Applied Soil Ecology 115 (2017) 10–18

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

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

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ARTICLE INFO

*Article history:*
Received 20 December 2016
Received in revised form 24 February 2017
Accepted 2 March 2017
Available online 27 March 2017

*Keywords:*
Arbuscular mycorrhizal fungi
Native range
454 GS-FLX Titanium pyrosequencing
SSU rDNA
Virtual Taxa

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.



## 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

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http://dx.doi.org/10.1016/j.apsoil.2017.03.004
0929-1393/

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.

### 2.5. 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 5× 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