Species diversity and community composition of native arbuscular mycorrhizal fungi in apple roots are affected by site and orchard management

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Abstract

Arbuscular mycorrhizal fungi (AMF) are beneficial microrganisms which establish mutualistic symbioses with the roots of most food crops, improving plant performance, nutrient uptake and tolerance to biotic and abiotic stresses. A better understanding of the factors affecting AMF occurrence and diversity is fundamental to implement sustainable agricultural managements effectively profiting from beneficial plant symbionts. Here, we investigated AMF occurrence, diversity and community composition in the roots of apple trees from 21 orchards in South Tyrol, as affected by location, management (organic vs integrated) and altitude, by PCR cloning and sequencing and PCR-DGGE of partial 18S rRNA gene. The screening of 448 clones from 21 clone libraries allowed the identification of 6 native AMF at the species level: *Glomus indicum*, *Sclerocystis sinuosa, Funneliformis mosseae*, *Rhizoglomus irregulare*, *Septoglomus constrictus* and *Claroideoglomus lamellosum*. The most abundant genera were represented by *Glomus* (29.7 % of the sequences), *Paraglomus* (19.4 %), *Claroideoglomus* (17.2 %), *Sclerocystis* (16.1 %) and *Rhizoglomus* (12.3 %). *Septoglomus*, *Diversispora* and *Funneliformis* sequences corresponded to less than 4 % of total sequences. Although the degree of root colonization was unaffected by treatments, ANOSIM analysis of PCR-DGGE clusters revealed significant differences in apple root AMF diversity between sites and agricultural managements. Species richness was significantly higher in organically managed orchards than in integrated ones. Our findings provide insights into important factors affecting native AMF communities of apple trees, which could be exploited in sustainable fruit production systems, where beneficial soil biota boost biogeochemical cycles, energy fluxes and crop productivity.

Keywords: Apple trees; Native AMF communities; Mycorrhizal colonization; Small Ribosomal Subunit (SSU rDNA); Clone libraries; PCR-DGGE

1. Introduction

Soil microorganisms are essential elements of soil health, fertility and productivity in sustainable and organic farming systems, playing key roles in the completion of biogeochemical cycles and availability of mineral nutrients, carbon sequestration and soil structure improvement, pest and disease control (Pimentel et al., 1997). Arbuscular mycorrhizal (AM) fungi (AMF, Phylum Glomeromycota) are beneficial microorganisms establishing mutualistic symbioses with the roots of most food crops, including cereals, legumes and fruit plants, and improving plant performance, nutrient uptake and tolerance to biotic and abiotic stresses (Smith and Read, 2008). AMF are obligate biotrophs, obtaining sugars from their host plants while providing soil mineral nutrients - such as P, N, S, K, Ca, Fe, Cu and Zn - absorbed and translocated by means of large and ramified extraradical hyphal networks that spread from plant roots into the surrounding soil (Giovannetti et al., 2015). Moreover, AMF deliver important agroecosystem services, such as soil aggregation and carbon sequestration (Gianinazzi et al., 2010), and are considered important soil biological indicators (Creamer et al., 2016; Stone et al., 2016). AMF enhance plant performance and fitness also through the synergistic action of beneficial mycorrhizosphere bacteria, *i.e.* strictly associated to mycorrhizal hyphae, colonised roots and spores, which display multifunctional activities, from antibiotic, siderophores and indole acetic acid production to P-solubilisation, phytate mineralization and N-fixation (Barea et al., 2002; Philippott et al., 2013; Agnolucci et al., 2015). Thus, AMF represent environmentally-friendly biofertilisers and biostimulants, as they reduce the need of chemical fertilizers and pesticides in sustainable food production systems (Rouphael et al., 2015). In addition, they contribute to the production of safe and high-quality food, positively affecting the synthesis of health-promoting secondary metabolites in food crops (Battini et al., 2016a, b).

In spite of all the potential benefits of the symbiosis, AMF role has been often marginalised in agriculture. In field crops, several agronomic practices, such as continuous monocultures, deep ploughing, intensive fertiliser and pesticide use can decrease AMF occurrence, activity and diversity, often leading to a reduction of AMF benefits to crop production and soil quality (Douds and Millner, 1999; Jansa et al., 2003; Oehl et al., 2004, 2005b; Brito et al., 2012; Avio et al., 2013). Moreover, in fruit production the soil is usually replanted with the same tree species short after the preceding trees are removed. In organic farming systems, thanks to the fact that synthetic fertilizers, herbicides and pesticides are not applied, AMF diversity, activity and abundance have been reported to be higher than in conventional systems, supporting the view that AMF play a fundamental functional role in the maintenance of soil fertility and crop

production in low-input agroecosystems, compensating for the reduced use of agrochemicals (Mäder et al., 2002; Oehl et al., 2003, 2004; Gosling et al., 2006; Ryan and Tibbet, 2008; Mazzoncini et al., 2010; Verbruggen et al., 2010).

Different strategies have been devised to increase the mycorrhizal potential of soils in sustainable agriculture: among them, the inoculation with non-native AMF (Jeffries et al., 2003; Gianinazzi and Vosatka, 2004) has proved effective in improving root colonization, plant biomass production and P uptake (Lekberg and Koide, 2005). However, such an approach involves high costs of production and application when utilised on a large scale, and raises concerns about potential negative impacts of non-native invasive AMF inoculants on the composition and structure of native AMF communities, possibly leading to biodiversity losses (Schwartz et al., 2006). A different approach focuses on the enhancement of native AMF by means of mycotrophic crops able to maintain or increase native mycorrhizal potential of soils and root colonization and growth of the subsequent crops (Kabir and Koide, 2002; Karasawa and Takebe, 2012; Lehman et al., 2012; Njeru et al., 2015, 2014).

Little information is available on the impact of cultural practices on AMF occurrence, species richness and composition in apple crop production systems. Different orchard floor managements create different levels of soil disturbance and may therefore promote or depress AMF occurrence. Organic farming guidelines, for example, differently from conventional or integrated fruit production techniques (Kelderer, 2004), do not allow the use of chemical herbicides, but recommend, instead, the use of alternative techniques to manage the orchard floor by either superficial soil tillage, grass mowing or mulching. Similarly, while conventional and integrated management techniques allow the use of synthetic mineral fertilisers, organic farming guidelines recommend increasing soil fertility by enhancing soil organic matter. The use of straw mulches and compost have been reported to increase apple root colonisation, which was positively linked with soil pH and availability of P, K, Zn, Mn and C, suggesting that the relevant organic orchard floor management practices promoted functional AMF associations more effectively than conventional practices (Meyer et al., 2015). A recent work carried out using molecular methods showed that soil characteristics and farming systems affect AMF diversity and community composition in the roots of cultivated apple trees. In particular, a low soil available P content was associated with a higher AMF diversity in organically managed orchards, as compared with conventionally managed ones (Van Geel et al., 2015). In such a study, the native AMF colonizing apple roots were identified by 454-pyrosequencing of small subunit rRNA gene amplicons, with a taxonomic resolution at the family level. So far, the characterization of apple AMF communities at the species level has been performed only by conventional morphological methods, which show several shortfalls, mainly due to the use of spores extracted from the soil, whose integrity and quality may induce species misidentification (Cavallazzi et al., 2007; Miller et al., 1985; Purin et al., 2006).

92 Here we used two molecular methods, PCR-cloning and sequencing and PCR denaturating gradient gel
93 electrophoresis (PCR-DGGE) of partial 18S rRNA gene to characterize AMF communities of apple roots at genus and
94 species levels. We investigated AMF occurrence and diversity in the root systems of apple trees cultivated under
95 organic and integrated management from 21 orchards in the South Tyrol province (Italy), one of the most important
96 apple production districts in Europe, with more than 18,000 ha acreage and approx. 1 million tons apples harvested
97 yearly (Dalla Via and Mantinger, 2012). Our data provide knowledge of the factors which affect AMF colonization and
98 shape the native AMF community composition in apple roots, allowing the implementation of effective sustainable
99 management strategies that take advantage from beneficial plant symbionts.
100
101 **2. Materials and Methods**
102 **2.1. Soil and orchard management parameters**
103 The research was carried out in South Tyrol (Northern Italy). In May 2013, 21 commercial apple (*Malus domestica*
104 Borkh) orchards located either in the municipalities of Terlano (46°31' 59" N, 11°14' 47" E) or in that of Lagundo (46°
105 41′ 0″ N, 11° 8′ 0″ E) were randomly selected from a list provided by the local advisory service (The South Tyrolean
106 Advisory Service for Fruit and Grape growing, Lana, Italy). This list included drip-irrigated apple orchards planted
107 between 2007 and 2009, managed since planting following either the integrated fruit production (hereafter referred to as
108 "integrated") or the organic farming (hereafter referred to as "organic") guidelines.
109 In the municipality of Lagundo 3 organic and 3 integrated orchards were selected at low (298-334 m a.s.l.) and
110 at high altitude (591-660 m a.s.l.) while in the municipality of Terlano 6 organic and 3 integrated orchards were selected
111 at a low altitude (ranging from 243 to 299 m a.s.l.). Orchards were classified according to the area (Te, Terano; La,
112 Lagundo), management (O, Organic; I, Integrated) and altitude (L, Low altitude; H, High altitude) (Fig. 1).
113 The climate in area is warm temperate according to the Koppen-Geiger classification (Kottek et al., 2006).
114 Average (1993 to 2013) climatic parameters are reported in Table 1. Average annual and maximum temperatures at
115 high elevation were approx. 2 °C lower than at low elevation, while average minimum temperature was approx. 1 °C
116 lower. Soils in the area are mainly sandy loam or loamy sand and are classified as Calcaric Cambisol according to the
117 FAO Soil Taxonomy (IUSS Working Group WRB 2015) (Table 2).
118 The trees were always grafted on the same clonal rootstock (M9, T337 strain) and belonged to one of the
119 following varieties: Gala, Golden Delicious, Pinova, Modì and Red Delicious. Orchards were uniform in terms of
120 training systems (spindle bush) and planting distances (approx. 3 m between rows and 1 m between trees along the
121 row). Orchard floor management always included the presence of grassed alleys between rows (2.0 to 2.3 m wide) that
122 were mowed 3-4 times per year, while in the 0.7-1.0 m soil strip along the tree row, soil management differed between

123 organic and integrated orchards (see below).The organic and integrated orchards differed both for the type of protection
124 against aboveground pests and for soil management. In particular, in the organic orchards weed control was carried out
125 through mechanical removal of weeds (approximately five times per year) and soil fertility was maintained only by the
126 addition of organic fertilisers. In the integrated orchards, weed control in the soil strip under the trees was performed by
127 spring and autumn application of glyphosate; mineral fertilizer supply was carried out according to the nutrient budget,
128 considering expected yields of approximately 60 t ha^{-1} and soil fertility, in the following ranges, 30-80 kg N, 19-28 kg
129 P, 71-155 kg K.
130
131 **2.2. Root, leaf and soil sampling**
132 In each orchard, root and leaf were samples from 6 randomly chosen trees from different rows, all located in the central
133 part of the plot. Sampling was carried out between May 15th and June 6th, 2013. Six soil cores were collected at a depth
134 of 10-30 cm and at approx. 20 cm distance from the trunk, using a split tube sample of 5 cm-diameter (Eijkelkamp
135 Agrisearch Equipment, BV). Roots were gently cleaned from the soil and approx. 20 g of fine roots (<2 mm diam) per
136 tree were collected and stored at 4 °C in polyethylene bags, to be successively analysed for mycorrhizal colonization. A
137 single root sample per orchard was prepared for molecular analyses (100-125 mg each) pooling together root
138 subsamples from the individual trees. Samples were stored at -20 °C, until processed. Soil samples were oven-dried at
139 65 °C until constant weight and stored at room temperature until analysed. Ten leaves per tree (without petiols) were
140 sampled from the 6th or the 7th nodes of one year-old shoots, oven-dried at 65 °C until constant weight and stored until
141 analysed.
142
143 **2.3. Mycorrhizal colonization**
144 Mycorrhizal colonization was determined on 10 g thoroughly washed root samples. Roots were cleared with 10% KOH
145 in a 80 °C water bath for 15 min and stained with Trypan blue in lactic acid (0.05 %) after 10 min in 2 % aqueous HCl.
146 The percentage of AMF colonization was calculated from root samples from individual trees from each orchard using a
147 dissecting microscope at x25 or x40 magnification and the gridline intersect method (Giovannetti and Mosse, 1980).
148
149 **2.4. Soil and leaf analysis**
150 With the exception of soil N and C concentration (performed by an elemental analyzer, Thermo Scientific), soil
151 parameters were determined according to the methods of the Verband Deutscher Landwirtschaftlicher Untersuchungs-
152 und Forschungsanstalten (VDLUFA, 1991). Leaf N concentration was determined by Elemental Analyser (Flash 2000,
153 Thermo Scientific), while leaf K, Ca, P and magnesium (Mg) concentrations were determined by inductive coupled

plasma-optical emission spectrometry (ICP-OES, Spectro Ametek, Arcos and Spectro Ciros CCD) after microwave digestion (Milestone UltraWAVE) with 69 % of ultrapure nitric acid.

2.5. Statistical analyses of soil, leaves and mycorrhizal colonization

Data of mycorrhizal colonization (after angular transformation) and leaf mineral nutrient concentrations were analysed by two-way-ANOVA. The sub-dataset from Lagundo was used to assess the interactive effects of orchard management (organic vs. integrated) and orchard altitude (low vs. high). The sub-dataset obtained at low altitude was used to assess the interactive effects of orchard management (organic vs. integrated) and site (Terlano vs. Lagundo). Mycorrhizal colonization data were linearly correlated with soil parameters using the entire dataset. The coefficient of mycorrhizal colonization data variation (CV) within each farm was also calculated. The statistical analyses were conducted using the dedicated software SigmaPlot Centurion XV (StatPoint Technologies, Inc).

2.6. Molecular analyses

2.6.1. DNA extraction from roots

Genomic DNA was isolated from root material by grinding with mortar and pestle in liquid nitrogen and subsequently using DNeasy Plant Mini Kit (Qiagen Milan, Italy), before performing cloning and sequencing analyses. Moreover, in order to obtain DNA containing less inhibitors possibly interfering with the amplification procedure of the PCR-DGGE technique, we extracted DNA from roots using the PowerSoilTM DNA Isolation Kit (MO BIO Laboratories, Solana beach, CA, USA). The isolated DNA was stored at -20 °C for subsequent analyses.

2.6.2. Cloning and sequencing

DNA (10-20 ng) extracted from roots was used as template. Partial small subunit (SSU) ribosomal RNA gene fragments were amplified in volumes of 25 µl with 0.125 U of GoTaq Flexi DNA Polymerase (Promega, Milan, Italy), 0.4 µM of each primer (NS31 /AML2, Simon et al., 1992; Lee et al., 2008), 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$ and 1× manufacturer's reaction buffer. The thermal cycler (Eppendorf Mastercycler personal, Eppendorf, Milan, Italy) was programmed as follows: a manual “hot start” at 94 °C for 3 min, 30 cycles at 94 °C for 30 sec, 58 °C for 40 sec, 72 °C for 55 sec and a final extension step at 72 °C for 10 min. Reactions yields were estimated using a 1 % (*w/v*) agarose I (Euroclone, Milan, Italy) in TBE 1X buffer (Euroclone) gels stained with ethidium bromide (0.5 µg ml^{-1}).

NS31/AML2 amplicons from apple root samples were purified using Wizard® SV Gel and PCR Clean-up system (Promega), then ligated into pGem-T Easy vector (Promega) to transform XL10-Gold Ultracompetent *Escherichia coli* cells (Stratagene, La Jolla, CA, USA).

The structure and composition of the AM fungal communities were determined using PCR-RFLP screening of clone libraries. Putative positive clones were amplified by using standard SP6/T7 amplifications, followed by a nested PCR using NS31/AML2 primers as described above. Twenty-five amplicons per clone library were digested by HinfI and Hsp92II restriction enzymes (Promega) and run on 2 % agarose at costant 50 V for 2 h. A 100 bp DNA ladder (Promega) was used as a molecular weight marker. DNA profiles were visualized under UV illumination and captured as TIFF format file by Liscap program for Image Master VDS System (Pharmacia Biotech).

A total of 448 clones were examined. Recombinant plasmids of representative clones of each RFLP pattern were purified by Wizard® Plus SV Minipreps (Promega) and sequenced using T7 vector primers at BMR Genomics s.r.l. (University of Padova, Italy).

Sixty-nine unique sequences of the clones generated in this study have been deposited in EMBL Nucleotide Sequence Database (www.ebi.ac.uk/embl/) under the accession numbers from LT600783 to LT600851.

2.6.3. PCR-DGGE analysis

To analyse AMF communities by PCR-DGGE, a semi-nested PCR approach was used. Initially a 550 bp fragment of the 18S rRNA gene was amplified using the primer NS31 (Simon et al., 1992) in combination with the primer AM1 (Helgason et al., 1998). Amplification reactions were performed in a final volume of 50 µl, using 10-20 ng of DNA, 5 µl of 10X *Ex Taq* Buffer (Takara Biotechnology, Milan, Italy), 1.25U of *TaKaRa Ex Taq* (Takara Biotechnology), 0.2 mM of each dNTP (Takara Biotechnology), 0.5 µM of each primer (Eurofins genomics, Milan, Italy) and an aliquot of 0.3 µg μl^{-1} acetylated bovine serum albumin (BSA, Promega). PCR amplifications were carried out using an iCycler-iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Milan, Italy) with the following conditions: 94 °C initial denaturation for 1 min; 35 amplification cycles of 30 s at 94 °C, 30 s at 66 °C, 30 s at 72 °C; final extension at 72 °C for 5 min. The presence of amplicons was confirmed by electrophoresis in 1.5 % (w/v) agarose gels in TBE 1X buffer (Tris-borate-EDTA, pH 8.0), stained with 0.5 µg mL^{-1} ethidium bromide.

Amplification products from the first PCR reaction were then diluted 1:10 and 1 µl was used as template in a second PCR using the NS31 and the Glo1 (Cornejo et al., 2004) primers. A GC clamp (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGG -3') was added to the 5' end of the forward primer NS31. PCR amplifications were performed as previously described except for the addition of BSA and the annealing temperature of 52 °C.

For the DGGE analysis, 20 µl of the PCR products plus 20 µl of buffer 2X made with 70 % glycerol, 0.05 % xylene cyanol and 0.05 % bromophenol blue, were separated in a 8 % polyacrylamide-bisacrilamide (37.5:1) gel with a 35-56 % urea-formamide gradient, using the DCode™ Universal Mutation Detection System (Bio-Rad). A composite

mix of AMF 18S rRNA gene fragments from *Funneliformis mossae* AZ225C, *Funneliformis coronatum* IMA3 and *Rhizoglomus intraradices* IMA5 was added in the middle and at both ends of each gel as DGGE marker (M). Gels were run and profiles were visualized as described in Agnolucci et al. (2013).

DGGE bands were excised from polyacrylamide gels and DNA was extracted by eluting in 50 µl ddH_2O at 4°C overnight. One µL of the supernatant diluted 1:100 was used to re-amplify the 230 bp DNA fragment of the 18S rRNA gene according to the PCR protocol described above, except that the primer NS31 was used without GC clamp. PCR products were then purified by UltraClean PCR CleanUp Kit (MO-BIO Laboratories) according to the manufacturers' protocol, quantified and 5' sequenced at the BMR Genomics (Padova, Italy).

2.7. Bioinformatics

Sequences from *E. coli* libraries and DGGE bands were edited in MEGA 6.0 and their similarities were determined using the Basic Local Alignment Search Tool (BLASTn) provided by NCBI. The detection of chimeric sequences was performed using USEARCH 6.0 (http://fungene.cme.msu.edu/FunGenePipeline/chimera_check/form.spr). Sequences were aligned with those corresponding to the closest matches from GenBank as well as with sequences from major clades of Glomeromycota using MUSCLE as implemented in MEGA6. Phylogenetic trees were inferred by Neighbour-joining analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method. The confidence of branching was assessed using 1000 bootstrap resamplings.

Richness and composition of AMF communities obtained from clone libraries were evaluated for each treatment. We determined the rarefaction curves with Past software to estimate whether the number of screened sequenced were sufficient to capture AMF diversity of each host. Estimates of community diversity were determined as Richness (S), bias-corrected Chao1 richness, Shannon-Weaver (Hs) and dominance index of Simpson (D). The indices were calculated using PAST 3.0 and 1000 bootstraps were used to determine confidence intervals. Non parametric Kruskal Wallis test was used to determine differences in the diversity indices among AMF communities in the roots of apple plants cultivated in the two sites (Terlano vs Lagundo), under different managements (organic vs integrated) and at different altitudes (low and high). AMF communities were also evaluated by permutational multivariate analysis of variance (PERMANOVA), to test the effect of the management (organic vs integrated), site and altitude. Non parametric tests and PERMANOVA were performed in SPSS version 20 software (IBM Corp., Armon, NY Inc, USA) and PAST 3.0, respectively.

DGGE profiles were digitally processed with BioNumerics software version 7.5 (Applied Maths, St-Martens-Latem, Belgium) following the manufacturer's instructions. The lanes were normalized to contain the same amount of total signal after background subtraction and the gel images were straightened and aligned to give a densitometric

curve. DGGE markers were used for digital gel normalization to allow comparison between gels. Bands were assigned using the auto search bands option and then checked manually and their positions were converted to Rf % values. All profiles were compared using the band matching tool with a position tolerance and optimization of 0.5 and 0 % respectively.

Similarities between DGGE patterns were calculated by determining Dice's similarity coefficients for the total number of lane patterns from the DGGE gel. The similarity coefficients were then used to generate the dendrogram utilizing the clustering method UPGMA (Unweighted Pair Group Method Using Arithmetic Average). DGGE profiles were also analysed using non-metric multidimensional scaling analysis (NMDS) performed from a data matrix based on presence/absence of bands (Bray-Curtis coefficient). The significance of data was assessed by the two way ANOSIM method (analysis of similarities; 999 permutations) (PAST 3.0) with geographical site (Terlano vs Lagundo) and management (organic vs integrated) as variability factors. Note that the stability index R describes the extent of similarity between each pair in the ANOSIM, with values close to unity indicating that the two groups are entirely separate and a value of zero indicating that there is no difference between the groups.

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 from the number of fragments. The overall diversity index of Shannon-Weaver (Hs) and the dominance index of Simpson (D) were calculated using the equations $Hs = -\sum(P_i \times \ln P_i)$ and $D = \sum P_i^2$ respectively, where the relative importance of each OTU is $P_i = n_i N^{-1}$, 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 $E = H (\ln S)^{-1}$. Two-way ANOVA was applied to diversity indices with site (Terlano vs Lagundo) and management (organic vs integrated) as variability factors, after checking the normal distribution of data. One-way ANOVA was used for Lagundo diversity indices with management as the variability factor. The means were compared by the Tukey's test ($P < 0.05$). Analyses were carried out with the SPSS version 20 software.

3. Results

3.1. Soil fertility and mycorrhizal colonization

Soils texture in the Lagundo area had either a silty loam or a sandy loam texture, while those in the Terlano area were either loamy or sandy loam. In general, as indicated by the standard error values (Table 2), there was a relatively low variability of N concentration among soils belonging to different treatments as well as within each treatment. All soils were well endowed with P and Mg, while soil K availability ranged from moderate-low to normal values. In Lagundo only, soils managed according to organic guidelines had higher pH (P=0.02), organic matter (OM) (P=0.01) and C

concentration (P=0.03) than those managed according to the integrated guidelines (Table 2). Soils at higher elevation also had higher soil OM (P=0.05) and soil C (P=0.06) than those at lower elevation (Table 2). When only data belonging to low elevation were analyzed, no significant differences in soil characteristics between the two orchard managements were detected.

No significant effect of altitude and management was recorded on leaf nutrient concentration (Table 3). Leaf P and K were significantly higher (P=0.01 and 0.001, respectively) and leaf Mg significantly lower (P=0.02) in orchards located in Terlano than in Lagundo (Table 3).

In all sampled orchards, apple roots were well colonized by AMF (Table 3). The percentage of colonized root length, ranging from 41 to 60 %, was not significantly affected by site, management and altitude. We did not find significant effects of treatments on the degree of mycorrhizal colonization variation among and within orchards (Table S1). The level of mycorrhizal colonization was not significantly correlated with most measured soil parameters, but a negative linear correlation with soil organic matter (r=-0.49, P=0.03) was found.

3.2. Identification of native AMF colonizing apple roots

The DNA extracted from apple roots was successfully amplified using the primer pair NS31/AML2, obtaining a fragment of the expected size (~550 bp). A total of 448 clones from the 21 clone libraries were screened by PCR-RFLP analysis, obtaining 19 different RFLP patterns. For each RFLP group several clones originating from different libraries were sequenced, giving 17 Glomeromycota sequences. All non-redundant sequences from the 21 clone libraries (69 out of 212) and 27 references from GenBank were used for neighbour-joining phylogenetic analyses (Fig. 2). After RFLPs, BLASTn and phylogenetic analyses the sequences were grouped into 17 OTUs supported by a bootstrap value >87 %.

Among the 17 OTUs, we retrieved sequences belonging to 8 out of the 19 Glomeromycota genera (Redecker et al., 2013). In particular all the genera of Glomeraceae were found (*Glomus*, *Septoglomus*, *Rhizoglomus*, *Sclerocystis*, *Funneliformis*) together with the genera *Claroideoglomus*, *Diversispora* and *Paraglomus*, belonging to Claroideoglomeraceae, Diversisporaceae and Paraglomeraceae, respectively. The most abundant genera were represented by *Glomus*, accounting for 29.7 % of the sequences, followed by *Paraglomus* (19.4 %), *Claroideoglomus* (17.2 %), *Sclerocystis* (16.1 %) and *Rhizoglomus* (12.3 %). *Septoglomus*, *Diversispora* and *Funneliformis* sequences corresponded to less than 4 % of total sequences. Twelve OTUs were ascribed to Glomeraceae family: within the genus *Glomus* we retrieved seven OTUs, of which only one (Glo7, 5.8 % of total sequences) was identified as *Glomus indicum*; the remaining six OTUs (Glo1 to Glo6) represented sequences of uncultured *Glomus* species (Table 4). In the genera *Sclerocystis, Funneliformis* and *Rhizoglomus,* OTUs named Scle, Fun and Rhi were identified as *Sclerocystis sinuosa, Funneliformis mosseae* and *Rhizoglomus irregulare* (synonym *Rhizophagus irregularis*, formerly known as

309 *Glomus irregulare*), respectively. OTUs Sept1 and Sept2 and Cl1, Cl2 and Cl3 assigned to *Septoglomus* and
310 *Claroideoglomus*, respectively, matched to sequences of either known (Sept2, *Septoglomus constrictus* and Cl1,
311 *Claroideoglomus lamellosum*), or unknown species (*Septoglomus* sp., *Claroideoglomus* sp.), while OTUs belonging to
312 *Paraglomus* (Par) and *Diversispora* (Div) matched only unknown Glomeromycota. MaarjAM database
313 (http://maarjam.botany.ut.ee/, accessed on March/2016) was used to confirm the assignment of our OTUs to sequences
314 of Glomeromycota (Table 4). Rarefaction analyses indicated that the number of analysed sequences was generally
315 sufficient to capture the AMF diversity in the roots of most orchards, since the curves almost reached the asymptote
316 (Fig. S1).
317 The bands of interest from the DGGE profiles, excised and sequenced to determine their affiliation, identified
318 the same AMF species as those described above, except Claroideoglomeraceae and Paraglomeraceae (data not shown).
319
320 **3.3. AMF community diversity in apple roots as affected by site, management and altitude**
321 ***3.3.1. Analysis of clone libraries***
322 The cloning and sequencing method allowed the identification of 14 and 15 OTUs in Terlano and Lagundo orchards,
323 respectively, 12 of which shared between the two sites (97.5 and 88.8 % of the total sequences of the two sites,
324 respectively). Fun (*F. mosseae*) and Glo6 were found only in Terlano apple roots, while Sept2 (*S. constrictus*), Glo2
325 and Cl3 were retrieved only in Lagundo. Two ways PERMANOVA analyses revealed differences in AMF community
326 composition between the two sites (P=0.02) and between organic and integrated managements (P=0.02). Moreover, in
327 Lagundo, where orchards were located at two different altitudes (300 m and 600 m) two ways PERMANOVA analysis
328 showed differences among AMF communities in relation to management (P=0.003) and altitude (P=0.003). In total,
329 orchards under organic management hosted 13 and 6 OTUs at low and high altitude, respectively. In organic orchards
330 growing at higher elevation the most common species (Par, Glo7, Rhi, Scle) present at both sites and under different
331 managements were detected together with Cl1 and Div (species found in all organic orchards at low altitude) (Fig. 3).
332 By contrast, in integrated orchards 10 and 9 OTUs in total were found at low and high altitude, respectively. At high
333 altitude, together with the most common species, a particular OTU (Cl3, 31 % of the sequences) was retrieved (Fig. 3).
334 Among diversity indices, S and Chao-1, indicating observed and estimated AMF richness, were significantly
335 higher in organic orchards (6.33 ± 0.55 and 7.14 ± 0.88, respectively) than in integrated ones (4.00 ± 0.01 and 4.00 ±
336 0.01, respectively) in Terlano (P=0.027 and P=0.034, respectively). In Terlano 13 OTUs were retrieved in organic
337 management, in contrast with only 6 OTUs found in integrated ones. No differences among diversity indices were
338 detected among orchards in Lagundo.
339

340 ***3.3.2. Analysis of PCR-DGGE profiles***

341 AMF 18S rDNA fragments of approx. 230 bp were successfully amplified from all the samples, by the semi-nested
342 PCR approach. DGGE analyses of PCR products showed profiles characterized by a high number of intense and clearly
343 defined fragments.

344 AMF community composition was assessed by cluster analysis of DGGE profiles (Fig. 4). The dendrogram
345 showed two main subclusters with a low similarity, 20 %. In particular, the first subcluster included all Terlano samples
346 (except one), while the other one included all Lagundo samples (except one). The latter was formed by two subclusters
347 in which organic and integrated samples were separated with a similarity of 36 %. The analysis of DGGE profiles did
348 not separate samples originating from orchards growing at different altitudes (Fig. 4). Terlano samples showed a more
349 complex clustering, with no clearcut separation by management.

350 These findings were confirmed by the NMDS analysis of the DGGE AMF community profiles (Fig. 5).
351 ANOSIM revealed significant differences in AMF community composition of Lagundo samples compared with that of
352 Terlano (R=0.786, P=0.0001) and between samples from organic and integrated sites (R=0.227, P=0.05).

353 AMF DGGE profiles were also analysed to assess S, Hs, D and E diversity indices. Richness (S) and Hs
354 indices were significantly different between the two geographical sites, while a significant interaction was observed for
355 Hs and D (Table 5). The relevant one-way ANOVA revealed significant differences between organic and integrated
356 samples in Lagundo, where S and Hs were significantly higher in the organic management (P=0.049 and P=0.029
357 respectively) (Table 6).

358

359 **4. Discussion**

360 Our data showed that, in South Tyrol orchards, geographical area, altitude and management shaped AMF species
361 community composition in apple roots, which however always maintained high levels of mycorrhizal colonization.
362 Here, for the first time, we identified at the species and genus level native AMF colonizing apple roots and
363 characterized their diversity and community composition utilizing two molecular methods - PCR cloning and
364 sequencing and PCR-DGGE.

365

366 **4.1. Mycorrhizal colonization of apple roots**

367 The percentage of colonized root length of apple trees ranged from 41 to 60 %, comparing well with previous data on
368 mycorrhizal colonization of apple seedlings, that ranged from 40 to 60 % after inoculation with *F. mosseae* and *Glomus*
369 *macrocarpum*, respectively (Miller et al., 1989) and with findings obtained by Meyer et al. (2015) in conventionally and
370 organically-managed apple orchards in South Africa. Other authors reported more variable values, ranging from 24 to

371 68 % in micropropagated apple trees, depending on soil pH and the identity of the inoculated AMF species (Cavallazzi
372 et al., 2007). In our work, mycorrhizal colonization of apple roots was uncoupled from soil parameters, except for
373 organic matter, confirming previous findings on the decrease of mycorrhizal colonization levels in an organic apple
374 orchard after a straw mulch floor management treatment (Meyer et al., 2015).

375 Overall, mycorrhizal root length was not significantly affected by site, altitude and management in the apple
376 orchards investigated. As to site, Miller et al. (1985) in a survey of 18 apple rootstock plantings in USA found a high
377 variability in mycorrhizal colonization, ranging from 5 to 75 %, with the lowest levels observed in California and
378 Washington and the highest in Virginia, Pennsylvania, Georgia and Iowa. Such data are expected, given the different
379 range of geographical distances of the apple orchards analysed, entailing the most divergent environmental conditions,
380 at the subcontinental level.

381 Most studies investigating the effects of altitude on mycorrhizal colonization have been carried out at very high
382 elevation gradients (from 1,500 to 5,300 m), in alpine habitats of Tibet, Andes, Rocky Mountains, Alps and Mount Fuji
383 (Read and Haselwandter, 1981; Wu et al., 2007; Lugo et al., 2008; Schmidt et al., 2008; Gai et al., 2012). Overall, these
384 works reported an adverse effect of increasing altitude on AMF colonization across a large altitude gradient. In our
385 study, we assessed the effects of elevation in a single site, Lagundo, from apple orchards cultivated at 300 and 600 m,
386 an altitudinal range probably too low to produce significant effects on AMF ability to colonize apple roots, which,
387 indeed, showed high percentages of mycorrhizal root length, 35-52 %.

388 The type of farming practices has long been known to affect mycorrhizal colonization in different crops, such
389 as wheat (Ryan et al., 1994; Mäder et al., 2000), vetch-rye and grass-clover (Mäder et al., 2000), onion (Galván et al.,
390 2009), maize (Douds et al., 1993; Bedini et al., 2013), soybean (Douds et al., 1993). Very few studies investigated the
391 effects of organic vs. conventional management regimes on the establishment of mycorrhizal symbiosis in apple roots.
392 The absence of differences in the percentage of mycorrhizal root length in our experimental apple orchards may be
393 ascribed to the uniformity of the orchard floor managements, which included the presence of grassed alleys between
394 rows. AMF-host weed species may have contributed to the maintenance of wide and infective extraradical mycelial
395 networks establishing linkages among the roots of cover plants and apple trees (Atkinson, 1983; Giovannetti et al.,
396 2004; Njeru et al., 2014). The application of glyphosate in the small soil strip along the tree rows and that of mineral
397 fertilizer supply in the integrated orchards did not have a significant impact on the ability of AMF to colonize apple
398 roots. This finding is consistent with previous studies reporting variable and unpredictable effects of such chemicals on
399 AMF symbioses in different environments and geographical locations (Gosling et al., 2006).
400
401 **4.2. Identification of native AMF colonizing apple roots**

The screening of 448 clones from 21 clone libraries allowed us to affiliate DNA sequences with 8 out of the 19 Glomeromycota genera, which encompassed all the genera of the family Glomeraceae described so far: *Glomus*, *Septoglomus*, *Rhizophagus*, *Sclerocystis*, *Funneliformis*. In addition, members of the families Claroideoglomeraceae, Diversisporaceae and Paraglomeraceae were found.

Overall, the most common species belonged to the genus *Glomus*, in agreement with previous findings obtained by morphological description of AMF spores in a survey of 18 apple rootstock plantings in USA (Miller et al., 1985). Some of the species retrieved in the quoted study occurred also in our apple roots, such as *F. mosseae* and *S. constrictus* (formerly *Glomus mosseae* and *Glomus constrictum*, respectively) and *S. sinuosa*, while no species of the genus *Gigaspora*, common in the USA apple plantings, were found. Our data differ from those obtained in a survey of apple orchards in Santa Catarina, south region of Brazil, where 15 species of the genus *Acaulospora* were retrieved and described after spore sievings from the soil, using morphological methods (Purin et al., 2006). Only few AMF species were in common, belonging to *F. mosseae*, *Claroideoglomus* spp. and *S. sinuosa* (formerly *Glomus sinuosum*). Another interesting work taxonomically characterized AMF species by morphological identification of spores retrieved from soil sieving and trap cultures in Brazil (Cavallazzi et al., 2007): the species with the highest number of spores was *Acaulospora mellea*, followed by *Scutellospora heterogama*, *Gigaspora decipiens* and *Acaulospora spinosa*, none of which occurred in apple roots of South Tyrol orchards. A molecular study, utilising 454-pyrosequencing of small subunit rRNA gene amplicons, identified, at the family level, the AMF colonizing roots of cultivated apple in central Belgium (Van Geel et al., 2015). In the work, 73 % and 19 % of OTUs were affiliated with the Glomeraceae and Claroideoglomeraceae, in agreement with our findings, 70 % and 18 %, respectively. In addition, the authors detected only a few OTUs belonging to the families Gigasporaceae, Diversisporaceae and Acaulosporaceae, while the percentage of OTUs affiliated with Paraglomeraceae was much lower in Belgian apple orchards, compared with South Tyrol ones, 1 % vs. 19 %. A recent work, carried out in an experimental apple orchard near Sint-Truiden, Belgium, confirmed the previous data, with 72 % of OTUs belonging to Glomeraceae, 26 % to Claroideoglomeraceae and 2 % to Paraglomeraceae (Van Geel et al., 2016).

The absence or rarity of specific taxa, such as Acaulosporaceae and Gigasporaceae, from the three European apple orchards investigated so far, compared with the frequency of their retrieval from USA and Brazil, may be ascribed to a number of complex and interacting factors - soil, environment, climate, agronomic practices, history of the sites etc. - beyond geographical position. Although the number of experimental data on apple from the two continents is still too small to deduce general trends, recent studies at the global scale revealed that differences in AMF communities diversity do occur among different continents and climatic zones (Kivlin et al., 2011; Öpik et al., 2013; Davison et al., 2015). It is interesting to note that a survey of AMF species distributed all over the world in protected areas reported a

433 general predominance of Acaulosporaceae and Gigasporaceae in tropical or subtropical forests in Brazil and Argentina
434 (Turrini and Giovannetti, 2012).

435 In our study we found sequences (uncultured *Clareidoglomus*-Cl2, *Paraglomus*-Par and *Glomus*-Glo1
436 sequences, corresponding to 18 % of total OTUs), matching those retrieved in apple roots from German orchards
437 (JN644447, unpublished), in roots of other fruit tree plants, such as *Pyrus pyrifolia* (AB695049) (Yoshimura et al.,
438 2013) and *Citrus* rootstocks (JQ350797) (Wang and Wang, 2014). It is tempting to speculate that fruit trees may show a
439 preference for specific fungi, possibly recruiting AMF species in relation to their functional significance.
440

441 **4.3. AMF community diversity in apple roots as affected by site, altitude and management**

442 Present results show that root AMF community composition of apple trees cultivated in two production sites, Terlano
443 and Lagundo, differed significantly and were affected by agricultural management, organic vs. integrated, and altitude.
444 Such findings are supported by consistent data obtained by combining two molecular methods, cloning and sequencing
445 and PCR-DGGE, and utilizing two different primer pairs to amplify the same region of the partial 18S rRNA gene. To
446 the best of our knowledge, this is the first application of such a comprehensive approach for the characterization of root
447 AMF diversity of fruit trees, allowing the differentiation of AMF communities colonizing apple roots in different
448 agricultural conditions.

449 The differences detected in root AMF community composition between the two apple production sites, as
450 revealed by cloning and sequencing analysis, were ascribed to specific taxa occurring only in Terlano (Fun and Glo6) or
451 Lagundo (Sept2, Glo2 and Cl3), to their relative abundance and to the different distribution of shared OTUs between
452 the two sites. Such site differences were consistently detected by PCR-DGGE community profiles analysis, which
453 separated the two relevant clusters with a very high dissimilarity (80 %), and confirmed by NMDS analysis. Our data
454 are in agreement with a previous work, carried out at the regional scale, investigating the distribution of 6 AMF species
455 in 154 agricultural soils across Switzerland, which showed that AMF communities were strongly affected by
456 geographical distance (max. 294 km) (Jansa et al., 2014). Consistent findings were also reported by van der Gast et al.
457 (2011), who investigated AMF community diversity across England (max. 250 km). Although the maximum distances
458 among the sampled apple orchards were much lower than those described above (max. 27 km), our work identified
459 different AMF species and genera in the two sites, Terlano and Lagundo. Such distribution patterns may be the result of
460 biogeographic history, reflecting the dispersal of AMF taxa over time, and the variable climatic and environmental
461 conditions (Morton et al., 1995). Recent molecular works (Hazard et al., 2013; De Beenhouwer et al., 2015; Van Geel et
462 al., 2015) suggested a key role of soil environment in shaping AMF diversity and distribution patterns. Further in-depth
463 and comprehensive studies should be performed in order to separate geographical distance effects from those due to the

characteristics of each sampling site, i.e. soil properties, use of pesticides and chemical fertilizers, environs, even when comparing sites at the regional and local scale. In addition, beyond geographical, soil and environmental variables, host plant identity should be taken into account, given its essential role played in the selection of AMF symbionts functionally established in the roots (Helgason et al., 2002; Gollotte et al., 2004; Sýkorová et al., 2007). Actually, at local scale, a marked host preference has been recently found in maize plants, which hosted completely different AMF communities, as compared with those of the preceding cover crops (Turrini et al., 2016).

No differences in AMF community composition were found by PCR-DGGE cluster analysis between orchards located at 300 m and 600 m altitude in Lagundo, in contrast with cloning and sequencing, that allowed the detection of a particular OTU (Cl3-*Claroideoglomus* sp., 31 % of the sequences) only in the 600 m high orchards. Such divergent data may be ascribed to the different primers utilized during DNA amplification by the two diverse molecular techniques: indeed, Glo1 primer, used in PCR-DGGE, did not allow the amplification of Claroideoglomeraceae, as shown by band sequencing. The OTU Cl3 is infrequent, probably fitting better to the colder environment and to the higher levels of organic matter and soil C found in the 600 m Lagundo orchards. Indeed, the occurrence of infrequent AMF species at high altitude was previously reported (Liu et al., 2011; Sýkorová et al., 2007), while several new species have been described in soils from Swiss Alps (Oehl and Sieverding, 2004; Oehl et al., 2005a, 2006, 2011).

In this work, the relative abundance of single AMF species colonizing apple roots showed significant differences between organic and integrated management, as assessed by PERMANOVA, both in Terlano and in Lagundo orchards. Differences were found also by the analysis of PCR-DGGE AMF community profiles, carried out by NMDS and ANOSIM. Diversity indices showed higher AMF richness in organically managed apples compared with integrated ones, using both molecular approaches. Although scarce information is available on AMF diversity in apple orchards, as affected by management, our findings compare well with those obtained by Purin et al. (2006), who, by a morphological approach, found a higher AMF richness in organic compared with conventional orchards in Brazil. A few other studies, performed on plant species other than apple, reported the effects of organic farming on the composition of AMF communities. For example, Oehl et al. (2004), in the DOC field experiment in Switzerland found that organic management enhanced AMF spore diversity and abundance, while Bedini et al. (2013) showed a progressive increase of AMF richness and composition during the transition from conventional to organic agriculture. Other authors, using molecular techniques, showed that the diversity of AMF species composition in maize and potato roots was higher in organic sites, compared with conventional ones, in agricultural fields throughout the Netherlands (Verbruggen et al., 2010).

The experimental data collected so far on the influence of organic and conventional managements on AMF diversity, either in the soil or in the roots, using either morphological or molecular approaches, are limited and do not

allow us to infer consistent response patterns. Indeed, the words “organic” and “conventional” may have different meanings in the different experimental studies, as the diverse farming systems often encompass heterogeneous local soil properties, environmental conditions and host plants. Thus, AMF diversity or community composition may be affected by a number of uncontrolled variables, such as the use of weed cover, soil tillage, cover crops, manure, quality and quantity of herbicides, pesticides and fertilizers. Whereas floor management in the orchard alleys was similar in organic and integrated orchards, the two management systems differed mainly by the weed control and fertilizer supply to the soil in the strip centres on the tree row: we speculate that the use of glyphosate and mineral fertilizers in integrated managed orchards may have selectively modulated the abundance and composition of AMF taxa able to tolerate such chemicals.

5. Conclusions

In this work, we utilized a multimodal approach to study AMF communities living in symbiosis with apple roots in South Tyrol orchards, under different geographical and environmental conditions. High levels of mycorrhizal colonization were detected across the different variables, while AMF diversity and community composition were affected by geographical area, altitude and farming system management, as detected by PCR cloning and sequencing and PCR-DGGE. In particular, species richness was significantly higher in organically managed orchards than in integrated ones. We identified, for the first time, the native AMF communities of apple roots at the species and genus level, detected infrequent taxa and retrieved some environmental sequences matching those obtained from other fruit plant species. Our findings provide insights into factors affecting native AMF communities of apple trees, which could be exploited to implement sustainable fruit production systems, where beneficial soil biota can boost biogeochemical processes fundamental for energy fluxes, ecosystem functioning and crop productivity.

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Authorship and contributorship M.G., F.S. and M.T. conceived and designed the experiments. F.S. and E.T. carried out mycorrhizal colonization analyses and determined plant and soil parameters, M.A., M.P. and A.T. performed molecular and data analyses. M.A., M.G. and A.T. wrote the paper. F.S., M.T. and E.T. provided agronomic expertise and apple root samples. M.A., M.G., F.S., M.T. and A.T. contributed reagents/ materials/analysis tools.

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733 **Table 1**

734 Main climatic parameters of the studied sites

		Altitude	
Parameter	Unit	Low	High
average annual temperature	°C	11.7	10.3
average minimum temperature (January)	°C	-3.8	- 5.1
average maximum temperature (July)	°C	29.2	27.5
annual rainfall	mm	714	522

735 Data are from the meteorological stations (average 1971-2013) of the Province of Bolzano-Bozen

736

737

738 **Table 2**

739 Main soil characteristics in the selected orchards according to their geographical site, management and altitude

Area	Management	Altitude	Organic matter (g kg^{-1})	pH (H_2O)	C (g kg^{-1})	N (g kg^{-1})	P (mg kg^{-1})	K (mg kg^{-1})	Mg (mg kg^{-1})
Lagundo	Organic	Low	58.7 ± 8.7	7.3 ± 0.1	34.1 ± 1.6	3.2 ± 0.1	57 ± 24	43 ± 21	380 ± 45
		High	81.7 ± 10.7	7.2 ± 0.1	34.1 ± 1.0	3.2 ± 0.1	108 ± 19	56 ± 23	293 ± 18
	Integrated	Low	38.7 ± 4.1	6.9 ± 0.2	25.1 ± 1.1	2.8 ± 0.3	79 ± 5	44 ± 13	360 ± 105
		High	48.7 ± 1.8	6.2 ± 0.5	31.1 ± 1.8	3.2 ± 0.1	61 ± 8	103 ± 37	187 ± 26
Terlano	Organic	Low	40.0 ± 6.7	7.3 ± 0.1	32.1 ± 4.7	2.6 ± 0.3	88 ± 16	86 ± 18	293 ± 14
	Integrated	Low	36.0 ± 5.5	6.4 ± 1.0	28.7 ± 2.9	3.0 ± 0.2	43 ± 8	70 ± 17	283 ± 38

740 Data are averages ± s.e. For clarity, statistics is reported only in the text.

741

Table 3

Percentage of root mycorrhizal colonisation and leaf nutrient concentrations in the selected orchards according to their geographical site, management and altitude

Area	Management	Altitude	Mycorrhizal colonization (%)	N (%)	P (%)	K (%)	Mg (%)	Ca (%)
Lagundo	Organic	Low	41.44 ± 12.23	3.64 ± 0.70	0.22 ± 0.01	0.95 ± 0.05	0.34 ± 0.01	1.23 ± 0.16
		High	34.97 ± 15.69	2.94 ± 0.29	0.21 ± 0.01	0.97 ± 0.05	0.29 ± 0.01	1.07 ± 0.04
	Integrated	Low	52.02 ± 14.33	3.21 ± 0.39	0.21 ± 0.01	1.02 ± 0.08	0.36 ± 0.05	1.17 ± 0.12
		High	48.19 ± 11.11	2.42 ± 0.09	0.21 ± 0.01	1.15 ± 0.06	0.25 ± 0.02	1.18 ± 0.10
Terlano	Organic	Low	60.01 ± 13.76	2.82 ± 0.10	0.24 ± 0.01	1.26 ± 0.04	0.3 ± 0.01	1.12 ± 0.06
	Integrated	Low	54.71 ± 23.41	3.01 ± 0.24	0.24 ± 0.01	1.2 ± 0.05	0.27 ± 0.03	0.9 ± 0.15

Data are averages ± s.e. For clarity, statistics is reported only in the text.

752 **Table4**

753 Sequence types of arbuscular mycorrhizal fungi, identified using NS31-AML2 primers pair, in the roots of apple plants

754 grown in South Tyrol orchards

OTU name	Identity NCBI database (%)	Identity Maarjam database (%)	Maarjam database virtual taxa	Taxonomic affiliation
Cl1[†]	AJ276087 (99)	FN869808 (99)	VTX00057	*Claroideoglomus lamellosum*
Cl2	JN644447 (99)	HE615004 (99)	VTX00056	*Claroideoglomus* sp.
Cl3	KF290671 (99)	KF290671 (99)	VTX00225	*Claroideoglomus* sp.
Div	FJ831643 (100)	FJ831643 (100)	VTX00062	*Diversispora* sp.
Fun	AJ306438 (99)	AY635833 (99)	VTX00067	*Funneliformis mosseae*
Glo1	JQ350797 (99)	KF386274 (99)	VTX00214	*Glomus* sp.
Glo2	KF467269 (99)	KF467269 (99)	VTX00135	*Glomus* sp.
Glo3	H4380136 (99)	HG004495	VTX00153	*Glomus* sp.
Glo4	JX144121 (99)	KC579423	VTX00304	*Glomus* sp.
Glo5	JN009364 (99)	JN009364 (99)	VTX00151	*Glomus* sp.
Glo6	JX144133 (99)	HG004465	VTX00301	*Glomus* sp.
Glo7	GU059539 (99)	GU059539 (99)	VTX00222	*Glomus indicum*
Par	AB695049 (100)	-	-	Uncultured Glomeromycota sp.
Scle	AJ33706 (99)	AJ33706 (98)	VTX00069	*Sclerocystis sinuosa*
Sept1	KF386332 (99)	KF386332 (99)	VTX00063	*Septoglomus* sp.
Sept2	FR750212 (99)	FJ831626 (99)	VTX00064	*Septoglomus constrictus*
Rhi	FJ009618 (99)	FJ009617 (99)	VTX00114	*Rhizoglomus irregulare*

755 [†]Names denote the most similar AM fungal species of sequenced clones: Cl1, *Claroideoglomus* sp.1; Cl2,

756 *Claroideoglomus* sp.2; Cl3, *Claroideoglomus* sp.3; Div, *Diversispora* sp.; Fun, *Funneliformis mosseae*; Glo1, *Glomus*

757 sp.1; Glo2, *Glomus* sp.2; Glo3, *Glomus* sp.3; Glo4, *Glomus* sp.4; Glo5, *Glomus* sp.5; Glo6, *Glomus* sp.6; Glo7, *Glomus*

758 sp.7; Par, *Paraglomus* sp.; Scle, *Sclerocystis sinuosa*; Sept1, *Speptoglomus* sp.1; Sept2, *Speptoglomus* sp.2; Rhi,

759 *Rhizoglomus irregulare*.

760 †

761

762 **Table 5**

763 Richness (S), Shannon-Weaver (Hs), Simpson (D) and Evenness (E) indices calculated from AMF DGGE profile
764 associated with apple plant roots growing in orchards cultivated in two geographical sites (Terlano vs Lagundo) and
765 under two managements (organic vs integrated) (mean ± standard error)

Geographical area	Management	S	Hs	D	E
Lagundo	Organic	9.83 ± 0.87	2.19 ± 0.08	0.12 ± 0.01	0.97 ± 0.00
	Integrated	6.83 ± 1.01	1.72 ± 0.16	0.20 ± 0.03	0.93 ± 0.02
Terlano	Organic	10.5 ± 1.63	2.18 ± 0.17	0.13 ± 0.02	0.96 ± 0.01
	Integrated	12.3 ± 1.33	2.38 ± 0.08	0.09 ± 0.01	0.95 ± 0.01
Analysis of variance (P values)					
Geographical area		**0.033**	**0.049**	0.069	0.734
Management		0.666	0.392	0.252	0.126
Geographical area x Management		0.086	**0.044**	**0.033**	0.171

766

767

768

Table 6

Richness (S), Shannon-Weaver (Hs), Simpson (D) and Evenness (E) indices calculated from AMF DGGE profile associated with apple plant roots growing in orchards cultivated in Lagundo under two managements (organic vs integrated) (mean ± standard error)

Geographical area	Management	S	Hs	D	E
Lagundo	Organic	9.83 ± 0.87	2.19 ± 0.08	0.12 ± 0.01	0.97 ± 0.00
	Integrated	6.83 ± 1.01	1.72 ± 0.16	0.20 ± 0.03	0.93 ± 0.02
Analysis of variance (P values)					
Management		**0.029**	**0.028**	**0.049**	0.069

778 **Captions**

779 **Fig. 1.** a) Maps showing the location of apple orchards in South Tyrol, Italy. ● Integrated managed orchards at high
780 altitude, ● Organically managed orchards at high altitude, ● Integrated managed orchards at low altitude, ●
781 Organically managed orchards at low altitude. Source of the air photo on the right: Google earth V 7.1.2.2041 image©
782 2016 DigitalGlobe http://www.earth.google.com. Pictures of organically b) and integrated c) managed apple trees in the
783 study site.

784

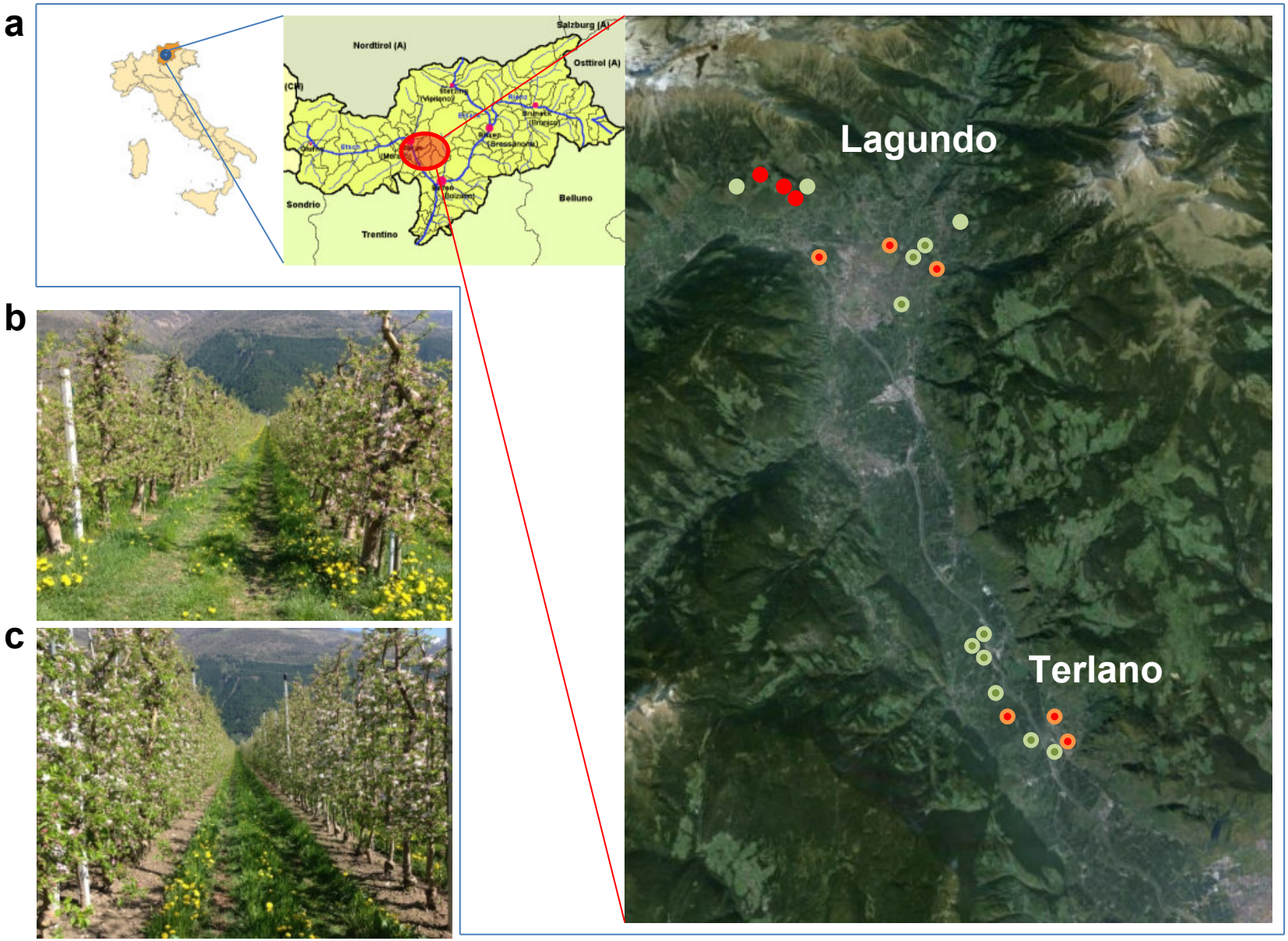
785 **Fig. 2.** Neighbor-Joining phylogenetic tree of glomeromycotan sequences derived from apple roots of orchards growing
786 in South Tyrol (Italy). Bootstrap values are shown when they exceed 75 % (1,000 replications). The analysis is based on
787 partial nuclear small subunit ribosomal RNA gene sequences (SSU; ~ 550bp; NS31/AML2 fragment) and involved 96
788 nucleotide sequences. Different sequence types are indicated in brackets and names are reported in Table 1. AMF
789 family are also reported. Sequences obtained in the present study are shown in bold and their accession numbers are
790 prefixed with site/management/altitude clone identifiers (Te, Terlano; La, Lagundo; O, Organic; I, Integrated; L, Low
791 altitude; H, High altitude). The tree is rooted with a reference sequence of *Corallochytrium lymacisporum* (L42528).
792

793 **Fig. 3.** Relative abundance (%) of AMF phylotypes detected in the roots of the different orchards cultivated in two
794 geographical sites (Lagundo and Terlano), under two managements (organic and integrated) and at two altitudes (Low,
795 300 m and High, 600m).

796

797 **Fig. 4.** Dendrogram obtained by UPGMA (Unweighted Pair Group Method Using Arithmetic Average) based on AMF
798 DGGE profiles obtained from apple plant roots growing in orchards cultivated in two geographical sites (Terlano and
799 Lagundo) and under two managements (■ Organic, ■ Integrated) (Te, Terlano; La, Lagundo; O, Organic; I, Integrated;
800 L, Low altitude; H, High altitude).
801

802 **Fig. 5.** Non-metric multidimentional scaling plot of DGGE analysis. Each point on the plot represents the AMF
803 community composition associated with apple plant roots growing in orchards cultivated in Terlano area (blue symbols)
804 or Lagundo area (orange symbols). The stress value is 0.2, the ANOSIM values (R) indicates significant differences
805 between Lagundo and Terlano (0.786, P=0.0001) and between organic and integrated (0.227, P=0.05) (Te, Terlano; La,
806 Lagundo; O, Organic; I, Integrated; L, Low altitude; H, High altitude).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Nordtirol (A)
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Osttirol (A)
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Trentino
Belluno
Lagundo
Terlano
b
c

Fig. 1

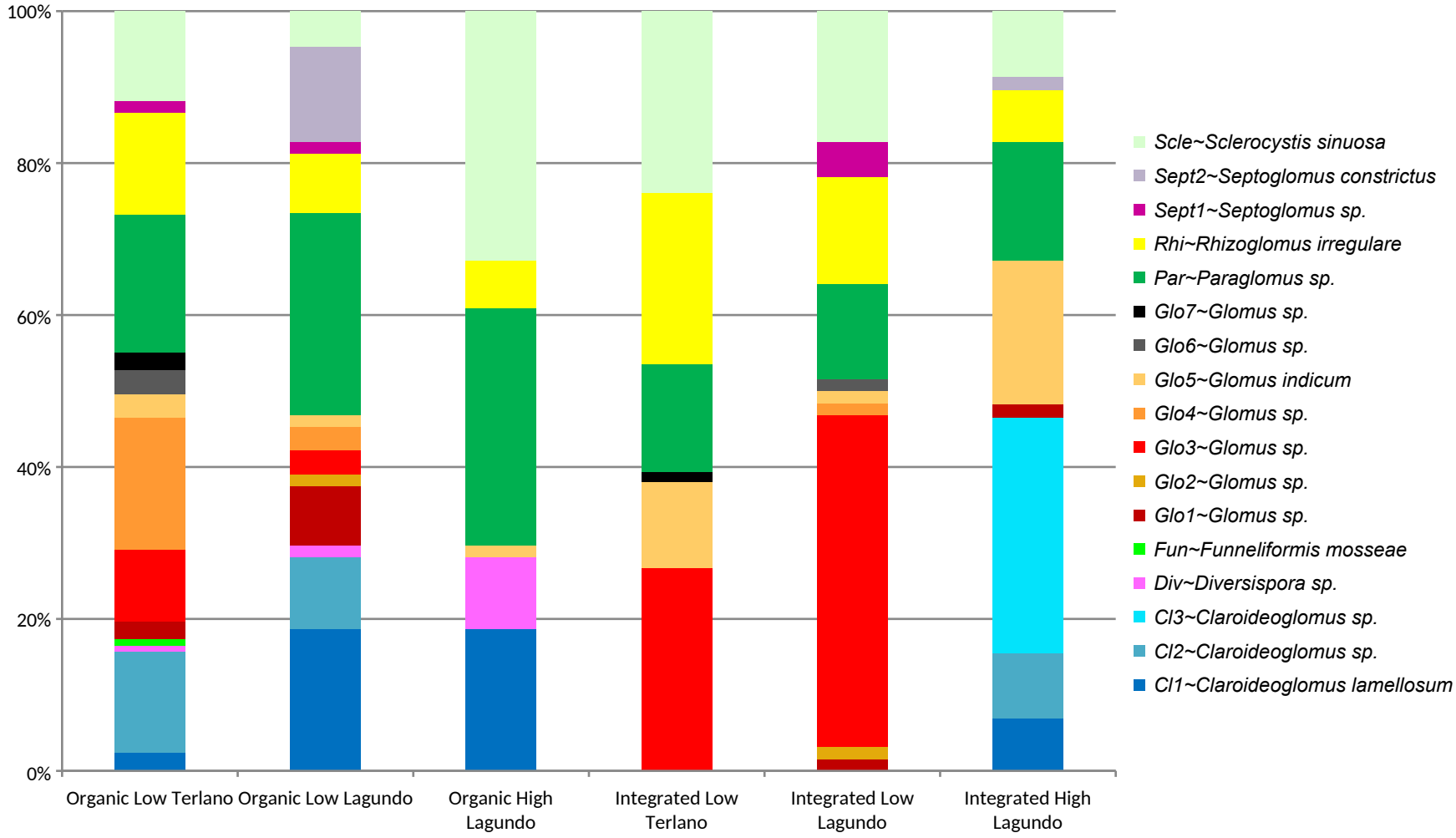
100%
80%
60%
40%
20%
0%
Organic Low Terlano
Organic Low Lagundo
Organic High Lagundo
Integrated Low Terlano
Integrated Low Lagundo
Integrated High Lagundo
Scle~Sclerocystis sinuosa
Sept2~Septoglomus constrictus
Sept1~Septoglomus sp.
Rhi~Rhizoglomus irregulare
Par~Paraglomus sp.
Glo7~Glomus sp.
Glo6~Glomus sp.
Glo5~Glomus indicum
Glo4~Glomus sp.
Glo3~Glomus sp.
Glo2~Glomus sp.
Glo1~Glomus sp.
Fun~Funneliformis mosseae
Div~Diversispora sp.
Cl3~Claroideoglomus sp.
Cl2~Claroideoglomus sp.
Cl1~Claroideoglomus lamellosum

Fig. 3

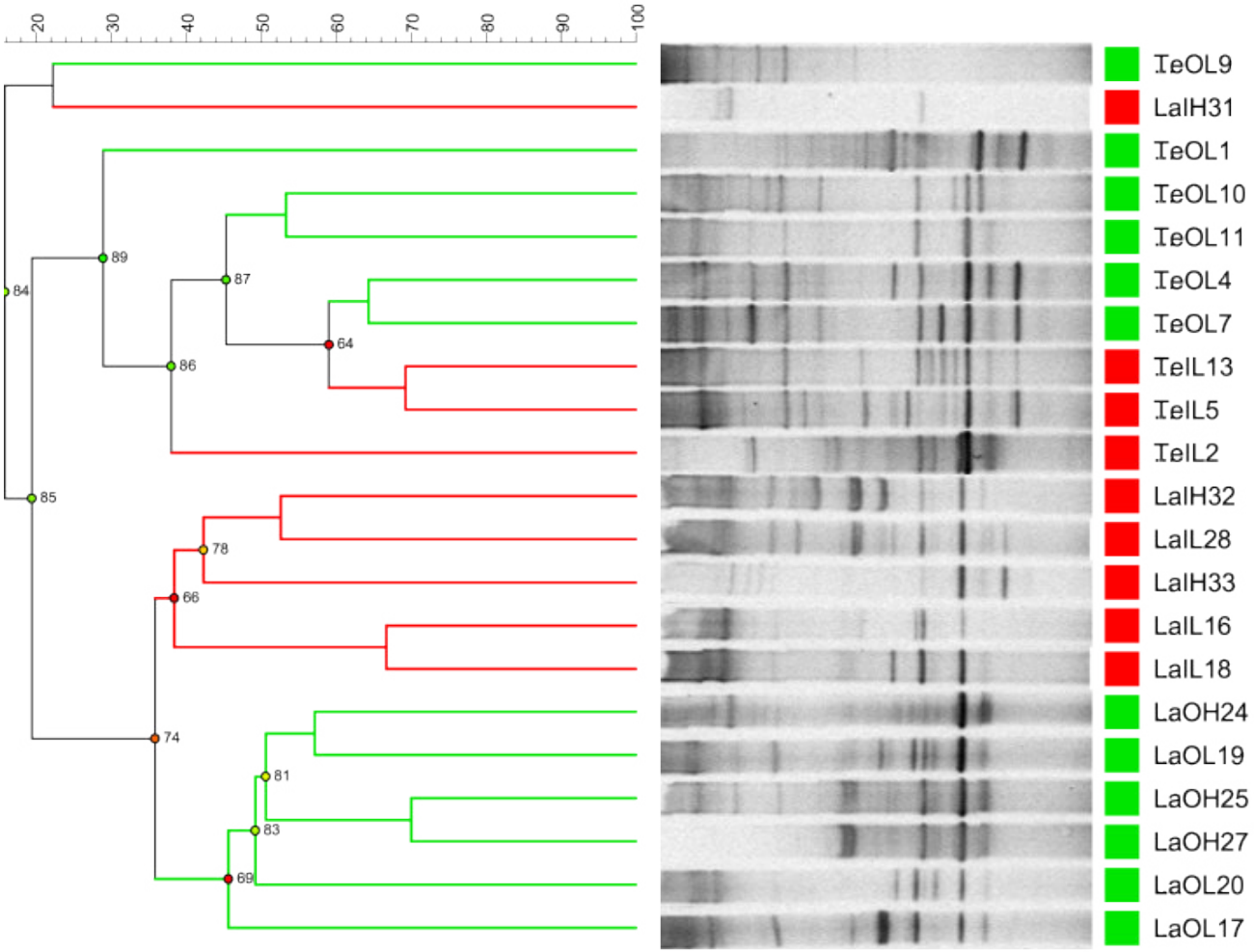
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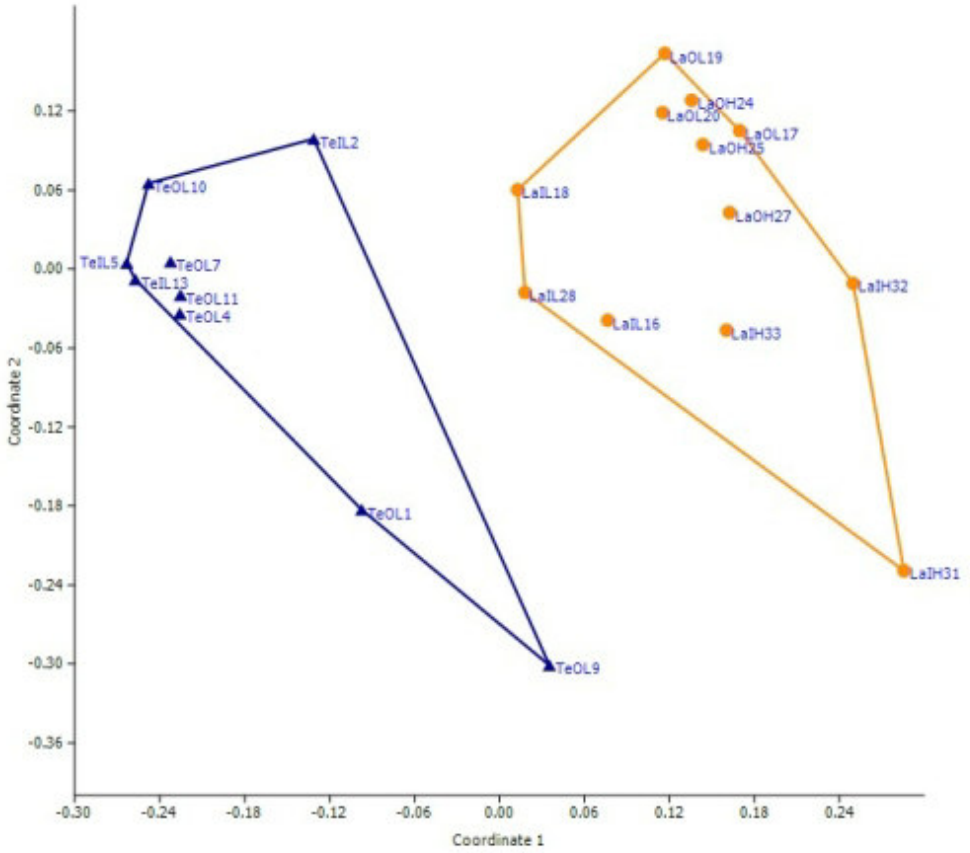
Fig.4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TeOL10
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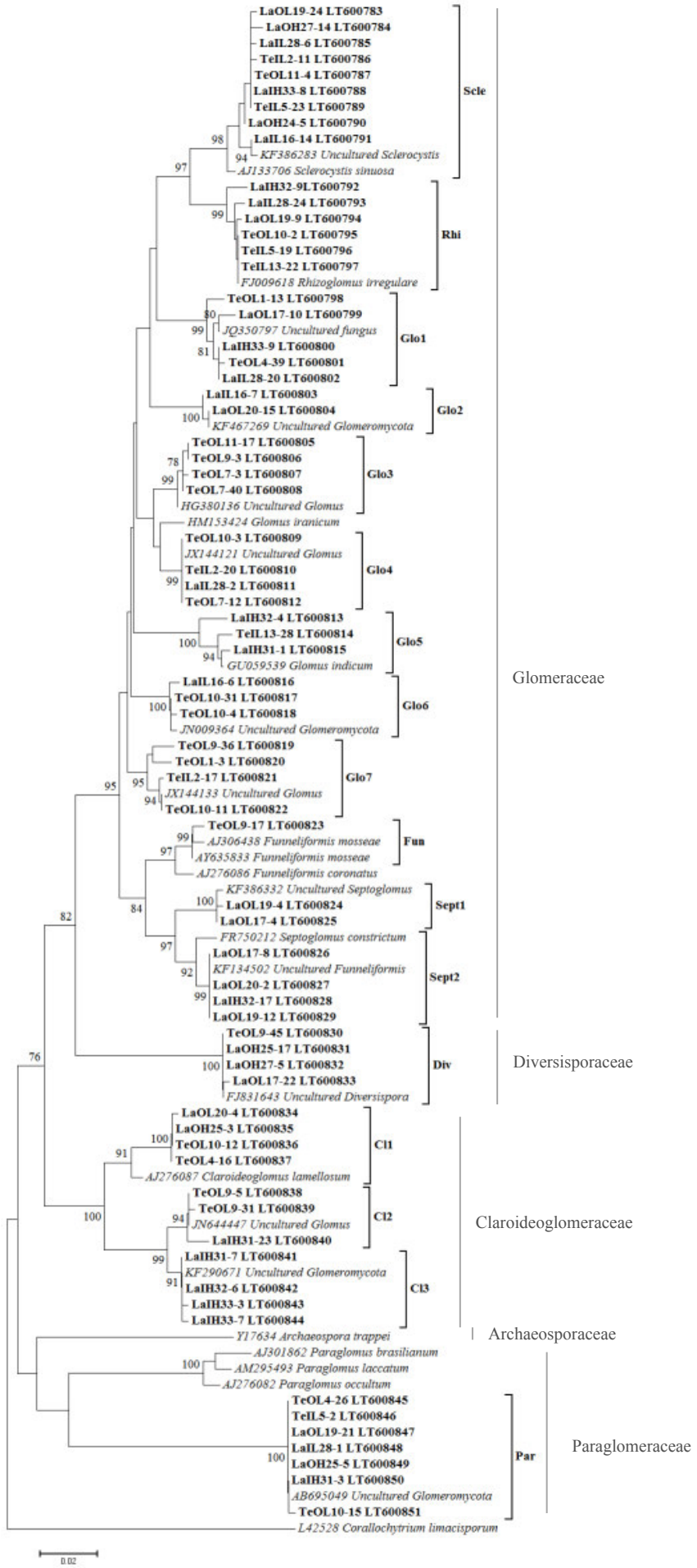
Coordinate 1

Fig. 5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KF386283 Uncultured Sclerocystis
AJ133706 Sclerocystis sinuosa
Scle
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LaIL28-24 LT600793
LaOL19-9 LT600794
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FJ009618 Rhizoglomus irregulare
Rhi
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TeOL4-39 LT600801
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Glo1
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Glo2
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Glo3
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Glomeraceae
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AJ276086 Funneliformis coronatus
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Sept1
FR750212 Septoglomus constrictum
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Sept2
TeOL9-45 LT600830
LaOH25-17 LT600831
LaOH27-5 LT600832
LaOL17-22 LT600833
FJ831643 Uncultured Diversispora
Div
Diversisporaceae
LaOL20-4 LT600834
LaOH25-3 LT600835
TeOL10-12 LT600836
TeOL4-16 LT600837
AJ276087 Claroideoglomus lamellosum
Cl1
TeOL9-5 LT600838
TeOL9-31 LT600839
JN644447 Uncultured Glomus
LaIH31-23 LT600840
Cl2
LaIH31-7 LT600841
KF290671 Uncultured Glomeromycota
LaIH32-6 LT600842
LaIH33-3 LT600843
LaIH33-7 LT600844
Cl3
Claroideoglomeraceae
Y17634 Archaeospora trappei
Archaeosporaceae
AJ301862 Paraglomus brasilianum
AM295493 Paraglomus laccatum
AJ276082 Paraglomus occultum
TeOL4-26 LT600845
TeIL5-2 LT600846
LaOL19-21 LT600847
LaIL28-1 LT600848
LaOH25-5 LT600849
LaIH31-3 LT600850
AB695049 Uncultured Glomeromycota
TeOL10-15 LT600851
Par
Paraglomeraceae
L42528 Corallochytrium limacisporum
0.02

Fig. 2