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Investigating the endobacteria which thrive in arbuscular mycorrhizal fungi

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1 Investigating the endobacteria which thrive in Arbuscular Mycorrhizal Fungi

2

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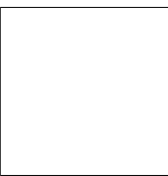
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12 Running head: The Endobacteria Thriving in Arbuscular Mycorrhizal Fungi

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2Summary

3The study of the so-called “unculturable bacteria” is still considered a challenging task.
4However, the current availability of culture-free approaches and the improvement in their
5sensitivity allows the identification and characterization of such microbes in complex
6biological samples.

7In this chapter we report how endobacteria thriving inside arbuscular mycorrhizal fungi
8(AMF), which are themselves obligate biotrophs of plants, can be studied using a
9combination of *in vitro* culture, molecular biology and microscopy techniques.

10

11**Key words:** Endobacteria, Arbuscular mycorrhizal fungi, *Candidatus* Glomeribacter
12gigasporarum, Mollicutes-related endobacteria, Transmission electron microscopy,
13Fluorescence *in situ* hybridization, Real-time quantitative PCR, Bacterial enrichment

2



11. Introduction

2Thousands of microbes are commonly associated with plant roots, forming the so-called
3root microbiota which plays a pivotal role on plant life. Among them, a group of soil fungi
4that colonize the roots of most land plants, the arbuscular mycorrhizal fungi (AMF), has a
5key role improving mineral nutrition and protection of their host plant. AMF, which
6belong to the ancient phylum Glomeromycota (1), have been considered as the oldest
7group of fungi capable to positively interacting with plants: they have been hypothesized
8to be crucial for the terrestrialization of first land plants c. 450 Mya (2). In addition to some
9distinctive features (AMF are asexual, multinucleated and obligated biotrophs),
10Glomeromycota may harbour endobacteria in their cytoplasm (3). Two types of
11endobacteria have been so far described and identified in AMF: a rod-shaped Gram-
12negative β -proteobacterium called *Candidatus Glomeribacter gigasporarum* (CaGg) (4) and a
13coccoid bacterium which represents a still enigmatic taxon of Mollicutes-related
14endobacteria (Mre) (5). Differently from Mre, that show a wide distribution across the
15Glomeromycota, the presence of CaGg is limited to the Gigasporaceae family. CaGg has
16been deeply investigated: its genome sequence has revealed that the endobacterium is
17nutritionally dependent on the fungus and has a potential role in providing its host with
18essential factors like vitamin B12 (6). The fungus, on the contrary, is not obligately
19dependent on the endobacterium (7). However the removal of CaGg causes some
20morphological changes in *Gigaspora* spores and a reduced proliferation of the presymbiotic
21fungal hyphae. By contrast, information on Mre is extremely limited: based on 16S rRNA
22gene sequences, this novel bacterial taxon is sister to a clade encompassing the
23Entomoplasmatales and Mycoplasmatales (5) and shows high level of sequence variability.
24Interestingly, CaGg and Mre have been simultaneously detected in some *Gigaspora*
25*margarita* isolates (fam. Gigasporaceae) hosting what has been described as a new fungal
26microbiota (8). Thus, single or multiple bacterial populations can thrive inside AMF that,
27being themselves obligate symbionts, need a host plant to complete their life cycle.
28Consequently, this complex “Russian dolls-like” organization is difficult to dissect with
29traditional culture-based techniques. Further, the endobacteria so far described in AMF
30are considered unculturable microbes, thus they cannot be obtained in pure culture.

In this chapter, we present several techniques that can be applied to investigate the endobacteria thriving in AMF. First, we describe three methods which have been set up to obtain the fungal material and, thereby, their associated endobacteria. The in pot culture is the recommended method for *G. margarita* routine propagation and the obtainment of large amounts of spores. The Millipore sandwich allows to produce clean and intact extraradical mycelium, while the root organ cultures (ROCs) can be used to obtain *in vitro* spores and mycelium. As for bacteria living inside insect (9), the demonstration of the intracellular localization is the first criterion to speak about endobacteria. Therefore, as a second step, we illustrate the protocols to process the biological material for transmission electron and confocal microscopy, in order to detect the endobacteria and describe their morphology. Third, we present some molecular techniques that make possible a direct investigation of the endobacteria from their fungal hosts. Having as a pre-requisite the availability of specific primer pairs, the PCR permits to identify the endobacteria, whereas the real-time quantitative PCR (qPCR) allows to assess their abundance. Fluorescence *in situ* hybridization (FISH) permits to reveal and simultaneously localize the presence of endobacteria in AMF spores. Last, we describe a filtering based protocol first described by Ghignone and colleagues (6) that allows to obtain a spore lysate enriched in the endobacterial component with a limited carryover of fungal nuclei. All the techniques described in this chapter have been applied with success to the study of the endobacteria thriving in AMF (5-8, 10-12).

The presence of endobacteria thriving inside the cytoplasm of fungi has been reported few times so far. In addition to Glomeromycota, endobacteria have been described inside other group of fungi, such as Mucoromycotina. Among them, *Rhizopus microsporus*, a rice pathogenic fungus whose pathogenicity is related to the internal presence of a strain of *Burkholderia rhizoxinica* (13); *Mortierella elongata*, a filamentous fungus which hosts a *Burkholderia*-related endobacterium (14); *Endogone*, one of the oldest plant-associated fungi which host Mre in fruiting body-forming spores (15). All these findings strongly suggest that the presence of endobacteria in the cytoplasm of fungi is more widespread than expected. Novel bacterial populations still wait to be discovered and characterized. As a consequence, the application of protocols which allow to unambiguously detect and

1

1 identify these bacterial dwellers of fungi will dramatically improve the knowledge of such
2 complex symbiotic systems.

3 2. Materials

4 Prepare all solutions using analytical reagents and deionized water (unless indicated
5 otherwise). Use sterile consumables and reagents, or, prior to use, autoclave them at 120°C
6 for 20 min. Wear personal protective equipment, carefully handle dangerous reagents and
7 diligently follow all waste disposal current regulations when disposing of waste materials.

8

9 2.1 Biological materials and growing media

10 The fungal material employed in the experiments (*see* sections 2.1 and 3.1) consists of
11 monoxenic inocula of the AMF *G. margarita* BEG34, purchased from specialized companies
12 and/or in-house propagated following the in pot culture technique (*see* subsection 3.1.1).

13

14 2.1.1 Pot cultures

15 1. Plastic pots (0.9 L volume).

16 2. Sterilized quartz sand (oven-sterilized at 180°C for 3 h).

17 3. *Trifolium repens* (clover) seeds (*see* Note 1).

18 4. Clean collected AMF spores.

19 5. Long Ashton fertilization solution (16), modified (low phosphate): 0.75 mM MgSO₄
20 7H₂O, 1 mM NaNO₃, 1 mM K₂SO₄, 2 mM CaCl₂ 2H₂O, 32 μM Na₂HPO₄, 0.025 mM Fe
21 Na EDTA, 0.005 mM MnSO₄ 12H₂O, 0.00025 mM CuSO₄ 5H₂O, 0.0005 mM ZnSO₄
22 7H₂O, 0.025 mM H₃BO₃, 0.0001 mM Na₂MoO₄ 2H₂O.

23 6. Sieves (aperture 100 μm).

24

2

1

12.1.2 Spore sterilization

2 1. Ultrapure water.

3 2. Chloramine T.

4 3. Streptomycin sulphate.

5

1

12.1.3 *Lotus japonicus* seeds sterilization and germination

- 2 1. Sulfuric acid (99.99%).
- 3 2. Sterile water.
- 4 3. Agar-H₂O (0.6% Agar) plates, diameter 9 cm.

5

62.1.4 Millipore sandwich method

- 7 1. Agar-H₂O (0.6% Agar) plates, diameter 9 cm.
- 8 2. Magenta boxes.
- 9 3. Sterile cellulose nitrate membranes (pore size 45 µm).
- 10 4. Sterilized quartz sand (oven-sterilized at 180°C for 3 h).
- 11 5. 1:2 diluted modified Long Ashton solution (*see* subsection 2.1.1).
- 12 6. Sterilized AMF spores.
- 13 7. 3-4 days germinating *L. japonicus* seedlings.

14

152.1.5 *In vitro* fungal propagation under root organ culture (ROC) condition

- 16 1. *Cichorium intybus* (chicory) transformed root cultures (*see* Note 2).
- 17 2. Sterilized AMF spores.
- 18 3. Minimal (M) Medium (17): 3 mM MgSO₄ 7H₂O, 0.79 mM KNO₃, 0.87 mM KCl, 1.22
- 19 mM Ca(NO₃)₂ 4H₂O, 35.0 µM KH₂PO₄, 21.7 µM Na Fe EDTA, 4.5 µM KI, 30.3 µM MnCl₂
- 20 4H₂O, 9.2 µM ZnSO₄ 7H₂O, 24.0 µM H₃BO₃, 0.5 µM CuSO₄ 5H₂O, 0.01 µM Na₂MoO₄
- 21 2H₂O, 40 µM glycine, 0.3 µM Thiamin HCl, 0.5 µM Pyridoxin HCl, 4 µM Nicotinic Acid,
- 22 277 µM Myo-inositol, 10 g/L Sucrose, Phytagel 4 g/L, pH 5.5.

23

2

12.2 Morphological analyses

22.2.1 Transmission electron microscopy

3 1. Ultramicrotome with glass and diamond knives.

4 2. 1% toluidine blue (w/v).

5 3. Polypropylene spot plate (well Ø 2 cm).

6 4. Hot plate.

7 5. Uranyl acetate solution: prepare a saturated solution by dissolving uranyl acetate in
8 ddH₂O.

9 6. NaOH (pellets).

10 7. Lead citrate solution.

11 ● Solution A (Lead nitrate stock solution): add 31.25 g in 500 mL of doubled-
12 distilled water (ddH₂O). Add 10 drops of HNO₃ 10N (HNO₃ 10N: 630 g in 1
13 L of ddH₂O).

14 ● Solution B (Sodium citrate stock solution): add 41.50 g in 500 mL of ddH₂O.
15 Add 5 drops of solution A.

16 ● Solution C (NaOH 1N): add 0.2 g in 5 mL of ddH₂O.

17 Prepare the solution as follows: 2.1 mL solution A + 2.1 mL solution B. Mix. Add
18 0.8 mL solution C. Mix.

19 Solution A and B can be stored at room temperature. Solution C must be freshly
20 prepared every time.

21 8. Philip CM10 transmission electron microscope (FEI, Hillsboro, OR, USA).

22

232.2.2 Confocal microscopy

1

1 1. SYTO 9[®] Green-Fluorescent Nucleic Acid Stain (Life Technologies, Carlsbad, CA,
2 USA), 5 mM solution in DMSO. Store at -20°C and protect from light. Freshly prepared
3 working solution for bacterial (50 nM–20 µM) and eukaryotic (10 nM–5 µM) cell
4 visualization: make a 1:1000 dilution in ultrapure water. Vortex to mix. Store on ice and
5 protect from light.

6 2. Leica TCS-SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

7

82.3 Molecular analyses

92.3.1 DNA extraction

10Rapid DNA extraction

11 1. 10X PCR buffer (Sigma).

12 2. Sterile 1.5 mL plastic pestles.

13CTAB-based DNA extraction

14 1. 2X CTAB (Cetyltrimethylammonium bromide) extraction buffer: 100 mM Tris-HCl,
15 pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB (w/v). Adjust the volume of the solution
16 with ultrapure water. Autoclave for 20 min at 120°C.

17 2. Proteinase K: prepare a 1 mg/mL stock solution in ultrapure water. Aliquot and
18 store at -20°C. Prepare working concentration (1 µg/mL) in ultrapure water.

19 3. RNase A (100 mg/mL). Prepare working solution (40 µg/µL) in ultrapure water
20 following the manufacturer's instructions.

21 4. Polyvinylpyrrolidone (PVP).

22 5. Phenol:chloroform:isoamyl alcohol (25:24:1).

23 6. Chloroform.

24 7. Cold 2-propanol.

2

1

1 8. 70% ethanol.

2

32.3.2 PCR

4 1. PCR reagents: 10 μ M of suitable primers (*see* **Tab. 1**), 2.5 mM of each dNTP, 5X
5 Phusion[®] HF Buffer, Phusion[®] DNA Polymerase (2U/ μ L) (Thermo Fisher Scientific,
6 Waltham, MA, USA), ultrapure water.

7 2. PCR purification and clean-up: Wizard[®] SV Gel and PCR Clean-Up System
8 (Promega, Fitchburg, Wisconsin, USA).

9

102.3.4 Cloning

11 1. Cloning vector: pGEM[®]-T Easy Vector Systems (Promega, Fitchburg, WI, USA).

12 2. Chemically competent bacterial cells: One Shot[®] TOP10 Chemically Competent
13 *Escherichia coli* (Life Technologies, Carlsbad, CA, USA).

14 3. Fresh LB plates (9 cm diameter) with ampicillin (final concentration 100 mg/mL).

15

162.3.3 Real-time qPCR

17 1. 48-well StepOne[™] Real time PCR system and StepOne[™] software (Life
18 Technologies, Carlsbad, CA, USA) or similar Real time PCR equipment.

19 2. Qubit[®] 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA)

20 3. PCR reagents: 3 μ M of each suitable primers (*see* **Tab. 1**), 2X Power SYBR[®] Green
21 PCR Master Mix (Life Technologies, Carlsbad, CA, USA), ultrapure water.

22

232.4 Fluorescence *in situ* hybridization (FISH)

2

- 1 1. Sterile 1X and 10X phosphate buffered saline (PBS), pH 7.2.
- 2 2. Fixative solution: 4% paraformaldehyde (18). Heat up 45 mL of ultrapure water at
3 55-58°C (avoid exceeding 60°C). Add 2 g of paraformaldehyde and stir with a magnet.
4 If necessary, add a few drops of NaOH 10N while stirring continuously until powder
5 dissolves. Add 5 mL of 10X PBS. Cool on ice. Adjust pH to 7.2-7.4 with HCl. Filter the
6 solution with 0.45 µm filter. Store the solution a few days at 4°C, or 2-3 weeks at -20°C.
7 Avoid repeated freeze-thaw cycles.
- 8 3. Microscope slides with 8 individual wells (well Ø 6 mm) and large cover slides.
- 9 4. Low melting point agarose.
- 10 5. 50%, 75% and 100% ethanol.
- 11 6. Coplin jars.
- 12 7. Hybridization oven.
- 13 8. Proteinase K (*see* subsection 2.3.1.2). Prepare working solution (10 µg/mL) in
14 ultrapure water.
- 15 9. Tween 20.
- 16 10. Suitable labeled oligonucleotide probes (*see* **Tab. 2**). Probes are 5'-end labeled with
17 fluorochromes like fluorescein isothiocyanate (FITC) or cyanine dyes (Cy3 or Cy5).
18 Resuspend in ultrapure water the probes to obtain a 500 ng/µL probe stocks. Aliquot
19 and store the stocks in the dark at -20°C. Dilute probe stocks at the working
20 concentration of 50-70 ng/µL with ultrapure water and aliquot in individual tubes (50
21 µL per tube) to avoid repeated freeze-thaw cycles. Store in the dark at -20°C.
- 22 11. Sterile 20X saline-sodium citrate (SSC) buffer (3 M NaCl and 0.3 M sodium citrate,
23 pH 7). Aliquot and store at -20°C.
- 24 12. 100% formamide. Aliquot and store at -20°C (*see* **Note 3**).

1

1 13. 50X Denhardt's solution: dissolve the Denhardt's powder in ultrapure water
2 according to the manufacture's instruction. Store at -20°C for up to two years. Prepare
3 25X concentrate dilutions as working solution. Aliquot and store at -20°C.

4 14. Antifade mounting medium: 1,4-Diazabicyclo[2.2.2]octane (DABCO) solution (25
5 mg/mL). Dissolve 250 mg DABCO in 10 mL 1X PBS. Add 90 mL Glycerol. Adjust pH
6 to 8.6 with HCl or NaOH. Store the solution at 4°C.

7 15. Leica TCS-SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

8

92.5 Bacterial enrichment

10 1. 0.9% NaCl in ultrapure water (w/v). Autoclave for 20 min at 120°C.

11 2. Sterile 1.5 mL plastic pestles.

12 3. Sterile 3 µm cellulose nitrate filters.

13 4. Sterile syringes and syringe filter holders.

14 5. RQ1 RNase-Free DNase (1 U/µL) (Promega, Fitchburg, WI, USA).

15

163. Methods

173.1 Biological materials and growing media

183.1.1 Pot cultures

19The in pot culture is the recommended method for *G. margarita* routine propagation and
20for the obtainment of large amounts of spores, since it represents a "nearly natural"
21method to obtain AMF material under controlled conditions.

22 1. Place the oven-sterilized quartz sand in 0.9 L pots.

23 2. Soak the sand with the modified low phosphate Long Ashton solution and let it
24 drain. Inoculate 100 *G. margarita* spores under the sand surface by pipetting.

2

1

1 3. Spread 80-100 *T. repens* seeds on the sand surface and cover them with a thin sand
2 layer.

3 4. Put the pots in a climatic chamber with a photoperiod of 16 h and a temperature of
4 23°C during the day and 21°C during the night. Keep the pots in culture for at least 3
5 months.

6 5. During the entire culturing period, fertilize the pots once a week with the modified
7 low phosphate Long Ashton solution. Water the pots with water whenever needed.

8 6. After 3 months of cultivation, collect the newly formed spores from the sand soil by
9 putting a 100 mL aliquot of substrate in a beaker and adding water. Shake the beaker
10 so that spores are temporarily kept in suspension and immediately pour the water in a
11 100 µm aperture sieve. Recover the sieve content in a large glass Petri dish by washing
12 it with water. Manually collect the spores under a stereomicroscope by pipetting with a
13 P1000 pipette or by individually collecting them with laboratory tweezers (*see Note 4*).

14

153.1.2 Spore sterilization

16The entire procedure should be performed under a biological hood.

17 1. Prepare a solution containing 3% chloramine T and 0.03% Streptomycin sulphate in
18 ultrapure water. Shake well until the powders are completely dissolved. Typically, 50
19 mL of solution are prepared to sterilize up to 2000 AMF spores.

20 2. Place the collected spores in a tube and remove the residual water. Add the
21 sterilization solution and shake the tube. Typically, 1.5 mL of sterilization solution is
22 used for 100 spores (*see Note 5*).

23 3. Place the tube horizontally, so that spores are not pelleted at the bottom, and wait 10
24 minutes.

25 4. Remove the sterilization solution by pipetting and add the same volume of ultrapure
26 water; shake well and wait 5 minutes.

2

1

1 5. Remove the water by pipetting and add the same volume of sterilization solution.

2 Shake well, place the tube horizontally and incubate 10 minutes.

3 6. Remove the sterilization solution by pipetting and add the same volume of ultrapure

4 water; shake well and wait 5 minutes.

5 7. Remove the water by pipetting and add the same volume of ultrapure water; shake

6 well and wait 10 minutes.

7 8. Repeat step 7. After having waited 10 minutes, remove by pipetting all traces of

8 water.

9Spore are now ready for subsequent treatments. If their intended use is DNA extraction,
10place tubes in liquid nitrogen and store the frozen spores at -80°C until use. If they will be
11employed for further vital manipulation, store them at 4°C for at most one week.

12

133.1.3 *Lotus japonicus* seeds sterilization and germination

14The procedure should be performed under a chemical hood and wearing suitable gloves
15until step 4, since sulfuric acid is toxic and corrosive.

16 1. Extract *L. japonicus* seeds from pods and place them in a plastic tube (*see Note 6*).

17 2. Add sulfuric acid to the tube so that seeds are completely soaked.

18 3. Mix well by vortexing and leave the seeds soaked for 3 minutes.

19 4. Eliminate the sulfuric acid by pipetting and rinse the seeds with sterile water for 10

20 min.

21 5. Repeat step 4 twice.

22 6. Under a biological hood, take individual seeds with flame-sterilized tweezers and

23 place them on Agar/H₂O plates. Put approx. 5 seeds per plate.

2

1 7. Incubate plates containing the sterilized seeds in the dark at 22°C for 4 days, then put
2 them in the light at the same temperature until the cotyledons become green (*see Note*
3 7).

4

53.1.4 Millipore sandwich method

6The Millipore sandwich method is the technique of choice to obtain clean and intact *G.*
7*margarita* extraradical mycelium, allowing at the same time the collection of colonized root
8portions. The entire procedure should be performed under a biological hood.

- 9 1. Fill the Magenta boxes with quartz sand for 1/3 of their volume. Autoclave for 20
10 min at 120°C.
- 11 2. Pick an autoclaved cellulose nitrate membrane with flame-sterilized tweezers and
12 place it on an Agar-H₂O plate to let it moisten.
- 13 3. Take a germinated *L. japonicus* seedling (*see* subsection 3.1.3) and place it on the
14 membrane, with the shoot apex going beyond the edge.
- 15 4. Collect with a pipette 20-25 *G. margarita* spores and place them below the seedling
16 rootlet; eliminate the excess liquid by pipetting.
- 17 5. Pick a second cellulose nitrate membrane with flame-sterilized tweezers and
18 superpose it to the first one to close the sandwich.
- 19 6. Open the Magenta box and soak the sand with the 1:2 diluted modified Long Ashton
20 solution.
- 21 7. With long, thick laboratory tweezers dig a groove in the sand, at the center of the
22 Magenta box.
- 23 8. With long, thick laboratory tweezers take the previously prepared sandwich from
24 the Agar-H₂O plate and place it vertically in the groove, with the plantlet apex upside.
25 Gently close the groove and soak again with the 1:2 diluted modified Long Ashton
26 solution (*see Note 8*).

1

1 9. Eliminate the excess of 1:2 diluted modified Long Ashton solution by pipetting and
2 close the Magenta box.

3 10. Incubate the Magenta boxes in a climatic chamber with a photoperiod of 16 h and a
4 temperature of 23°C during the day and 21°C during the night.

5 11. After 30 days, disassemble the sandwiches and collect the material under a
6 stereomicroscope.

7 12. For the external mycelium, peel the root surface and place the collected hyphal
8 bundle in tubes; for mycorrhizal roots, cut the root fragments in small pieces with a
9 sharp scalpel and collect the material in tubes. Freeze the samples in liquid nitrogen
10 and store them at -80°C until use.

11

123.1.5 *In vitro* fungal propagation under root organ culture (ROC) conditions

13 Root organ cultures can be used to monoxenically produce *in vitro* *G. margarita* BEG34
14 spores and mycelium (*see Note 9*).

15 1. Propagate clones of root-inducing T-DNA-transformed roots previously established
16 by subculturing them every 4 weeks on minimal (M) medium in round Petri dishes (9
17 cm diameter). Keep the dishes in the dark at 26°C.

18 2. Using flame-sterilized tweezers, transfer a 4-5 cm long T-DNA transformed root
19 fragment in the center of a round Petri dish (9 cm diameter) containing M medium.
20 Gently sink the root explant below the medium surface with tweezers to avoid
21 desiccation.

22 3. Transfer about 10 sterilized *G. margarita* spores in the plate, all around the
23 transformed root explant.

24 4. Carefully seal the dishes with Parafilm and incubate them in the dark at 26°C.

2

1

1 5. A new spore generation is produced within 1.5-2 months as a result of mycorrhizal
2 colonization. To keep the spores sterile, collect them under a biological hood using
3 flame-sterilized tweezers.

4

53.2 Morphological analyses

63.2.1 Transmission electron microscopy

7In order to preserve fungal structures and organelles, as well as the small endobacteria,
8single spores were processed by using cryo-methods, that is, high-pressure and freeze-
9substitution preparation. Subsequently, spore samples were infiltrated with
10Epon/Araldite resin and then embedded in resin blocks. For details on the cryo-
11preparation and the subsequent resin infiltration and polymerization refer to (8). In this
12section, we provide details on the processing of the samples for transmission electron
13microscopy starting from the sectioning of the resin blocks.

14 1. Pre-warm the hot plate to about 50°C.

15 2. After embedding, the resin blocks are sectioned by using an ultramicrotome. Cut the
16 blocks into semithin sections (1 μm) with a glass knife.

17 3. Place the sections on a microscope slide. Stain the sections with 1% toluidine blue
18 (w/v). Let the microscope slide dry on the hot plate (about 50°C). Observe the stained
19 sections under a light microscope for the orientation of the sample within the block.
20 Select a small area of the section for ultrathin sections.

21 4. Cut the selected area of the block into ultrathin sections (70 nm) with a diamond
22 knife. Treat ultrathin sections as floating sections until the end of the counterstaining
23 step.

24 5. Prepare a humid chamber to prevent the sections from drying out. Place a wet paper
25 towel inside a covered Petri dish (\varnothing 9 cm). Put a spot plate on the paper towel.

26 6. Fill a spot plate well with 500 μL of uranyl acetate solution. Lay down the sections
27 on the solution for 20 min. Cover the Petri dish and put it on the hot plate (about 50°C).

2

1

1 Place a piece of aluminium foil on the cover of the dish to keep the samples in the dark
2 during the staining.

3 7. Rinse twice the sections in water for 10 min.

4 8. Fill a spot plate well with 500 μ L of lead citrate solution. Lay down the sections on
5 the solution for 2-3 min. In order to prevent excessive lead citrate precipitation by
6 exposure to CO₂, add NaOH pellets (~10 g) near the spot plate. Cover the Petri dish
7 during the staining.

8 9. Rinse twice the sections in water for 10 min.

9 10. Mount the sections on a 200- or 300-mesh copper grids.

10 11. Observe under a transmission electron microscope (*see Fig. 1a, b*).

11

123.2.2 Confocal microscopy

13 Prepare a fresh aliquot of the SYTO 9[®] Green-Fluorescent Nucleic Acid Stain working
14 solution (1:1000 dilution in ultrapure water). Store on ice and protect from light.

15Spore processing

16 1. Transfer sterilized spores (1-3 spores per slide) on a microscope slide (*see Note 10*).

17 2. Add a 40-100 μ L drop (depending on the size and number of spores) of SYTO 9[®]
18 directly on the spores.

19 3. Add a large cover slide covering the entire microscope slide. Slightly press the cover
20 slide down until you crash the spores.

21 4. Incubate the microscope slide for 5 min in the dark.

22 5. Observe under a confocal microscope (*see Note 11*) (*see Fig. 1c*).

23Bacterial suspension processing

24 1. Transfer 10 μ L of filtered bacterial suspension on a microscope slide (*see section 2.6*).

2

1

1 2. Add 10 μL drop of SYTO 9[®] directly on the bacterial suspension and mix gently by
2 pipetting.

3 3. Add a large cover slide covering the entire microscope slide.

4 4. Incubate the microscope slide for 5 min in the dark.

5 5. Observe under a confocal microscope (*see Note 11*).

6

73.3 Molecular analyses

8 In order to avoid contaminations carry out all steps under a biological hood (unless
9 indicated otherwise). Prior to use, clean all the instruments (*i.e.* micropipettes, tube racks,
10 centrifuge, etc.). Use sterile filter tips.

11

123.3.1 DNA extraction

13 Rapid DNA extraction

14 1. Prepare a fresh aliquot of extraction buffer with 10X PCR buffer:ultrapure water (1:1
15 dilution).

16 2. Place one to ten sterilized spores in 1.5 mL tube.

17 3. Crush the spore in a volume of 30 μL (single spore), 50 μL (pool of 5 spores) or 70 μL
18 (pool of 10 spores) of freshly prepared extraction buffer.

19 4. Incubate crashed spores at 95°C for 15 min.

20 5. Centrifuge the crude extract at 16,000 g for 10 min.

21 6. Collect the supernatant and store -20°C.

22 CTAB-based DNA extraction

23 The procedure should be performed under a chemical hood.

2

1

1 1. Pre-warm water bath at 65°C.

2 2. Prior starting extraction, add 1% (w/v) PVP to the 2X CTAB. Prepare 1 mL per
3 sample of extraction buffer and pre-warm to dissolve PVP at 65°C.

4 2. Place one to ten sterilized spores or add up to 100 µL of bacterial suspension (*see*
5 section 3.6) in a 2 mL tube.

6 3. Add 900 µL of extraction buffer, 10 µL of proteinase K (10 µL/mL) and 3 µL of
7 RNase (25 ng/µL). Crush the spore(s) (if extracting from spores) with a 1.5 mL plastic
8 pestle.

9 4. Incubate in the water bath at 65°C for 1 h.

10 5. Centrifuge at 9,500 g for 10 min.

11 6. Transfer the supernatant in a new 2 mL tube without disturbing the pellet (if
12 present).

13 7. Add 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1). Mix by inverting the
14 tube until the solution becomes lactescent and homogeneous.

15 8. Centrifuge at 6,000 g for 10 min.

16 9. Transfer the supernatant in a new 2 mL tube without disturbing the interface.

17 10. Add 1 volume of chloroform. Mix by inverting the tube 10-15 times.

18 11. Centrifuge at 6,000 g for 10 min.

19 12. Transfer the supernatant in a new 1.5 mL tube without disturbing the interface.

20 13. Add 2/3 of the volume of cold 2-propanol. Mix by inverting the tube 10-15 times.

21 14. Incubate the tube on ice for 30-40 min.

22 15. Centrifuge at 9,500 g for 10 min.

23 16. Carefully discard the supernatant carefully to avoid dislodging the pellet.

24 17. Add 200 µL of 70% ethanol.

2

1

1 18. Centrifuge at 9,500 g for 1 min.

2 19. Remove the supernatant without disturbing the pellet.

3 20. Resuspend the pellet in 30-50 μ L of ultrapure water.

4

53.3.2 PCR

6 1. Carry out individual PCR reactions in a final volume of 20 μ L containing 1X
7 Phusion[®] HF Buffer, 375 μ M of each dNTP, 750 nM of each primer (*see Tab. 1*), 0.02
8 U/ μ L Phusion[®] DNA Polymerase, 1-4 uL (from rapid DNA extraction) or 40-50 ng
9 (from CTAB extraction) DNA template. Bring the mix to the final volume with
10 ultrapure water.

11 2. Use the following cycling conditions:

12 - Initial step of 98°C for 4 min.

13 - Cycles (cycle conditions vary according to the primer pair used):

▪ 14CaGgADf-CaGgADr: 35 cycles at 98°C for 13 s, 69°C for 30 s, 72°C for 55 s

▪ 15GlomGIGf-GlomGIGr: 30 cycles at 98°C for 10 s, 58°C for 25 s, 72°C for 30 s

▪ 16109F-1184R: 30 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 45 s (*see Note 12*)

▪ 17AML1-AML2: 35 cycles at 98°C for 10 s, 58°C for 30 s, 72°C for 35 s

▪ 18ITS1f-ITS4: 35 cycles at 98°C for 10 s, 57°C for 30 s, 72°C for 30 s

▪ 19Efgigf-Efgig2r: 30 cycles at 98°C for 10 s, 60°C for 25 s, 72°C for 20 s

▪ 20rpoBf-rpoBr: 35 cycles at 98°C for 10 s, 60°C for 30 s, 72°C for 35 s

21 - Final extension step of 72°C for 7 min.

2

1

1 3. Purify PCR products directly from an amplification reaction or extract DNA
2 fragments from agarose gel by using Wizard[®] SV Gel and PCR Clean-Up System
3 following the manufacturer's instruction.

4 4. Clone purified PCR products using the pGEM[®]-T Easy Vector System following the
5 manufacturer's instruction (*see Note 13*).

6 5. Insert the cloned vector into One Shot[®] TOP10 Chemically Competent *E. coli*
7 following the manufacturer's instruction.

8 6. Plate and grow transformed *E. coli* cells in the selective medium containing
9 ampicillin.

10 7. Screen clones for insert length by PCR. Select positive clones.

11 8. Sequencing.

12

133.3.3 Real-time qPCR

14 Real-time qPCR is widely used for cultivation-independent detection and quantification of
15 microorganisms. The estimation of the starting target quantities based on the amplification
16 threshold cycle in each sample allows microbes (or nuclei for multinucleate organisms) to
17 be quantified when single-copy genes are considered. The present application of qPCR can
18 be used to quantify the abundance of endobacteria in a fungal sample, to determine the
19 bacterial–fungal ratio (number of endobacteria vs number of fungal nuclei detected) and
20 to relatively quantify different endobacterial populations when simultaneously present in
21 a fungal sample (*see Note 14*). In order to avoid contaminations carry out all steps under a
22 biological hood. Use sterile filter tips.

23 1. Prepare serial dilution of the plasmids containing the target DNA sequences (*see*
24 subsection 3.3.4). Quantify plasmids with the Qubit[®] 2.0 fluorometer and estimate the
25 copy number/ μL based upon the molecular weight of the template. Generate serial
26 plasmid dilutions so that a 10^6 to 10^1 plasmid copies are present in 1 μL of sample
27 solution (*see Note 15*).

2

1 2. Serially dilute by 10 fold the fungal sample(s) to be tested in qPCR (*see Note 16*).

2 3. Carry out individual real-time qPCR reactions in a final volume of 20 μ L containing
3 2X real time mix, 150 nM of each primer (*see Tab. 1*) and 1 μ L of appropriate DNA
4 dilution. Bring the mix to the final volume with ultrapure water. Prepare three
5 technical replicates for each sample.

6 4. Use the following cycling conditions: initial step of 95°C for 3 min, 40 cycles of 95°C
7 for 15 s followed by 60°C for 40 s, with fluorescence measurement during the 60°C step
8 (*see Note 17*). At the end of the amplification add a melting curve analysis as follows:
9 55 to 95°C with a heating rate of 0.5°C per 10 s, with continuous fluorescence
10 measurement (*see Note 18*).

11 5. As a first qPCR run, perform a standard curve using plasmid serial dilutions to
12 calculate the PCR efficiency. Do the same with the serially diluted fungal sample(s) to
13 check that PCR efficiency is comparable to that obtained from plasmid standards (*see*
14 **Note 19**). Those fungal sample(s) dilutions falling into the standard curve dynamic
15 range can be selected for the quantification of the endobacteria/fungal nuclei (*see Note*
16 **20**).

1 6. Perform the quantification assay. Set up the qPCR plate so that both fungal sample(s)
2 and plasmid serial dilutions are amplified in the same run. The number of target DNA
3 sequences present in each PCR mixture is calculated by comparing the crossing points
4 of the sample TCs with those of the standard plasmids using the StepOne™ software. If
5 diluted fungal sample(s) were used for the quantification run, multiply the figure
6 obtained by the dilution factor to retrieve the actual number of target DNA sequences
7 present in the starting sample. If diluted fungal sample(s) were used for the
8 quantification run, multiply the figure obtained by the dilution factor to obtain the
9 actual number of target DNA sequences present in the starting sample.

10

113.4 Fluorescence *in situ* hybridization (FISH)

12 1. Prior to hybridization, fix sterilized spores in fresh and cold 4% paraformaldehyde
13 buffered with PBS. Incubate the spores at 4°C for 3-6 h or, alternatively, at room
14 temperature for 1-2 h.

15 2. Remove the fixative and wash the spores three times in 1X PBS. Process the spores
16 immediately for the next hybridization steps, or suspend them in 50% ethanol in 1X
17 PBS and store at -20°C until use.

18 3. Prepare a 2% agarose solution in ultrapure water (w/v) in a small autoclaved flask.

19 4. Transfer and immobilize fixed sterilized spores (*see Note 10*) on 8-well microscope
20 slide with a 20-30 µL drop of 2% agarose (1-3 spores per well).

21 5. Dehydrate immobilized spores: plunge the entire microscope slide in an ethanol
22 series (use Coplin jars): 3 min each, first in 50% ethanol, then 75% and 100%. Let the
23 ethanol to evaporate but avoiding desiccation of the agarose drop.

24 6. Crush the spores to allow the penetration of the probes into the cytoplasm during
25 the hybridization: crush the spores by adding a cover slide on the spore-embedded
26 agarose drop, and by pressing slightly. Gently remove the cover slide.

1

1 7. Carry out a pre-hybridization treatment with proteinase K (1 $\mu\text{g}/\text{mL}$) for 10 min:
2 add 50-70 μL of proteinase K on each well (agarose drop) (*see Note 21*).

3 8. Remove proteinase K and carry out the following steps: rinse with 1X PBS for 5 min;
4 wash with 1% Tween20 in 1X PBS (freshly prepared) for 5 min; rinse twice with 1X PBS
5 for 5 min.

6 9. Pre-warm the hybridization buffer to 46°C.

7 Carry out the next steps in the dark and avoid exposing probes to the light.

8 10. During the pre-hybridization steps freshly prepare the hybridization buffer (at 35%
9 formamide stringency) in a 2 mL tube, one tube per microscope slide, as follows:

- 10700 μL of 100% formamide (final concentration 35%) (*see Note 3*)
- 11200 μL of 20X SSC (final concentration 2X)
- 12100 μL of Denhardt's solution (final concentration 1.25X)
- 131000 μL of ultrapure water (according to the volume of formamide)

14 Store on ice.

15 11. Deposit a 60 μL drop of the hybridization buffer on each well. Add 3 μL of each
16 probe (*see Tab. 2*) at the working concentration of 50-70 $\text{ng}/\mu\text{L}$ per well, directly on the
17 surface of the drop. Avoid using more than three probes (labeled with different
18 fluorochromes) at the same time (*see Note 22*). Gently mix with a pipet tip without
19 disturbing the attached agarose drop.

20 12. Prepare the humid chamber to prevent probe and buffer evaporation during the
21 hybridization: fold a paper towel and place it into a 50 mL tube. Pour the towel with
22 the remaining hybridization buffer.

23 13. Place the microscope slide horizontally inside the 50 mL tube, over the moist towel,
24 and close it tightly (the humid chamber must not dry out). Incubate in the
25 hybridization oven at 46°C for 1 h and 30 min.

2

1

1 14. After hybridization, remove the hybridization buffer and rinse the samples twice
2 with 2X SSC for 10 min and once with 0.1X SSC for 10 min. Let the microscope slide
3 dry vertically.

4 15. Mount the microscope slide with a 20-30 μ L drop of DABCO per well. Add a large
5 cover slide to cover all wells (*see Note 23*). Remove excess DABCO.

6 16. Observe the microscope slide under a confocal microscope (*see Note 11*) (*see Fig.*
7 **1d**). Store the microscope slide at -20°C in the dark for several months.

8

93.5 Bacterial enrichment for genome sequencing

10 1. Distribute 1000-1200 sterilized spores in 1.5 mL tubes (about 100 spores per tube) (*see*
11 **Note 24**).

12 2. Resuspend the spores in 400 μ L of 0.9% NaCl.

13 3. Crush the spores using a 1.5 mL plastic pestle until the spore walls are well-smashed
14 and the 0.9% NaCl becomes opaque and with a slightly pasty consistency. Use the
15 same pestle to crush spores from the same AMF isolate.

16 4. Bring final volume to 1 mL, adding 600 μ L of 0.9% NaCl.

17 5. Using flame-sterilized forceps, place the 3 μ m filter in the filter holder and connect
18 the syringe to the filter holder. Use a new 1.5 mL tube to collect the filtrate.

19 6. Transfer the suspension into the syringe and filter the crushed spores. Push
20 thoroughly twice in order to allow the bacteria to pass through the filter pores. Remove
21 the filter and place a new one in the filter holder. Repeat the passage with the other
22 100-spore batches. Maintain the tubes separated.

23 7. Centrifuge at 9,500 g for 10 min.

24 8. Gently remove the supernatant to avoid losing the pellet.

25 9. Resuspend the pellet in 30 μ L of 0.9% NaCl.

2

1

1 10. Stain with SYTO 9[®] 10 µL of filtered bacterial suspension and observe under a
2 confocal microscope (*see* subsection 3.2.2). Fungal nuclei should not be present.

3 11. Treat bacterial suspension with RQ1 RNase-Free DNase A according to the
4 manufacturer's instruction: incubation at 37°C for 30 min followed by 10 min at 65°C to
5 inactivate the enzyme. The 100-spore batch tubes are still separated. Store at -20°C.

6 12. Pool the bacterial suspension and extract genomic DNA with a CTAB-based
7 method (*see* subsection 3.3.1).

8 13. Check DNA extraction for fungal contamination using primers specific for AMF
9 and bacteria (*CaGg* and/or *Mre*) (*see* subsection 3.3.1). Fungal primers should not
10 provide any PCR amplification (*see* **Fig. 2**).

11 14. Check quantity and quality of extracted genomic DNA.

12 15. Sequencing.

13

14

15

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19 University of Turin (Local project 60%).

20

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12

13Notes

14**Note 1** *Trifolium repens* is used here as host plant for *G. margarita* propagation since it has a
15small size, suitable for climatic chamber cultivation, and provides a good yield in terms of
16AMF spores in a relatively short time. However, other host plants, such as *Sorghum bicolor*
17and leek (*Allium porrum*), can be used with success.

18**Note 2** *Cichorium intybus* T-DNA-transformed roots are used in this protocol, and they
19have been obtained as described by Fontaine and colleagues (19). However, both *Daucus*
20*carota* and *Medicago truncatula* transformed roots can be used to set-up *in vitro* cultures of
21AMF, and the protocol to obtain the ROCs of such plant species is described by Bécard
22and Fortin (17) and Boisson-Dernier and colleagues (20), respectively.

23**Note 3** Avoid to subject formamide to freeze-thaw cycles. After thawing an aliquot, it
24should be stored at 4°C and shortly used. Adjust the concentration of the formamide
25depending on the stringency necessary for the used probes.

1**Note 4** If, after collection, the spores still look very dirty (*i.e.* several residues are attached
2to the spore surface), a mild sonication can be added prior to perform the sterilization (for
3not more than 30-40 sec).

4**Note 5** Depending on the spore amount being sterilized the suitable tube should be
5chosen. As an example, use 1.5 mL tubes to sterilize individual batches of 100 spores each,
6and 50 mL tubes when groups of 1000 spores are treated together.

7**Note 6** Depending on the seed amount being sterilized the suitable plastic tube should be
8chosen. As an example, use 2 mL tubes to sterilize individual batches of 20 seeds each, and
915 mL tubes when up to 100 seeds are treated together.

10**Note 7** After 4 days of germination in the dark, check whether the germination occurred
11and, if so, place the Petri dishes in the light, otherwise wait two-three days more. The
12seedlings are ready to be transplanted when cotyledons become green. From that moment,
13they can be kept in the Petri dish prior to use for at most one week.

14**Note 8** The sandwich, composed by two cellulose nitrate membrane containing the *L.*
15*japonicus* seedling and the *G. margarita* spores should be handled with extreme care; it
16should be placed in the Magenta box so that approximately 2/3 of its height is embedded
17in the sand.

18**Note 9** The mycorrhizal efficiency is fast reducing in subsequent ROC cycles for this
19specific AMF isolate. Furthermore, the population of the endobacterium *Candidatus*
20*Glomeribacter gigasporarum* is dramatically reduced in successive spore generations
21obtained with this cultivation method, and this effect is further amplified when single
22spore inocula are employed (7). Thus, it is recommended not to perform more than one
23ROC cycle to monoxenically produce *G. margarita* BEG34, unless the aim of the experiment
24is to obtain a cured line of the fungus, which is devoid of endobacteria (7).

25**Note 10** When transferring the spores on a microscope slide, remove with a micropipette
26or absorb with blotting paper the remaining liquid (*i.e.* PBS, ethanol-PBS mixture, water).

Note 11 FITC and SYTO 9[®] fluorescence is excited at 488 nm and imaged with an emission window at 500 – 540 nm. Cy3 fluorescence is excited at 546 nm and imaged at 550 – 600 nm. Cy5 fluorescence is excited at 633 nm and imaged at 640 – 700 nm.

Note 12 If the primer pair 109F-1184R (5) primer pair does not succeed, use a semi-nested PCR approach. Carry out a first PCR with 109F and 1387R [5'-GGGCGGWGTGTACAAGGC-3', (21)]: cycling conditions were the same mentioned for 7109F-1184R but with 55 s of extension in the cycles. Then apply a semi-nested PCR using the reverse primer 1184R: cycling conditions were the same mentioned for 109F-1184R but with 27 cycles. Use semi-nested approach for particularly difficult templates (*i.e.* templates from scarce or poor quality starting material).

Note 13 Certain DNA polymerases add a single adenine to the 3' ends of amplified DNA fragments. The pGEM[®]-T Easy linearized Vector contains a single 3' terminal thymidine at each end which binds to the A overhang added by the polymerase. However, the Phusion[®] DNA Polymerase used in this protocol, as the other DNA polymerases that have a proofreading function, produce greater than 95% blunt-end fragments. Thus, PCR fragments generated with such proofreading enzymes should be tailed at 72°C for 15 min with dATP (200 µM final concentration) prior to cloning into the pGEM[®]-T Easy Vector.

Note 14 The primer pairs GlomGIGf-GIGrA (10, 7) and RpoBRTf-RpoBRTTr (11) were used to detect and quantify *CaGg* inside *G. margarita* BEG34, whereas the primer pair Efgig2f-Efgigr (11) was used to target the fungal host DNA. The same qPCR approach was used to relative quantify the two bacterial populations (Mre and *CaGg*) hosted inside *G. margarita* CM23, using the primer pairs CMsAD1f-CMsAD2r (8) and CaGgAD7f-CaGgAD6r (8) designed to specifically target the 16S rRNA gene of Mre and *CaGg*, respectively.

Note 15 Since the endobacteria AMF are to date considered unculturable microbes, like their fungal host, plasmids carrying the target DNA inserts were used for the construction of the standard curve for each target gene.

Note 16 The maximum dilution to be used in qPCR assays depends on the concentration of the starting material. For the quantification to be reliable, the fungal samples to be

1quantified must generate threshold cycles (TCs) that fall in the dynamic range established
2with the standard curve generated by plasmid amplification (*see Note 18 and 19*).

3**Note 17** qPCR primers here described were designed so that their melting temperature is
4between 65 and 70°C and the amplified fragment for each primer pair is comprised
5between 80 to 150 bp. If these parameters are not respected, change annealing temperature
6and time accordingly.

7**Note 18** Melting curve analysis is necessary to assess that only a target-specific
8amplification occurs, and the absence of primer-dimer formation.

9**Note 19** The amplification efficiency (E) can be obtained from the slope of the generated
10standard curve using the following formula: $E = (10^{(-1/\text{slope})} - 1) * 100$. This value should
11be between 90% and 105% for each primer pair and on each tested template (whether
12coming from plasmid or from total DNA extraction). Since the precision of microbial
13quantification using qPCR relies on the assumption that the unknown sample and the
14standard solutions share a comparable PCR efficiency, this should be verified prior to
15perform qPCR quantification.

16**Note 20** The qPCR output recorded for each sample is represented by threshold cycle (TC),
17which is the intersection between an amplification curve and the threshold line in the
18qPCR graph. The dynamic range represents the TC interval in which the linearity of the
19target quantity with the TCs has been verified for those specific reaction conditions, and
20within which the absence of an inhibition effect and the sensitivity of the amplification are
21assessed. Thus, the fact that the sample TCs fall in the dynamic range established with the
22standard curve assures the reliability of the quantification.

23**Note 21** Prior to hybridization, prepare negative controls treating the samples with RNase
24A (40 µg/µL).

25**Note 22** In addition to *CaGg*- and/or *Mre*-specific probes, use a non-specific probe, such as
26the universal bacterial probe EUB338 (18), as positive control. Use also a negative control
27probe which specifically targets other bacterial taxa, such as the *Buchnera*-specific probe
28ApisP2a (22) Koga *et al.*, 2003). As further negative control, use non-sense probes, such as

1the probe non-BLOsADf2, which is the reverse complement of the probe BLOsADf2 (12).
2Non-sense probes have no known rRNA target, thus ensuring that non-specific probe
3incorporation into the samples does not occur: they must not provide any fluorescent
4signal.

5**Note 23** DABCO drops should spread when the cover slide is placed. If not, slightly press
6the cover slide down until DABCO is homogeneously distributed. Due to the thickness of
7the agarose drops, it could happen that the cover slide does not adhere well to the
8microscope slide. If so, to avoid DABCO and samples from drying out, use nail polish to
9seal the agarose drop-formed space.

10**Note 24** The number of spores to be used as starting material to carry out the bacterial
11enrichment can vary depending on the goal of the experiment (*i.e.* bacterial genome
12sequencing, FISH on bacterial suspension, DNA extraction, etc.), the abundance of the
13endobacteria within the spores, and the size of the spores. This protocol describes the steps
14necessary to prepare the material for the genome/metagenome sequencing of the
15endobacteria from *Gigaspora margarita* (BEG34 and MR104) and *Racocetra verrucosa*
16(VA105B) isolates. 1000 (for BEG34) - 1200 (for MR104 and VA105B) spores used as
17starting material allow the obtainment of about 1 ug of enriched bacterial DNA. *G.*
18*margarita* and *R. verrucosa* produce relatively big spores (mean spore Ø 321 and 308 µm,
19respectively), but different AMF isolates or species could require a lower/higher spore
20number to be used as starting material for a bacterial enrichment and following
21genome/metagenome sequencing.

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Table 2
List of the oligonucleotide probes used in FISH experiments

Target gene	Organism	Probe	Probe sequence (5'-3')	Fluorochrome	References
16S rRNA gene	<i>Ca Gg</i>	CaGgADf1	CTATCCCCCT CTACAGGAYAC	Cy5	Desirò et al. [9]
	<i>Mre</i>	BLOsADf2	ATCCRTAGACC TTCMTCCTTC	Cy3	Desirò et al. [15]
	Bacteria	EUB338	GCTGCCCTCCC GTAGGAGT	Fluorescein	Amann et al. [26]
	<i>Buchnera</i>	Apis2Pa	CCTCTTTGGG TAGATCC	Fluorescein	Koga et al. [27]
None	None	non-CaGgADf1	GTRTCCTGTAG AGGGGGATAG	Cy5	Desirò et al. [9]
		non-BLOsADf2	GAAGGAKGAAGGT CTAYGGAT	Cy3	Desirò et al. [15]

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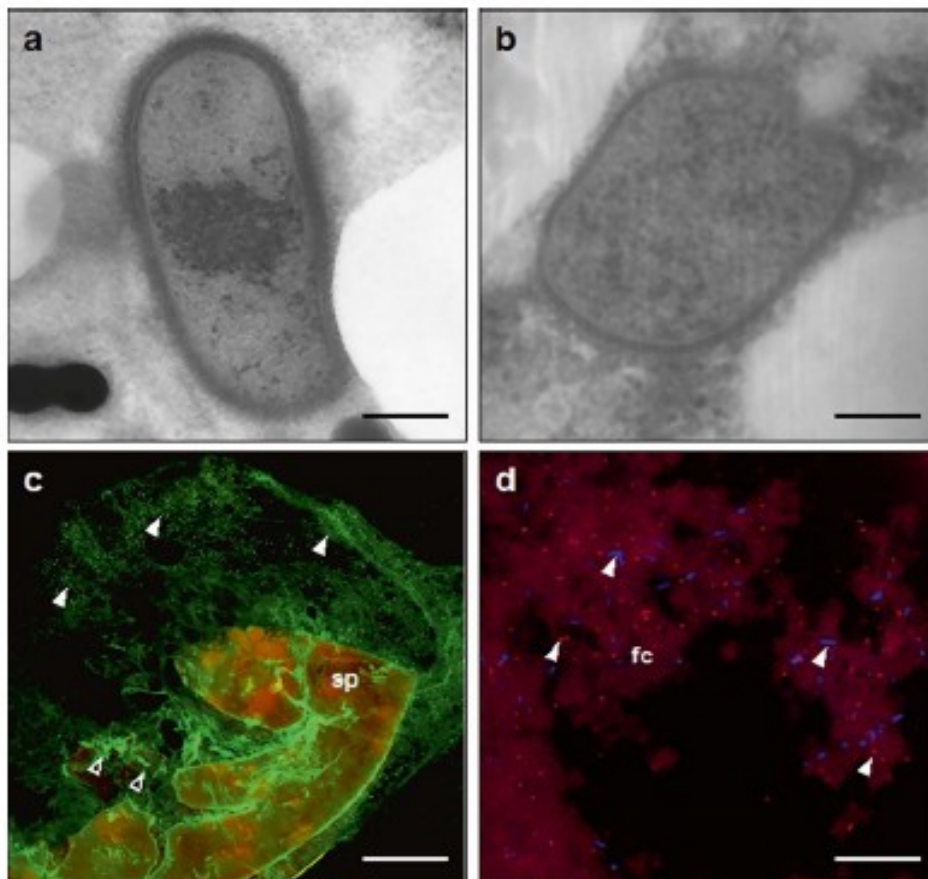


Fig. 1 Transmission electron and confocal microscopy of *Gigaspora margarita* (a, c, d) and *Rhizophagus clarus* (b) spores. Ultrastructure of (a) the rod-shaped *Candidatus Glomeribacter gigasporarum* and (b) the coccoid *Molluscites*-related endobacterium as seen under a transmission electron microscope. (c) Crushed *G. margarita* spore (sp) after staining with SYTO 9[®]: the cytoplasm spreads over the slide forming a halo rich in endobacteria (arrowheads). Fungal nuclei (empty arrowhead) are trapped inside the cytoplasm. (d) FISH on a crushed spore of *G. margarita*: the double labelling with the *CaGg*-specific probe CaGgAD11 (blue) and the *Mre*-specific probe BLOsADf2 (red) confirms the simultaneous presence of the two endobacterial types in the same AMF spore; bacteria are seen as rod-shaped or coccoid fluorescent spots (arrowheads). Fungal cytoplasm (fc). Scale bars, (a) 0.13 μ m; (b) 0.12 μ m; (c) 150 μ m; (d) 13 μ m

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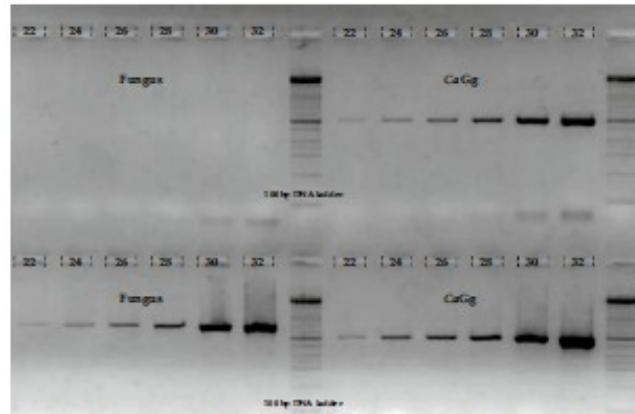


Fig. 2 Agarose gel electrophoresis patterns of the PCR amplification targeting the 18S rRNA gene of *G. margarita* (left) and the 16S rRNA gene of *CaGg* (right). The fungal and bacterial detection was carried out using the primer pairs AML1-AML2 [23] and GlomGIG1-GlomGIGr [13], respectively (see Subheading 3.3.2). After a rapid DNA extraction (see Subheading 3.3.1), the samples in the upper part of the gel were subjected to bacterial enrichment prior to amplification (see Subheading 3.5), whereas the ones in the lower part of the gel were directly amplified. Six subsamples for each assay were prepared. Each subsample was amplified with a different number of cycles (22, 24, 26, 28, 30, 32, respectively). As expected, the greater was the cycle number, the higher was the amount of amplicons obtained. Interestingly, any fungal amplification was observed from the samples enriched in endobacteria, suggesting a limited or absent carryover of fungal nuclei. These results confirmed the efficiency of the bacterial enrichment procedure here described