681323.1)
35 (LN554967)
Pseudomonas fluorescens (NR 102835.1)
41 (LN554976)
4 (LN554985)
28 (LN554965)
Bacterium endosymbiont host Mortierella elongata (AB558494.1)
47 (LN554961)
Methylibium sp. (GQ369048.1)
13 (LN554953)
Ideonella sp. (AB049105.1)
Herbaspirillum sp. (HQ830498.1)
19 (LN554948)
Duganella nigrescens (EF584756.1)
Janthinobacterium sp. (JQ316390.1)
40 (LN554975)
Massilia aurea (HE587996.1)
12 (LN554950)
23 (LN554951)
7 (LN554982)
26 (LN554970)
Sinorhizobium meliloti (NR 074279.1)
Agrobacterium radiobacter (AJ389904.1)
5 (LN554980)
6 (LN554981)
Rhizobium rhizogenes (KC196497.1)
22 (LN554952)
Rhizobium giardinii (AM181755.1)
16 (LN554947)
17 (LN554946)
Uncultured proteobacterium (EU297365.1)
Uncultured delta proteobacterium (UN038854.1)
Uncultured delta proteobacterium (EU297071.1)
Uncultured Myxococcales (EU665119.1)
38 (LN554968)
10 (LN554969)
21 (LN554949)
Paenibacillus castaneae (JX840390.1)
42 (LN554977)
Bacillus firmus (KC520574.1)
Bacillus subterraneus (HF585027.1)
Bacteroidetes bacterium (FN554387.1)
31 (LN554960)
Uncultured bacterium (EU133643.1)
Mre host Gigaspora margarita (KF378702.1)
Mre host Ambispora fennica (FJ984643.1)
27 (LN554964)
Mre host environmental Glomeromycota liverwort-associated (JN791184.1)
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2 (LN554978)
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25 (LN554963)
30 (LN554959)
Mre host Funneliformis mosseae (FJ984709.1)
Mre host Gigaspora margarita (KF378701.1)
3 (LN554979)
Mre host Funneliformis caledonius (FJ984651.1)
29 (LN554958)
Mre host Funneliformis mosseae (FJ984704.1)

Fig. 7