Wild Camellia japonica specimens in the Shimane prefecture (Japan) host previously undescribed AMF diversity

Andrea Berruti[1], Sonia Demasi[2], Erica Lumini[1], Nobuo Kobayashi[3], Valentina Scariot[1,2], Valeria Bianciotto[1]

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[1]Institute for Sustainable Plant Protection, UOS Torino – National Research Council, Torino (Italy)

[2]Department of Agricultural, Forest, and Food Sciences – University of Torino, Grugliasco (Italy)

[3]Faculty of Life and Environmental Science – Shimane University, Matsue (Japan)

Corresponding author:

Dr PhD Andrea Berruti

Institute for Sustainable Plant Protection

National Research Council

Viale Mattioli, 25

10125 Torino, Italy

Phone: +39 011 6705951

Fax: +39 011 6705062

e-mail: andrea.berruti@unito.it

Abstract

The native range of the broadleaf evergreen *Camellia japonica* L. includes natural non-model ecosystems that have been largely overlooked in the investigation of the diversity of arbuscular mycorrhizal fungi (AMF). Despite a recent overview of the AMF assemblages associated in the naturalized range of *C. japonica*, no such survey has ever been carried out within the native range of this plant species. For this reason, we examined through 454 sequencing the diversity and structure of AMF assemblages in camellia roots and surrounding soil from four locations within the Shimane prefecture (Japan), a region that harbors native *C. japonica* trees. The specific objectives were as follows: (i) to evaluate the differences between the root-colonizing and the soil-dwelling AMF community through different measurements of diversity and (ii) to evaluate if and how deeply the small-scale environmental changes affect the structure of AMF assemblages.

We found that a large number of AMF (~90%) could not be assigned to previously known phylotypes, suggesting the occurrence of several undescribed taxa. Diversity was generally higher in roots than in soil samples and the level of dominance was low. Almost 70% of soil-dwelling AMF were retrieved inside the roots and also community structure was very similar between the two niches. Most AMF clades/genera were infrequent and only *Rhizophagus*/*Sclerocystis* and *Glomus sensu lato* were very abundant in both root and soil samples. Above all, soil Fe and Mg content, soil C/N ratio, and the distance from the nearest source of saline water were consistently correlated with AMF community shifts at the local scale.

Keywords:

arbuscular mycorrhizal fungi, native range, 454 GS-FLX Titanium pyrosequencing, SSU rDNA, Virtual Taxa

1. Introduction

Obligate symbiotic fungi that form arbuscular mycorrhizae (AMF) are among the most important soil microorganisms. AMF facilitate mineral nutrient uptake from the soil and promote water-stress tolerance and resistance to certain diseases, in exchange for plant-assimilated carbon (Smith and Read, 2008), and are therefore considered promising biofertilizers (Berruti et al., 2016a). Different AMF species and isolates differ in life-history (Maherali and Klironomos, 2012; Powell et al., 2009) and functional traits (Fitter et al., 2005; van der Heijden and Scheublin, 2007; Hoeksema et al., 2010). Recent studies have found evidence that AMF communities can be influenced by both environmental (Davison et al., 2011; Helgason et al., 2007; Kohout et al., 2015; Öpik et al., 2009; Torrecillas et al., 2013; Verbruggen et al., 2013) and stochastic factors (Dumbrell et al., 2010; Lekberg et al., 2012), with the contribution of the two varying depending on the ecological context (Caruso et al., 2012). Although several factors notably affect AMF communities, most taxa are ubiquitously found (Dumbrell et al., 2010; Fitter et al., 2005; Öpik et al., 2010, 2009) and apparently reveal very low endemism on the global scale (Davison et al., 2015). While less common AMF tend to associate with host plant species that occupy specific ecological niches, the generalist taxa interact symbiotically with a wide range of host plants, including both native and invasive species, in a broad spectrum of environments (Davison et al., 2011; Moora et al., 2011; Öpik et al., 2013, 2009). In addition, AMF distribution may vary in belowground compartments since spore production rate and amounts of AMF hyphae in roots and soil have been demonstrated to vary substantially in a taxon-specific manner (Hempel et al., 2007; Johnson et al., 2004; Parniske, 2008; Varela-Cervero et al., 2015).

The broadleaf evergreen *Camellia japonica* L. (Magnoliophyta, Theales, Theaceae Mirb.) is a mycorrhizal plant species (Berruti et al., 2013) that is traded worldwide as ornamental potted plant. Although naturalized in several European countries, its center of origin resides in Japan (Mondal, 2011). Our research group has already described the AMF communities associated to naturalized specimens of *C. japonica* in parks and gardens around the Lake Maggiore area in Italy (Borriello et al., 2015), and found a strong difference in the community composition between the root-colonizing and the soil-dwelling communities and among the three closely located sites analyzed. The data suggest that different combinations of edaphic properties have a pivotal role in shaping the AMF communities. However, no such study has ever been carried out within *C. japonica* native range, which includes natural non-model ecosystems. Wild plants and natural, undisturbed

systems have associated with a high diversity of so-called 'uncultured' AMF (Ohsowski et al., 2014) and could hide a number of taxa that have been previously overlooked.

In the present study, we specifically ask the following: (i) Do the root-colonizing and the soil-dwelling AMF assemblages also differ strongly within the native range of *C. japonica*? (ii) Are small-scale environmental changes more important than stochasticity in driving the structure of AMF assemblages within the native range of *C. japonica*? Are they the same ones found in the naturalized range? To answer these questions we examined the diversity and structure of AMF assemblages, using 454 GS-FLX Titanium pyrosequencing technology, from four different locations within the Shimane prefecture (Japan), a region that harbors native *C. japonica* trees.

2. Materials and Methods

2.1. Sampling sites

The research focused on four sites in the Shimane prefecture (Japan), each representative of different soil properties and microclimatic conditions (Table S1). The first sampling site was the Matsue Castle Park (Tonomachi, Matsue; 35.476174, 133.048735; site code - MATSUE), an evergreen oak forest that hosts centennial camellia trees, including specimens of about 400 years old. The second sampling was done in the area neighboring the Shimane University experimental fields (Kami-honjyocho, Matsue; 35.511772, 133.109521; site code - SHIMANE), another evergreen oak forest that hosts young camellias (~20 yrs). The third sampling site was located near the village of Sagiura (Taisha-cho, Izumo; 35.444486, 132.686531; site code - SAGIURA), in an evergreen forest of black pines, oaks, and young camellias (~30 yrs), on a hill over the sea. The last sampling site was an evergreen/coniferous forest (Koshibara, Matsue; 35.447749, 133.076278; site code - BAMBOO) that hosts young camellia specimens (~30 yrs) and is invaded by *Phyllostachys edulis* (Carrière) J.Houz. (moso bamboo).

2.2. Soil and root sampling

Two specimens of *C. japonica* were randomly selected in each of the four sites for a total of eight biological replicates. Samples were collected during the first week of May 2013, during camellia late flowering

period. Sampling operations consisted in digging to the first 5-20 cm and collecting fine feeder roots belonging to *C. japonica* and, separately, a portion of bulk soil (ca 1.5 Kg) surrounding the sampled roots. During the digging, the main root branches were carefully followed and young camellia roots were visually recognized and collected. Three root and soil samples were collected from each plant, for a total of 48 samples (24 root samples and 24 bulk soil samples). Bulk soil samples were sieved and roots were washed free of adhering soil, sonicated, and chopped into small fragments (~1 cm). The processed samples were in part frozen in liquid nitrogen and separately stored at -80°C for further molecular analyses. The remaining bulk soil sample were pooled in order to create a composite sample for each of the eight biological replicates and submitted to physicochemical analyses (eight composite samples in total, two biological replicates for each site).

2.3. Physicochemical analyses

Electrical conductivity and pH were measured with a pH-meter on 10 g of soil in aqueous extract (soil:water 1:5) according to the standard protocols EN13037 and EN13038. Total carbon and total nitrogen were measured on 0.03 g of soil after complete dry combustion (method ISO 10694) and analyzed with element analyzer NA2100 (CE INSTRUMENTS). Available phosphorous was measured with Olsen method (Olsen et al., 1954). Fe, Ca, Mg, and K were measured on 0.5 g of soil through atomic absorption spectrophotometry (AAnalyst 400; Perkin Elmer) after digestion with aqua regia ($HCl:HNO_3$ 3:1; EPA method 3051A).

2.4. DNA extractions from soil and roots

Two different extraction kits were used according to the different nature of the sample, soil or roots. DNA extractions (0.5 g of soil each, without roots) from the 24 soil samples were performed using a FastDNA Spin Kit for Soil (MP BIOMEDICALS), according to the manufacturer's recommendations. DNA extractions from the 24 root samples were performed using a DNeasy Plant Mini Kit (QIAGEN, Crawley, UK) on 0.1 g of fresh root material, according to the protocol for frozen samples.

Nested PCR and sequencing of the fungal ribosomal (rRNA) gene

Two sets of primers were used to amplify a region of the small subunit (SSU) of the Glomeromycota (the phylum that includes all AMF) ribosomal DNA. The Nested PCR approach used consisted in a first

amplification with Glomeromycota-specific primers AML1 and AML2 (Lee et al., 2008) and a following amplification round with tagged-primers AMADF (5′-GGGAGGTAGTGACAATAAATAAC-3', 121 nucleotides downstream from AML1 primer; newly designed by Desirò, 2013) and AMDGR (Sato et al., 2005) which specifically amplifies ~423 bp (size suitable for 454 GS-FLX System) of the V3-V4 variable domains within the 18S rDNA gene of AMF. PCR was carried out in 20 µl of a PCR reaction mix containing 2 µl of template DNA, 4 µl of 5X Phusion HF Buffer, 0.2 mM dNTPs, 0.5 µM of each primer, and 0.4 U of Phusion® High-Fidelity DNA Polymerase (FINNZYMES, Finland). Amplifications were carried out in 0.2 ml PCR tubes using a Biometra T Gradient thermocycler according to the following steps: 5 min initial denaturation at 94°C; 35 cycles of 1 min at 94°C, 1 min at 58°C and 57°C for the two Nested PCR rounds, respectively, 1 min at 72°C; and a final elongation of 10 min at 72°C. A negative control was included in the PCR to check for contamination. All PCR products were checked using 1.5% agarose gel stained with ethidium bromide (Sigma-Aldrich). The PCR products bearing the same tags and coming from the three root or soil samples taken from the same plant were pooled in order to create sixteen composite samples (eight biological replicates for both roots and soil) and purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega). These sixteen purified PCR products were equimolarly pooled and sequenced through 454 GS-FLX Titanium pyrosequencing technology (Beckman Coulter).

2.5. Sequence analyses and classification

Raw sequencing data were treated with the open-source mothur v1.33 for Mac (Schloss et al., 2009). Denoising of the flowgrams was performed using the PyroNoise algorithm (Quince et al., 2009). Tags and adaptors were trimmed off. Then, sequences with ambiguous nucleotides, shorter than 350 bp or with homopolymers longer than 13 bp were removed. Sequences were aligned and clustered according to a genetic distance matrix computed using the *dist.seqs()* command in mothur, setting the "countends" parameter to false. OTU picking was performed using the default Average Neighbor clustering method, implemented by mothur, setting a 3% dissimilarity cutoff. Although this distance cutoff is arbitrary and can be considered controversial, it was chosen on the basis of previous studies on AMF biodiversity (Borriello et al., 2015; Lumini et al., 2010). Moreover, a recent study has demonstrated how the choice of the OTU delineation method negligibly affects the interpretation of AMF community patterns (Lekberg et al., 2014). The most abundant unique sequence of

each cluster was selected as OTU representative. Pruning of OTUs with low numbers of sequences (<10) was carried out on a per-sample basis, as an OTU that is common in one sample may occur as a low-abundant contaminant in other samples due to tag switching (Carlsen et al., 2012) or slight cross-contamination. A search for similar sequences was conducted with Blast v2.2.29 (Zhang et al., 2000) on the latest release of the MaarjAM AMF Virtual Taxa (classified as VTXnnnnn, where "n" is a numeric code, e.g. VTX00113) type online database (Öpik et al., 2010) integrated with the SSU Silva database (Yilmaz et al., 2013) cleared of Glomeromycota sequences. Recently, two major reorganizations of the Glomeromycota classification were published (Oehl et al., 2011; Schüßler and Walker, 2010). In this study, for ease of data handling, the phylogeny derived from the work of Schüßler and Walker (Redecker et al., 2013) was basically adopted to affiliate OTUs to the corresponding taxonomy. Since the ~423bp ribosomal DNA fragment under study can make it difficult to clearly separate phylogenetically some of the genera described in the work of Redecker et al. (2013), sometimes clades were used (i.e. *Rhizophagus*/*Sclerocystis*, *Funneliformis*/*Septoglomus*, and *Glomus sensu lato*) in order to group sequences with a conservative approach. Non-Glomeromycota OTUs were removed from the dataset. Sequences were considered belonging to AMF when they had a minimum 90% homology with a VT type, minimum 93% homology with a Glomeromycota GenBank sequence, and did not better match any other accession from a different taxonomic group. Minimum sequence coverage required during the alignment was 93%. Potential chimeric sequences were identified and removed using the uchime algorithm (Edgar et al., 2011). A further chimera check was performed manually by individually blasting terminal chunks of sequences that had overall <97% homology with database accessions. Prior to statistical analyses, as a normalization step to reduce bias associated with different sequencing depths, all samples were subsampled down to the size of the smallest one.

2.6. Statistical data analyses

All statistical analyses were done using R v3.2.0 (R Development Core Team, 2015) and Past multivariate statistics software package v3.0 (Hammer et al., 2011). The R libraries *vegan* (Oksanen et al., 2015), *packfor* by Stephane Dray, and *indicspecies* (De Cáceres et al., 2010) were adopted. The number of OTUs, the exponential of the Shannon's diversity index (a biodiversity index based on the proportions of individuals), the number of OTUs per AMF clade/genus, the sample accumulation curves (a sampling effort

graphical assessment) and the individual sample rarefaction curves (a sequencing effort graphical assessment) were calculated for each sample. A one-way ANOVA (model = ~ sample type + Error(site)) was performed on OTU counts (after log transformation) and on the exponential Shannon's index, in order to determine the effect of sample type (soil or roots) on the AM fungal diversity. In order to untangle the relationship between diversity and physicochemical, climatic (mean annual temperature and precipitations), and spatial (latitude, longitude, distance from sea and distance from source of saline water) variables, Spearman's correlation coefficients were calculated between OTU counts of three AMF taxonomy levels (orders, families, and clades/genera) and environmental variables. The Non-metric Multi-Dimensional Scaling (NMDS), based on the Bray-Curtis distance (Wisconsin/square-root transformed OTU relative abundance dataset) was constructed to graphically assess the differences in the community composition between the two sample types. Soil physicochemical, climatic, and spatial variables (standardized as z-scores) were plotted as vectors in the Bray-Curtis NMDS and their squared correlation coefficients were calculated as indicator of goodness of fit, to assess their potential role in the community structuring and composition. In order to quantify the fractions of AMF community variance explained by the measured environmental variables and by the sample type, the partition of variation was performed. Only environmental variables that were measured at the plant level (i.e., soil variables) were submitted to forward-selection (function *forward.sel* in package *packfor*), using adjusted R^2 and alpha=0.05 as cutoffs according to Blanchet et al. (2008), in order to avoid collinearity among explanatory variables in the model and to search for parsimony. Subsequently, the amount of AMF community (Hellinger-transformed) variance potentially explained by forward-selected soil variables was computed. A PERMANOVA (999 permutations) based on the Bray-Curtis distance matrix was carried out to determine the effect of sample type (soil or roots), correcting for the random effect of the site factor (model = ~ sample type, strata = site). The heterogeneity of the communities was tested with a Beta-dispersion analysis. Finally, soil physicochemical variables were individually submitted to a k-means clustering which grouped soil physicochemical measures into two classes, corresponding to higher and lower values (Table S2). Indicator species for each resulting classes were detected using only OTUs with relative abundance >1% as input. To explore the differential distribution of OTUs between root and soil samples, the Similarity Percentage analysis (SIMPER) was carried out. For the same reason, multiple Kruskal-Wallis tests were performed.

205

206 **3. Results**

207 **3.1. Physicochemical properties of the soils**

208 The physicochemical properties of the soils are reported in Table 1. The most remarkable differences
209 were seen in available P, which was five-fold higher in MATSUE than in the other soil samples. Fe content
210 was more than double in SHIMANE soil than in the other samples. Noteworthy, soil total C was much higher
211 in BAMBOO, compared to the other locations, while total N was very low in SAGIURA.

212

213 **3.2. Sequencing output**

214 The 454 GS-FLX Titanium pyrosequencing yielded 101514 raw sequences. A preliminary removal of
215 short and low quality sequences resulted in 95249 Glomeromycota sequences and 1398 aspecific amplicons
216 (1247 belonging to Dikarya and 151 belonging to plants, annelids and unclassified Eukaryota). After removing
217 chimeric, rare, or artifact OTUs, 75829 good quality sequences were left (Table S3). Sequencing depth ranged
218 from 1858 to 9565 sequences, therefore, subsampling was carried out at 1858 reads (minimum sequencing
219 depth). Total OTU count was 254, ranging from 16 to 52 per sample. Overall, the root samples counted 216
220 OTUs while soil samples only 125. The 254 OTU representative sequences were registered in GenBank under
221 the following accession number string: **KT325597-KT325850**. Each rarefaction curve was able to reach the
222 asymptote at a much smaller number of sequences than the corresponding sample size, suggesting an optimal
223 sequencing effort for all samples (Fig. S1). All four Glomeromycota orders were retrieved, indicating a good
224 coverage of the biodiversity by the primers used, and the sequences were distributed in nine families (Fig. S2)
225 and 13 clades/genera (Fig. 1). Only 25 OTUs (9.84%) had a homology equal to or higher than 97% to a Virtual
226 Taxon type sequence. Even when considering only OTUs that were found in at least two samples (107 in total),
227 this value remained very low (16 OTUs, 14.95%). Overall, the Glomeraceae family was the most abundant
228 and diverse (88.4%, 222 OTUs), followed by Gigasporaceae (5.0%, 8 OTUs), Paraglomeraceae (3.6%, 4
229 OTUs), and marginal occurrence of Diversisporaceae (5 OTUs), Claroideoglomeraceae (6 OTUs),
230 Acaulosporaceae (5 OTUs), Ambisporaceae (2 OTUs), Archaeosporaceae (1 OTU), and Geosiphonaceae (1
231 OTU), together accounting for 2.9% sequences. The most abundant and diverse clade/genus was
232 *Rhizophagus*/*Sclerocystis* (51.4%, 124 OTUs), followed by *Glomus sensu lato* (36.1%, 93 OTUs), *Paraglomus*

(3.6%, 4 OTUs), *Scutellospora* (3.0%, 5 OTUs), *Gigaspora* (2.1%, 3 OTUs), *Claroideoglomus* (1.0%, 6 OTUs), and marginal occurrence of *Funneliformis*/*Septoglomus* (5 OTUs), *Diversispora* (4 OTUs), *Acaulospora* (5 OTUs), *Redeckera* (1 OTU), *Ambispora* (2 OTUs), *Archaeospora* (1 OTU), and *Geosiphon* (1 OTU), together accounting for 2.9% sequences. The root and the soil samples shared 87 OTUs, so 129 OTUs were only found in root samples while 38 OTUs were only found in soil samples. The four locations shared 14 OTUs (OTU001, OTU003, OTU004, OTU005, OTU006, OTU007, OTU008, OTU016, CHI001, OTU021, OTU024, OTU025, OTU035, and OTU045) that were phylogenetically related to ten Virtual Taxa (VTX00154, VTX00123, VTX00079, VTX00345, VTX00093, VTX00112, VTX00260, VTX00412, VTX00322, and VTX00111). Thirteen of these OTUs belonged to Glomeraceae (11 *Rhizophagus*/*Sclerocystis* and 2 *Glomus sensu lato*) and 1 to Gigasporaceae (*Scutellospora*). Of these, only three OTU representative sequences had homology higher than 97% with a Virtual Taxon type sequence (VTX00260, VTX00112, and VTX00093). No OTU was shared by all samples.

3.3. Effect of sample type on AMF community and diversity

The number of observed OTUs (richness) was significantly lower in the soil (p-value = 0.0043, Table 2, Fig. S3). No significant difference in the exponential Shannon diversity index was found between the root and the soil. The number of OTUs assigned to *Glomus sensu lato* was significantly lower in soil samples, while the opposite was true for *Ambispora* (Fig. S4), although the number of OTUs recorded for this genus was negligible. No OTU was significantly differentially distributed between root and soil samples, although some trends were noted (Table S4).

The NMDS ordination shown in Fig. 2 was based on the Bray-Curtis (stress: 0.142, 2D) distance matrix. A major overlap is visible between the 95% confidence ellipses of the two niches. The one-way PERMANOVA (Table 3) showed that the AMF community composition was not significantly affected by sample type. The test for homogeneity of multivariate dispersion proved that the results of PERMANOVA are reliable, since no significant heterogeneity of dispersion was detected (Table 3).

3.4. Explanatory variables of the AMF community structure and diversity

Soil physicochemical, climatic, and spatial variables that significantly (alpha = 0.05) fitted the NMDS ordination (Table S5) were plotted as vectors in the biplot in Fig. 2. Latitude, longitude, distance from saline or sea water, mean annual temperature and precipitation, Fe, Mg, and C/N ratio significantly correlated with the Bray-Curtis ordination.

Soil physicochemical variables that best explained AMF community variation are listed in Fig. 3. Overall, selected soil variables *per se* accounted for a highly significant portion (according to adjusted R^2 transformed into percentage, 50.21%) of the total community variance (Fig. 3). The fraction of variation explained by the sample type was instead low (3.29%) and not significant (p-value<0.10). Less than half AMF community variance (46.49%) remained unexplained.

Table 4 reports highly significant (p-value<0.01) Spearman's correlations between the environmental variables and the OTU richness of AMF orders, families, and clades/genera. Increasing distance from the sea and total C were shown to reduce the occurrence of Diversisporaceae diversity. Conversely, higher levels of soil EC, K, and pH were correlated with a higher diversity of this AMF family. *Diversispora* diversity also increased with pH. The ratio between total C and total N was negatively correlated with the diversity of Archaeosporales and Paraglomerales, while was positively correlated with *Rhizophagus*/*Sclerocystis* OTU count. Finally, the number of Paraglomerales OTUs increased with soil P content.

Indicator species analysis was carried out to test whether specific AMF OTUs could be predictive of particular soil physicochemical features. Measured soil variable values were clustered in two groups using the k-means algorithm. This made it possible to allocate each value to a rank/level called HIGH, for higher values, or LOW, for lower values. Table 5 reports the AMF OTUs that yielded consistent results (IndVal≥0.8, P<0.05) as indicator species for one of the two levels grouping the measured values of each soil physicochemical variable considered in this study. Three indicator OTUs were detected for lower pH, one for lower EC, one for both lower and higher available P, two for higher Fe, one for higher Mg, one for higher total C, and two for higher C/N ratio (Table 5).

4. Discussion

As previously demonstrated, the targeted amplification of sequences exclusively belonging to Glomeromycota from environmental samples is challenging, often resulting in frequent aspecific amplification and incomplete coverage of the AMF diversity (Alguacil et al., 2011; Kohout et al., 2014; Liu et al., 2011; Lumini et al., 2010; Van Geel et al., 2014). The use of AMADF forward primer, specifically designed to detect Glomeromycota by Nested PCR approach (Desirò, 2013), combined with the 454 GS-FLX Titanium pyrosequencing, proved to be successful, resulting in very high quality output. In fact, only 23.9% raw sequences were removed due to low quality (judged as candidate chimeras or potentially erroneous) and, unexpectedly, merely 1.4% sequences were the result of aspecific amplification. The coverage of the Glomeromycota phylum was wide, highlighting 254 OTUs, members of all four orders and nine out of the ten previously described families (Krüger et al., 2012; Redecker et al., 2013). Similarly, in a recent study in which the same protocol was adopted, only ~28% raw sequences were discarded due to low quality and the use of AMADF primer made it possible to achieve a near-complete coverage of the Glomeromycota phylum (Berruti et al., 2016b).

Overall, only less than 10% OTUs could be identified (≥97% homology) as known Virtual Taxa using the MaarjAM database (Öpik et al., 2010) as a reference. This corroborates that our knowledge of the diversity of the Glomeromycota phylum is still limited, with particular regards to natural systems, which have been largely overlooked so far (Ohsowski et al., 2014; Öpik et al., 2013). However, although our bioinformatic pipeline provided a very conservative approach for detecting and discarding most artifact OTUs, the complete removal of erroneous sequences cannot be achieved and diversity can consequently be somehow inflated (Lindahl et al., 2013), resulting in some spurious OTUs that cannot be affiliated to known reference sequences.

The per-sample OTU count (≥26) and the exponential Shannon index (>9.2) were generally high in both roots and soil, suggesting the presence of a very high level of AMF diversity. Accordingly, the per-sample relative abundance of the dominant taxon was always lower than 42%, meaning that the level of dominance was not high. Diversity indices were much higher than the ones found by Borriello et al. (2015) in *C. japonica* naturalized range. However, these authors have investigated AMF diversity based on Sanger sequencing that is often biased by its lower sequencing effort potential. If we add that 454 pyrosequencing can inflate diversity by bringing up to light several spurious OTUs with low read number, then OTU counts become incomparable between the two ranges. Conversely, the Shannon index and derived indices are more comparable, since they

315 are less affected by species count, being based on species proportions. In support, in our study the communities
316 were quite even, with the dominant OTU only being averagely 2 times more abundant than the second most
317 abundant OTU and 3.4 times more abundant than the third most abundant OTU, unlike in Borriello et al. (2015)
318 and Dumbrell et al. (2010) where these ratios were higher. In addition, the relative OTU counts seemed to be
319 higher in soil than in root samples in the work by Borriello et al. (2015) while we highlighted a diametrically
320 opposite situation, more similarly to other studies where plant species were growing in their native range soil
321 (Pivato et al., 2007; Saks et al., 2014).

322 Nonetheless, the difference in OTU richness between roots and soil did not reflect in a major difference
323 in AMF community structure, since the majority of soil-dwelling AMF (69.6% OTUs) were retrieved inside
324 the root as well. It might be argued that soil, being the reservoir from which AMF are recruited by the host
325 plant during specific time lapses, should logically harbor the highest level of diversity. An explanation could
326 be that the most abundant family found, i.e. the Glomeraceae, had a higher OTU number in roots than in soil,
327 presumably due to the fact that some of its members are less prone to extend very far from the roots (Maherali
328 and Klironomos, 2007) and is therefore more likely to be overlooked in soil samples. Another possibility is
329 that AMF taxa in soil might have been underestimated since AMF biomass, and consequently AMF DNA, is
330 expected to be an order of magnitude less than in roots (Olsson et al., 2010). This hypothesis is partially
331 supported by our results that show a generally lower number of good quality AMF reads in soil samples. In
332 addition, since the distribution of AMF nuclei is uneven among fungal structures (Gamper et al., 2008), the
333 occurrence of AMF taxa characterized by different life styles (e.g. profuse sporulation vs. soil hyphal growth)
334 might have partially biased the correct assessment of soil AMF richness due to a dilution effect. A possible
335 reason why root and soil communities were highly similar is that *C. japonica* might be more prone to
336 indiscriminately welcome inside its root apparatus most of the AMF taxa that are available from its native soil
337 potential, that might have co-evolved and could therefore be more specific. Moreover, the soil mycelial
338 network could be more shared between plant species clusters of the same geographic origin than between plant
339 species introduced from distinct regions, such as the ones hosted in parks and gardens of the Lake Maggiore
340 area (Borriello et al., 2015), where soil AMF diversity poorly matched root AMF diversity.

341 Diversity at the clade/genus level was also high in *C. japonica* native range, although most genera
342 were underrepresented and only *Rhizophagus*/*Sclerocystis* and *Glomus sensu lato* were very abundant in both

root and soil samples. Similarly, Glomeraceae of the *Rhizophagus*/*Sclerocystis* (VTX00412, VTX00219, VTX00223, VTX00074) and *Glomus sensu lato* (VTX00122) clades were dominant in the roots of *C. japonica* in its naturalized range (Borriello et al., 2015). However, these authors found evidence that *Paraglomus* was prevailing in the soil community. In addition, the globally distributed Diversisporales order (Öpik et al., 2010) was lacking in the naturalized range, differently than in the native range where it was more common (overall >5%).

Only two Virtual Taxa (the *Rhizophagus*/*Sclerocystis* VTX00093 and the *Paraglomus* VTX00239) were present in both the native range under study and the naturalized range of *C. japonica* investigated by Borriello et al. (2015). However, VTX00093 was common in the native range while very rare in the naturalized range while VTX00239 was not common in the native range while abundant and ubiquitous in the naturalized range. In another study on windmill palm, *Trachycarpus fortunei* (Hook.) H.Wendl., a higher number of shared Virtual Taxa (10) was detected between the native, the experimentally introduced, and the invasive range (Moora et al., 2011). However, unlike in the present study and in the work of Borriello et al. (2015), these authors have investigated the AMF diversity associated to seedlings at a relatively young stage (1-3 leaves).

As in *C. japonica* naturalized range (Borriello et al., 2015), the native range also demonstrated a high degree of heterogeneity in AMF community structure at the local scale. The variance partition analysis suggested that these changes were by a considerable extent mediated by soil variables. Above all, soil Fe content, C/N ratio, and Mg were consistently identified as the variables most likely involved in AMF community structuring. Previous studies strongly support the role of soil Fe (Moebius-Clune et al., 2013) and C/N (Dumbrell et al., 2009), as major determinants regulating the composition and structure of AMF communities. The increase of C/N ratio, which was previously described as negatively correlated with soil AMF biomass (Wang et al., 2012), caused a decline in the diversity of Archaeosporales and Paraglomerales, which are, in line with our results, notorious soil explorers (Hempel et al., 2007) and rather occasional (Alguacil et al., 2011) and patchy (Varela-Cervero et al., 2015) root colonizers. Conversely, *Rhizophagus*/*Sclerocystis* diversity benefitted from this condition. Accordingly, two *Rhizophagus*/*Sclerocystis* OTUs were highlighted as indicators of high C/N ratio. The concentration of Mg in the soil, and especially the ratio between soil Ca and soil Mg, are also commonly recognized as AMF community drivers (Schechter and Bruns, 2012, 2008). All soils examined in our study featured serpentine-like properties (Ca/Mg<1), with

SAGIURA showing the lowest ratio. One OTU of *Rhizophagus*/*Sclerocystis* was indicator of higher soil Mg. In the study on *C. japonica* naturalized range by Borriello et al. (2015), instead, a member of the *Glomus sensu lato* clade was found exclusively in high soil Mg condition, however Mg content was more than 10-fold higher than what found in the native range of *C. japonica*.

Some other variables, although poorly or less affecting the AMF community as a whole, had a clear effect on the diversity of specific taxonomic ranks or on the occurrence of single AMF taxa. The pH range examined in our study was rather narrow (5.78-6.43), however, similarly to another study (Fitzsimons et al., 2008), it was enough to be candidate as a potential driver of AMF community shifts. The viability and infectivity of different AMF taxa are known to vary in different pH ranges (van Aarle et al., 2002). In our study, pH positively affected the diversity of Diversisporaceae (in particular *Diversispora*). However, no *Diversispora* OTU was selected as indicator of higher pH. Instead, three *Rhizophagus*/*Sclerocystis* were found as indicator for lower pH. Soil N, P, and K showed a major involvement in AMF community structuring in *C. japonica* naturalized range (Borriello et al., 2015). In our study, total soil N did not influence AMF community structure while soil available P content, one of the major variables associated to the decline in AMF diversity in literature (Gosling et al., 2013; Lin et al., 2012; Sheng et al., 2013), unexpectedly showed to increase along with the OTU count of Paraglomerales. One *Paraglomus* OTU was indeed indicator of higher available P, along with a *Glomus sensu lato* OTU. One *Rhizophagus*/*Sclerocystis* OTU was instead indicator of lower available P. This is in line with Chen et al. (2014), who found that members of the Glomeraceae can be differentially distributed in response to different regimes of fertilization. Soil salinity (EC and proximity to seawater) positively affected Diversisporaceae diversity. Our results are in line with the work of Yamato et al. (2012), who, in the Tottori prefecture (Japan), which is adjacent to the location under study (Shimane prefecture), detected *Diversispora* members in association with high salinity, in the proximity of seawater. In previous studies, *Diversispora* was abundantly retrieved in a salt marsh environment (Estrada et al., 2013) and, interestingly, the species *Diversispora spurca* was demonstrated to have a role in salt stress alleviation in orange tree species (Zou and Wu, 2011).

It can be concluded that root-colonizing and soil-dwelling AMF communities associated to *C. japonica* in its native range are very similar and host a high number of previously undescribed AMF taxa. Multiple predictors (above all soil Fe, soil C/N, Mg, and distance from the nearest source of saline water) could explain

399 a considerable portion of the community variance and/or triggered important diversity shifts at several
400 taxonomic levels of AMF at the local scale. We elucidated the edaphic preference of several AMF taxa that
401 were shown to exclusively occur under certain environmental conditions. These taxa might be of interest when
402 looking for AMF inoculants suitable for agriculture under different soil conditions.
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Table captions

Table 1. Physicochemical properties of the soils sampled in the four different sites (Matsue, Shimane, Sagiura, and Bamboo). SD of means is provided.

Table 2. Species richness indicators calculated for the four sites (MATSUE, SHIMANE, SAGIURA, AND BAMBOO) and the two sample types (roots and soil). ANOVA p-values are reported for sample type (model = ~ sample type + Error(site)). SD of means is provided.

Table 3. Permutational multivariate analysis of variance (PERMANOVA) between sample types (model = ~ sample type, strata = site) using OTU-based Bray-Curtis distance matrix calculated on square-root/wisconsin standardized read counts. The tests for homogeneity of multivariate dispersion (Beta-dispersion) are also reported. Permutation number was set at 999.

Table 4. Highly significant ($p<0.01$) Spearman's correlations between the OTU richness of several taxonomic levels and environmental variables.

Table 5. Indicator species analysis for the k-means clusters (lower values and higher values) defined for each soil physicochemical variable. Specificity, sensitivity, and IndVal values, p-value, and OTU affiliation (taxonomy clade and closest virtual taxon type sequence) and units after subsampling are reported.

Table S1

Sampling site characteristics. Site code, location name, country, spatial variables (latitude, longitude, distance from the sea, distance from brackish or salt water), climatic variables (mean annual temperature, mean annual precipitations), biome, ecosystem type, plant approximate age, dominant canopy, and soil type are reported.

Table S2. K-means clusters (HIGH for higher values and LOW for lower values) used for indicator species detection for the measured soil physicochemical variables.

653 Table S3. OTU table with OTU taxonomic affiliation (order, family, clade/genus). Closest Virtual Taxa, first
654 hit sequence code, homology, alignment length, sample codes with site and sample type information, and per
655 sample OTU units after subsampling are reported. At the bottom of the table, total OTU counts, sequencing
656 depth prior to subsampling, and order, family, and clade/genus level OTU counts are also reported.
657
658 Table S4. SIMPER analysis and Kruskal-Wallis tests to assess differential distribution of OTUs between root
659 and soil samples. Average dissimilarity index, average abundance in roots and soil (including bar charts),
660 cumulative contribution in variation (proportion of variation), SIMPER p-value, Kruskal-Wallis p-value and
661 false discovery rate are reported.
662
663 Table S5. Squared correlation coefficients of the environmental variable fitting with the NMDS distribution
664 (ns = non-significant p-value, * = 0.010<p-value<0.050, ** = 0.001<p-value<0.010, *** = p-value<0.001).

Figure captions

Figure 1. Overall relative abundances of AMF clades/genera in root and soil samples. SE of the subsampled read counts are reported.

Figure 2. Non-metric Multi-Dimensional Scaling (NMDS) biplot based on Bray-Curtis distance matrix of the AMF communities found in each sample (filled circles = roots, open circles = soil). The vectors (arrows) graphically represent the significant correlations (at alpha = 0.05) of the NMDS axes with the measured environmental variables. Standard deviation of point scores was used to plot 95% confidence ellipses. Stress was 0.142.

Figure 3. Variance partitioning between the forward-selected environmental variables and the sample types (roots and soil). The fractions of variance explained by the forward-selected measured environmental variables, the sample type factor, and the residual variance are reported as adjusted R^2 (transformed into percentages). The level of significance of the explained fractions according to the modified F-test for multivariate datasets implemented in the function *forward.sel* of the package *packfor* in R v3.2.0 are also reported. The forward-selected environmental variables are listed and their individual statistical significance levels reported (* = 0.010<p-value<0.050, ** = 0.001<p-value<0.010, *** = p-value<0.001).

Figure S1. Rarefaction curves. The figure shows the OTU accumulation plotted as a function of the number of sequences (prior to subsampling) at the sample level. The sample name (e.g. MAT_1R) identifies the abbreviated site of origin (MAT=MATSUE, SHI=SHIMANE, SAG=SAGIURA, and BAM=BAMBOO), the sample number (1-8) and the sample type of origin (R for roots and S for soil).

Figure S2. Overall relative abundances of AMF families in root and soil samples. SE of the subsampled read counts are reported.

Figure S3. Sample accumulation curves. The figure shows the OTU accumulation plotted as a function of the number of root or soil samples.

Figure S4. Variation in the number of OTUs between root and soil samples according to a Kruskal-Wallis test.

Table 1.

Site	pH (units)	EC (mS/m)	Available P (mg/Kg)	Fe (mg/Kg)	K (mg/Kg)	Mg (mg/Kg)	Ca (mg/Kg)	Total C (%)	Total N (%)	C/N (ratio)
MATSUE	5.78±0.45	26.90±0.33	82.93±2.92	17673±3356	3463±683	2371±1147	1970±1102	4.96±1.00	0.33±0.07	15.21±0.02
SHIMANE	6.05±0.26	30.15±3.89	15.02±5.16	52695±6290	6352±382	1333±24	867±6	5.22±1.58	0.34±0.04	15.14±2.84
SAGIURA	6.43±0.09	38.68±0.54	4.59±0.10	23276±4468	5959±1108	3075±457	1371±49	2.89±0.05	0.16±0.03	18.95±3.53
BAMBOO	5.95±0.07	28.77±0.19	10.57±0.15	23784±2115	4363±490	1936±267	1511±489	10.11±7.05	0.46±0.28	21.35±2.27

Table 2.

Site	Sample type	OTU richness	exp(Shannon index)
MATSUE	**ROOTS**	37.0±7.1	11.3±2.9
MATSUE	**SOIL**	29.0±0.0	15.2±1.1
SHIMANE	**ROOTS**	46.5±4.9	17.7±0.8
SHIMANE	**SOIL**	30.5±0.7	13.0±2.5
SAGIURA	**ROOTS**	49.0±4.2	11.5±2.4
SAGIURA	**SOIL**	26.0±4.2	9.2±2.3
BAMBOO	**ROOTS**	46.0±4.2	15.0±0.3
BAMBOO	**SOIL**	30.5±20.5	12.9±11.6
p-values	**Sample type**	0.0043	0.5600

701 Table 3.

PERMANOVA	**pseudo-F**	**R^2**	**p-value**
OTU-based Bray-Curtis distance matrix			
sample type	0.981	0.065	0.211
Test for homogeneity of multivariate dispersion		**F-value**	**p-value**
among sample types		0.131	0.723

702

703 Table 4.

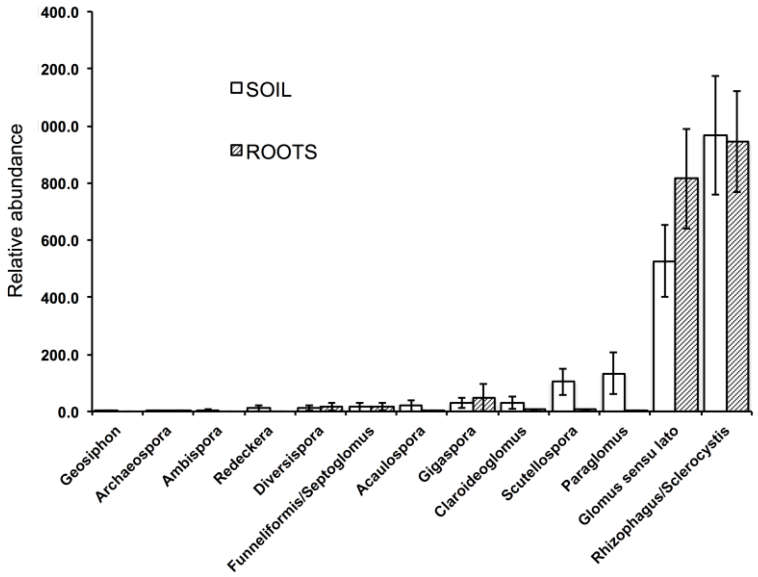
Taxonomic rank	**Soil physicochemical variable**	**R**	**p-value**
Diversisporaceae	**vs. Distance from sea**	-0.70	0.0025
	vs. pH	0.83	0.0000
	vs. EC	0.67	0.0045
	vs. K	0.64	0.0071
	vs. Total C	-0.71	0.0021
Archaeosporales	**vs. C/N ratio**	-0.67	0.0047
Paraglomerales, Paraglomeraceae, *Paraglomus*[#]	**vs. P**	0.75	0.0008
	vs. C/N ratio	-0.66	0.0053
Diversispora	**vs. pH**	0.72	0.0015
Rhizophagus/Sclerocystis	**vs. C/N ratio**	0.67	0.0046

704 [#]Paraglomerales is a monophyletic order that includes one monophyletic family (Paraglomeraceae) that includes only

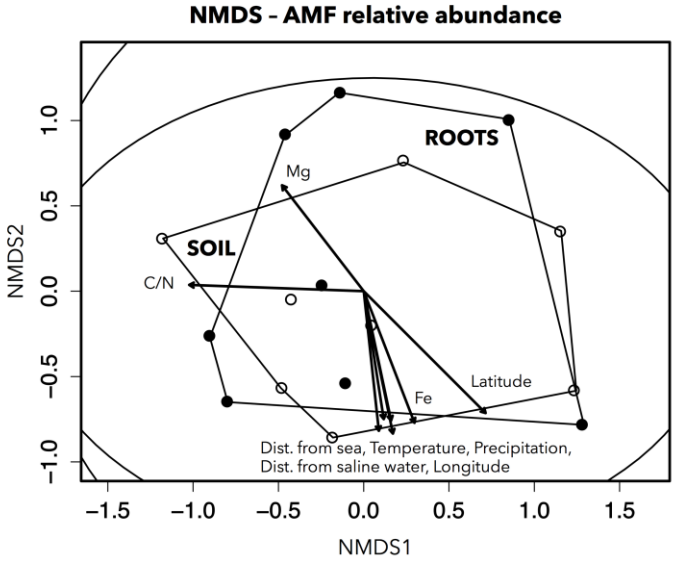
705 one genus (*Paraglomus*).

706 Table 5.

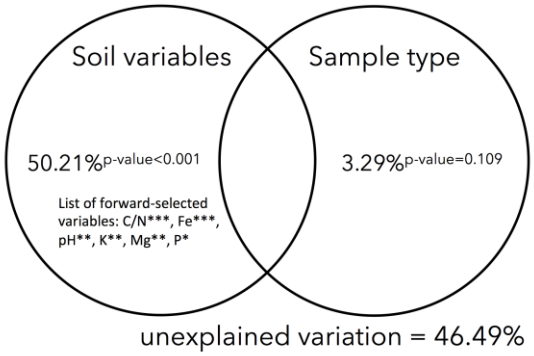
	Specificity	Sensitivity	IndVal	p-value	Clade	VT type (>97% ID)	units
Lower pH							
OTU012	1.000	1.000	1.000	0.0003	*Rhizophagus/Sclerocystis*	-	630
OTU005	0.823	1.000	0.907	0.0230	*Rhizophagus/Sclerocystis*		1730
CHI001	0.838	0.875	0.856	0.0089	*Rhizophagus/Sclerocystis*	-	656
Lower EC							
OTU012	1.000	0.800	0.894	0.0090	*Rhizophagus/Sclerocystis*	-	630
Lower Available P							
OTU002	1.000	0.833	0.913	0.0392	*Rhizophagus/Sclerocystis*	-	2755
Higher Available P							
OTU017	0.856	1.000	0.925	0.0019	*Paraglomus*	VTX00239	785
Higher Fe							
OTU011	0.993	1.000	0.996	0.0005	*Glomus sensu lato*	-	580
OTU010	0.970	1.000	0.985	0.0005	*Glomus sensu lato*	-	1033
Higher Mg							
OTU008	0.832	1.000	0.912	0.0087	*Rhizophagus/Sclerocystis*	VTX00112	944
Higher Total C							
OTU009	0.970	1.000	0.985	0.0092	*Rhizophagus/Sclerocystis*	-	764
Higher C/N ratio							
OTU002	0.939	1.000	0.969	0.0005	*Rhizophagus/Sclerocystis*	-	2755
OTU009	1.000	0.667	0.816	0.0072	*Rhizophagus/Sclerocystis*	-	764



707 Figure 1.



708 Figure 2.


Soil variables
Sample type
50.21%p-value<0.001
List of forward-selected variables: C/N***, Fe***, pH**, K**, Mg**, P*
3.29%p-value=0.109
unexplained variation = 46.49%

709 Figure 3.