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Analyzing the community composition of arbuscular mycorrhizal fungi colonizing the roots of representative shrubland species in a Mediterranean ecosystem

I. Sánchez-Castro, N. Ferrol, J.M. Barea*

Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain

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ABSTRACT

Community composition of arbuscular mycorrhizal (AM) fungi was analyzed in the roots of five representative shrub species (*Genista cinerea*, *Lavandula latifolia*, *Rosmarinus officinalis*, *Thymus mastichina* and *Thymus zygis*) growing in a typical semi-arid Mediterranean ecosystem. Roots from a well-preserved area of the ecosystem were extracted from soil and analyzed by nested PCR, single strand conformation polymorphism and sequencing of the NS31-AM1 and NS8-ARCH1311 regions of the small subunit of the ribosomal DNA (SSU rDNA). Ten AM fungal phylotypes were identified; eight belonged to the Glomeraceae and two to the Diversisporaceae. Only two of the phylotypes clustered with sequences of morphologically defined species and a high dominance by one AM group (*Glomus intraradices*) was detected. Our diversity analyses revealed that the AM fungal communities of *G. cinerea*, *L. latifolia* and *T. mastichina* did not significantly differ while the AM fungal communities of *R. officinalis* and *T. zygis* were distant from this cluster and from each other. The highest diversity was found in the roots of *T. zygis*. Our data indicate that co-occurring plant species may house distinct communities of AM fungi.

1. Introduction

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil inhabitants belonging to the phylum Glomeromycota which establish mutualistic symbiotic (mycorrhizal) associations with most land plants (Smith and Read, 2008). The ecological and physiological benefits of the AM fungi for their host plants have been well documented. Basically, AM fungi enhance plant uptake of minerals and water from beyond the rhizosphere, thereby ameliorating environmental stresses on the plant (Barea et al., 2005). A diverse community of AM fungi is important for the development and maintenance of plant diversity of terrestrial ecosystems contributing to plant community productivity (van der Heijden et al., 1998). These ecological impacts of AM fungi are particularly relevant for arid and semi-arid ecosystems where they would enable greater plant tolerance of environmental stresses characteristic of these ecosystems (Allen, 2007; Requena et al., 1996). Seasonal aridity characterises Mediterranean environments, typical of Southeast Spain, where the beneficial AM role, through a more efficient exploitation of soil nutrients and water, has been previously studied (Caravaca et al., 2003; Martínez-García et al., 2011; Requena et al., 2001).

Characterization of AM fungal diversity is a key issue because it has been demonstrated that not only the presence but also the AM fungal identity is ecologically relevant (Scheublin et al., 2007). Fungal isolates differ functionally resulting in different functional composition of communities which in turn determines plant community structure and ecosystem functioning (Öpik et al., 2008; van der Heijden et al., 2008). Thus, it is becoming increasingly important to gain a better understanding of the composition of the community of AM fungi in the ecosystem. Investigations of the diversity and functioning of natural AM fungal communities have traditionally been based on root colonization estimates and spore counts. Morphological spore analyses allow a taxonomical characterization of AM fungal communities but cannot be considered as an accurate measure of AM fungal diversity. Advances in molecular techniques now make it feasible not only to characterize AM fungi present as spores but also to directly identify some of the AM fungi *in planta* by using PCR-based methods to obtain AM fungal specific sequences. So far these molecular methods remain the most realistic approach to carry out diversity and population structure studies on AM fungi. These methodological approaches have been used to analyze the AM fungal communities actually colonizing key species in different ecosystems all over the world (Öpik et al., 2006). However, as stated by Öpik et al. (2010), more studies are needed for arid and semi-arid ecosystems, such as the Mediterranean ecosystems in Southeast Spain. These studies will also enable clearer prediction of the function of soil

* Corresponding author. Tel.: +34958181600; fax: +34958129600.
E-mail address: josemiguel.barea@eez.csic.es (J.M. Barea).

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ecosystems under arid environments that will facilitate the application of this knowledge.

Since mycorrhizal inoculation using native AM fungi has been recommended as an effective strategy for the revegetation of this type of environments (Requena et al., 2001), the identification of AM fungi able to colonize the root system of target plant species is a prerequisite to inoculation programs. Preliminary surveys on the AM fungal species composition in the rhizosphere of the plant species growing in the Mediterranean ecosystems present in South-East Spain were based on the use of conventional morphological approaches (Azcón-Aguilar et al., 2003; Barea et al., 2007; Ferrol et al., 2004). More recently, molecular methodologies have been used to study this type of ecosystems providing information on the molecular identities of the AM fungi present in the ecosystem and allowing, therefore, a broader generalization of the data (Alguacil et al., 2009, 2011; Martínez-García et al., 2011; Sánchez-Castro et al., 2008; Turrini et al., 2010). In the present study, we used several molecular techniques to characterize the diversity of AM fungal communities in the roots of five representative shrub species growing in a well-preserved area located in a typical Mediterranean ecosystem in South-East Spain.

2. Materials and methods

2.1. Field site and root sampling

A representative well-preserved homogeneous area (2° 50′ W, 37° 24′ N) of 100 m^2 was chosen within a semi-arid Mediterranean ecosystem located in Sierra de Baza Natural Park (Granada, Andalucía, Spain). The area experiences long and hot summer with scarce, erratic, but often torrential rainfalls during the winter season, with approximately 385 mm annual mean precipitation. There are strong differences in the day–night and summer-winter temperatures with a mean temperature of 6 °C in winter and 25 °C in summer. Soils are mainly calcareous cambisols. The vegetation is dominated by slow-growing shrubs, and the five most common species in the target area, *Genista cinerea*, *Lavandula latifolia*, *Rosmarinus officinalis*, *Thymus mastichina* and *Thymus zygis*, were selected for this study. In late May, when the highest AM fungal activity could be expected (Santos-González et al., 2007), a representative part of the root system of five randomly selected individuals of each species, located between 1 and 2 m from one another in the sampling area, were collected up to a depth of 20 cm, for each one of the five plant species within the target area, and placed in polyethylene bags. All samples were washed under running tap water and dried on paper. A small portion of the roots was used for the measurement of AM fungal colonization and the rest was frozen with liquid nitrogen and stored at −80 °C until DNA extraction was performed.

2.2. Degree of AM fungal colonization

Roots from all the different samples were cleared and trypan blue stained and the percentage of root length colonization by AM fungi determined (Phillips and Hayman, 1970) using the gridline intersect method (Giovannetti and Mosse, 1980).

2.3. DNA extraction and polymerase chain reaction

Genomic DNA was extracted from 150 mg of frozen fine roots from each sample representing about 3–6 m of root length or leaves using the DNeasy Plant Mini Extraction Kit (Qiagen Inc., Mississauga, ON, Canada) following manufacturer's instructions. A nested PCR approach was used to amplify the AM fungal DNA from the root samples. The first PCR step was performed with the universal eukaryotic primers NS31 and NS41 (Simon et al., 1992). Amplification products were then diluted (1:10) and used as template for the second PCR. The second round of amplification was performed with two different primer sets, NS8-ARCH1311 (Redecker, 2000; White et al., 1990) for Paraglomeraceae and Archaeosporaceae families and NS31-AM1 (Helgason et al., 1998) for the rest of AM fungal families. PCR amplifications were conducted in 25 μl reactions using 1 μM of forward and reverse primers, 0.2 mM dNTPs, and a Pure-Taq Ready-To-Go PCR bead (2.5 U pure *Taq* and 1.5 mM $MgCl_2$; Amersham Biosciences, Piscataway, NJ, USA). Cycling of the first PCR consisted in an initial denaturation of 95 °C for 5 min followed by 35 cycles of 94 °C (30 s), 50 °C (45 s) and 72 °C (1 min); the last cycle was followed by a final extension at 72 °C for 7 min. Conditions in the second PCR were similar except that we used 58 °C as annealing temperature for 1 min and the number of cycles was 30 instead of 35. All PCRs were performed in a Mastercycler Personal Thermocycler (Eppendorf, Hamburg, Germany).

2.4. Cloning of the PCR products

PCR products resulting from the second round of amplification of each sample were separated electrophoretically on 1.2% agarose gels, stained with ethidium bromide and visualized by UV illumination. The bands of expected size were excised with a scalpel and DNA isolated from the gel using the QIAEX Gel Extraction Kit (Qiagen) following the manufacturer's protocol. PCR products from each plant species were pooled and cloned into the pCR®2.1 vector following the protocol recommended by the manufacturer of the TA Cloning® Kit (Invitrogen Life Technologies, Karlsruhe, Germany) and transformed into One Shot® TOP10F′ Chemically Competent *Escherichia coli* cells (Invitrogen Life Technologies), resulting in five gene libraries. This pooling process does not reduce the number of AM-families detected (Renker et al., 2006). Within each resulting SSU rRNA gene library, putative transformants were screened by PCR using the primer set corresponding to the second PCR with the same conditions described above.

2.5. SSCP screening of the clones

In order to sequence the minimum number of clones, single strand conformation polymorphism (SSCP), an electrophoretic technique which allows detection of single mutations (Fischer and Lerman, 1979), was used to group the different clone types present in the gene libraries. A total of 250 positive clones, 50 from each plant species, were screened by this technique. Prior SSCP analysis, 4 μl of denaturing loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol) were added to 4 μl of the PCR amplification product of each clone. Samples were incubated at 95 °C for 3 min and immediately cooled on ice. After 3 min, samples were loaded into the gel. SSCP screening of the PCR products was performed using a 0.6 × MDE polyacrylamide gel (Cambrex Bio Sciences Rockland, ME, USA) with 0.6 × TBE buffer (Tris 0.6 M, boric acid 49.8 mM, EDTA 0.6 mM). The gels, 20 cm × 20 cm and 0.5 mm thick, were casted vertically and polymerized by 16 μl TEMED and 160 μl 10% ammonium persulfate. The gels were run at 4 W for 16 h at 20 °C in a Bio-Rad Protean II gel electrophoresis chamber (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For comparison of the SSCP patterns, 4 μl of pGEM® DNA molecular size marker (Promega Corp., Madison, WI, USA) were added to the gel. DNA in the gels was visualized by silver staining with the Bio-Rad Silver Stain Kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. Cluster analysis of SSCP patterns was done in the dendrogram type of the unweighted-pair group method with the Jaccard coefficient using InfoQuest FP software version 4.5 (Bio-Rad Laboratories, Inc.).

2.6. Sequencing of clones and sequence analysis

Clone types were defined according to SSCP patterns and at least one clone from each clone type was chosen for sequencing. The PCR product of each selected clone was purified using the Montage® PCR Centrifugal Filter Devices (Millipore Corporation, Billerica, MA, USA), according to the instructions of the manufacturer. Nucleotide sequences were determined by *Taq* polymerase cycle sequencing using an automated DNA sequencer (Perkin–Elmer ABI Prism 373). DNA fragments were sequenced in both strands. Sequences were edited with Sequence Scanner (Applied Biosystems) software, manually proofread, and corrected if necessary, checked for the presence of chimeras by using Ribosomal Database Project (RDP release 8.1) online Chimera Check program (http://rdp8.cmu.mse.edu/html/analyses.html). Sequence data were compared to gene libraries with BLAST (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/BLAST). The sequences were deposited in the EMBL Nucleotide Sequence Database under the accession numbers FM877490 to FM877526 (available online) and aligned to the SSU rDNA region of other AM fungal available in the public databases. Multiple sequence alignments of gene sequences were carried out using the program CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2). The Kimura two-parameter method was used to estimate distances and the phylogenetic analysis was performed by the neighbour-joining method by using PHYLIP version 3.67 (http://evolution.genetics.washington.edu/phylip.html) and a sequence of the zygomycete *Mortierella polycephala* as outgroup. The relative support of the different groups was determined based upon 1000 bootstrap trees. Results were verified by performing Parsimony analyses by using PHYLIP. Phylogenetic trees were drawn using TreeView version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.7. Definition of sequence phylotypes

Single morphospecies and even individual spores of the Glomeromycota contain multiple slightly different sequences of rDNA (Sanders et al., 1995). Thus, it is difficult to assign a single sequence to a particular species. Different sequence types or phylotypes, were defined as groups of closely related sequences, usually with a high level of bootstrap support in the phylogenetic analyses and of pairwise similarity (higher than 97%). The pairwise analysis within clusters was carried out using BioEdit software version 7.0.9.0. (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). We avoided splitting the lineages unless there was positive evidence for doing so.

2.8. Diversity indices and statistical analysis

Rarefaction curves were constructed by plotting the number of phylotypes observed against the number of screened clones using the analytical approximation algorithm embedded in the freely available software Analytical Rarefaction Program version 1.3 from Steven M. Holland (http://www.uga.edu/~strata/software/). The Shannon biodiversity index (H) was used to evaluate the genetic diversity (hereafter AM fungal diversity) and calculated by the formula $H' = -\Sigma(n_i/N)\ln(n_i/N)$, where n_i represents the number of sequences belonging to each phylotype and N the total number of phylotypes (Shannon and Weiner, 1963). The number of different phylotypes (Species Richness, S) was also considered for each plant.

Comparisons among plant species colonization percentage means were made using a least significant difference (LSD) test calculated at $P < 0.05$. A Principal Components Analysis (PCA) was run under statistical software SPSS 14.0 (SPSS Inc., Chicago, IL, USA) in order to discern the differences in AM fungal communities among the different samples.

3. Results

3.1. Degree of AM fungal colonization

The five plant species showed dominance of Arum-type structures with characteristic intercellular hyphae, intracellular arbuscules and vesicles. Paris-type structures were also detected in some samples. *T. mastichina* presented the highest degree of AM fungal colonization with 66.7 ± 9.3% followed by *G. cinerea* (63.0 ± 4.0%), *L. latifolia* (54.7 ± 10.7%), *R. officinalis* (52.3 ± 4.4%) and *T. zygis* (49.7 ± 7.3%). The degree of mycorrhization of plant roots in the different plant species did not differ significantly (data not shown).

3.2. PCR amplification and analysis of the clone libraries

Nested PCR on DNA from roots of *G. cinerea*, *L. latifolia*, *R. officinalis*, *T. mastichina* and *T. zygis* collected in the field resulted in a single band of the expected size for both primer sets (approximately 500 bp for NS8-ARCH1311 and 550 bp for NS31-AM1). No PCR product was obtained with DNA from leaves of the different plant species, confirming the specificity of the PCR primers for fungal DNA in plant roots (data not shown). PCR products were used to construct SSU rDNA libraries from roots of the five plant species. A total of 250 clones, 50 from each plant species, were analyzed by SSCP. In general, SSCP patterns were composed of two major bands corresponding to two DNA single strands, although most clones showed additional bands corresponding likely to different conformations of the DNA and/or small changes on the electrophoresis temperature (Welsh et al., 1997). Nevertheless, these additional bands did not present any difficulty in the interpretation of the SSCP patterns, which were perfectly reproducible. The different defined clone types showed clear distinct SSCP patterns in most of the cases. When the differences in the SSCP patterns were minor (Fig. 1), for example clone types G3, G5, G11, G13 and G17, they were considered distinct clone types by the software criteria in order to include the maximum of diversity. In total 51 different clone types were defined and at least one clone belonging to each of them was sequenced. Sequences derived from the same clone type were found to be almost identical, thus only one sequence per clone type was considered for subsequent analysis. Blast analyses revealed that 40 sequences had a high similarity (96–100% identity) to AM fungal sequences (Table 1) and 11 to sequences of ascomycetes, such as the *Tetracladium* sp., *Helminthosporium solani* and *Phoma* sp. All sequences derived from NS8-ARCH1311 primer set failed to reveal sequences of AM fungi. The distribution of the AM fungal SSCP profiles in the different plant species is shown in Fig. 1. Roots from *G. cinerea* presented 17 different SSCP profiles, from *L. latifolia* 15, from *R. officinalis* 21, from *T. mastichina* 15 and from *T. zygis* 19.

3.3. Phylogenetic analysis of the AM fungal sequences

Out of the 40 AM fungal sequences, three were found to be chimeras (clones G6, L3, M3; Fig. 1) and were excluded from further analyses. Thirty-five sequences closely matched to Glomeraceae and two to Diversisporaceae sequences (Fig. 2). The phylogenetic relationships among the sequences clearly revealed discrete sequence groups and enabled identification of 10 AM fungal sequence types or phylotypes: eight belonged to the Glomeraceae (GLO1, GLO2, GLO3, GLO4, GLO5, GLO6, GLO7 and GLO8) and two to the Diversisporaceae (DIV1, DIV2) (Table 1). All sequence clusters were supported by a bootstrap value of at least 80% both in the neighbour-joining and the maximum parsimony trees. Only two of the phylotypes clustered with sequences of morphologically defined species: GLO8 corresponded to *Glomus intraradices* and DIV1 to the *Glomus etunicatum-*

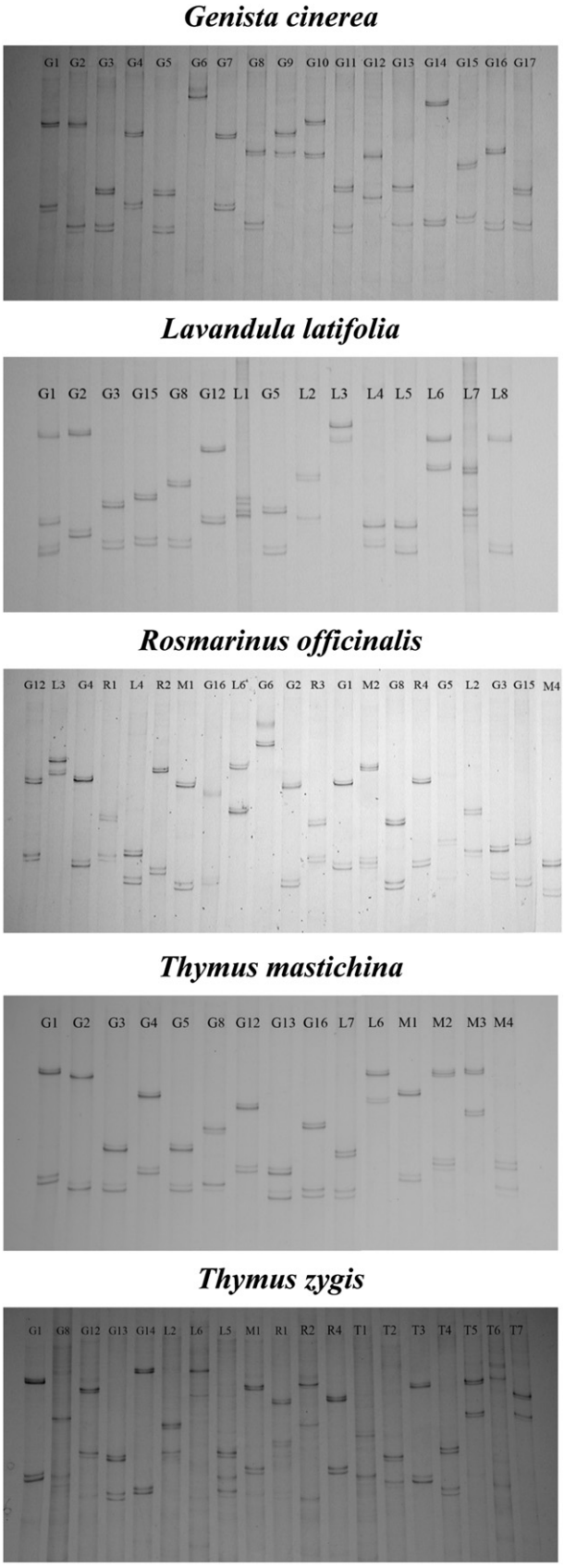

Fig. 1. SSCP patterns of SSU rDNA PCR products obtained from the roots of *G. cinerea*, *L. latifolia*, *R. officinalis*, *T. mastichina* and *T. zygis*. SSCP gels were run at 4W for 16 h at 20 °C.

Table 1
Characterization and abundance of clones of each sequence type obtained from each root sample.

Sequence type	Hosting plants (number of clones)	Phylotype	Closest relatives (accession no.) (% similarity)
G1	*G.c.* (2) *L.l.* (1) *T.m.* (10) *R.o.* (2) *T.z.* (1)	GLO8	*Glomus intraradices* (AJ536822) (99)
G2	*G.c.* (4) *L.l.* (1) *T.m.* (4) *R.o.* (3)	GLO8	*Glomus intraradices* (AJ418854) (99)
G3	*G.c.* (3) *L.l.* (1) *T.m.* (2) *R.o.* (1)	GLO1	Uncultured *Glomus* (AM946832) (99)
G4	*G.c.* (4) *T.m.* (3) *R.o.* (2)	GLO4	Uncultured *Glomus* (FN646059) (99)
G5	*G.c.* (4) *L.l.* (6) *T.m.* (1) *R.o.* (2)	GLO1	Uncultured *Glomus* (FN556639) (99)
G7	*G.c.* (4)	GLO4	Uncultured *Glomus* (FN646059) (99)
G8	*G.c.* (1) *L.l.* (2) *T.m.* (3) *R.o.* (4) *T.z.* (1)	GLO8	*Glomus intraradices* (AJ536822) (99)
G9	*G.c.* (1)	GLO6	Uncultured *Glomus* (FM955480) (99)
G10	*G.c.* (1)	GLO8	*Glomus intraradices* (AJ536822) (99)
G11	*G.c.* (1)	GLO1	Uncultured *Glomus* (FN556639) (99)
G12	*G.c.* (1) *L.l.* (1) *T.m.* (1) *R.o.* (6) *T.z.* (1)	GLO6	Uncultured *Glomus* (AJ496095) (98)
G13	*G.c.* (1) *T.m.* (1) *T.z.* (1)	GLO1	Uncultured *Glomus* (FN263130) (100)
G14	*G.c.* (1) *T.z.* (1)	GLO8	*Glomus intraradices* (AJ536822) (99)
G15	*G.c.* (1) *L.l.* (2) *R.o.* (1)	GLO8	*Glomus intraradices* (AJ536822) (99)
G16	*G.c.* (7) *T.m.* (3) *R.o.* (3)	GLO8	*Glomus intraradices* (AJ536822) (99)
G17	*G.c.* (1)	GLO1	Uncultured *Glomus* (EF393586)(99)
L1	*L.l.* (1)	GLO1	Uncultured *Glomus* (EF393586) (98)
L2	*L.l.* (1) *R.o.* (1) *T.z.* (3)	GLO5	Uncultured *Glomus* (AM946831) (97)
L4	*L.l.* (1) *T.m.* (1) *R.o.* (1)	GLO7	Uncultured *Glomus* (AM946912) (99)
L5	*L.l.* (2) *T.z.* (2)	GLO1	Uncultured *Glomus* (FN263130) (100)
L6	*L.l.* (2) *T.m.* (1) *R.o.* (1) *T.z.* (2)	GLO1	Uncultured *Glomus* (FM955481) (98)
L7	*L.l.* (1)	GLO6	Uncultured *Glomus* (AM946802) (99)
L8	*L.l.* (3)	GLO8	*Glomus intraradices* (AJ536822) (99)
M1	*T.m.* (2) *R.o.* (1) *T.z.* (11)	GLO4	Uncultured *Glomus* (FM955474) (99)
M2	*T.m.* (8) *R.o.* (1)	GLO8	*Glomus intraradices* (AJ536822) (99)
M4	*T.m.* (1) *R.o.* (1)	GLO1	Uncultured *Glomus* (FN263130) (99)
R1	*R.o.* (4) *T.z.* (2)	GLO6	Uncultured *Glomus* (AJ418893) (99)
R2	*R.o.* (2) *T.z.* (2)	GLO4	Uncultured *Glomus* (FN429381) (98)
R3	*R.o.* (1)	GLO6	Uncultured *Glomus* (AJ418893) (99)
R4	*R.o.* (1) *T.z.* (1)	GLO2	Uncultured *Glomus* (AM946840) (98)
T1	*T.z.* (2)	GLO1	Uncultured *Glomus* (FN263130) (99)
T2	*T.z.* (3)	GLO1	Uncultured *Glomus* (FN859966) (99)
T3	*T.z.* (1)	DIV1	*Glomus etunicatum-like* (AJ301860) (99)
T4	*T.z.* (1)	GLO3	Uncultured *Glomus* (GU238379) (99)
T6	*T.z.* (1)	DIV2	Uncultured Diversispora (FR728617) (97)

Table 1 (*continued*)

Sequence type	Hosting plants (number of clones)	Phylotype	Closest relatives (accession no.) (% similarity)
T5	*T.z.* (2)	GLO8	*Glomus intraradices* (AJ536822) (99)
T7	*T.z.* (1)	GLO8	*Glomus intraradices* (AJ536822) (99)

The first column lists the 37 sequence types detected by PCR-SSCP analysis. Clone identifiers relate to the plant where it was identified the first time and the clone number. Numbers in brackets in the second column indicate the number of clones of each sequence type detected in each plant. *G.c.* = *G. cinerea*; *L.l.* = *L. latifolia*; *R.o.* = *R. officinalis*; *T.m.* = *T. mastichina*; *T.z.* = *T. zygis*. The fourth column presents the closest relative organism corresponding to each sequence type. Accession number and percentage of similarity are presented in brackets.

like isolate W2423, which belongs to Diversisporaceae (Schwarzott et al., 2001).

3.4. Diversity analysis

To determine if the number of clones analyzed was sufficient to represent the diversity of AM fungi in the roots of the five plant species, sampling effort curves and rarefactions curves were constructed. In these curves, the numbers of clones analyzed were plotted against the cumulative number of phylotypes (Fig. 3). All the curves almost reached a plateau, suggesting that the analysis of about 40–45 AM fungal sequences per sample could provide a reasonable coverage of AM fungal diversity in the target ecosystem based on the proposed sampling regime.

Diversity indices based on the ten AM fungal phylotypes were calculated for each plant species. The highest diversity was found in the roots of *T. zygis* with a Shannon's diversity index of 1.81, followed by *R. officinalis*, *L. latifolia*, *G. cinerea* and *T. mastichina* (1.52, 1.28, 1.19 and 1.01, respectively). Concerning the total number of phylotypes found in the different plant species, 9 were found in *T. zygis*, 7 in *R. officinalis*, 5 in *L. latifolia* and *T. mastichina* and 4 in *G. cinerea*. Of the ten phylotypes identified, GLO8 was dominant, representing 41% of the total screened clones and occurring in all plant species. GLO2 (*R. officinalis* and *T. zygis*), GLO3 (*T. zygis*), GLO5 (*L. latifolia*, *R. officinalis* and *T. zygis*), GLO7 (*L. latifolia*, *R. officinalis* and *T. mastichina*), DIV1 (*T. zygis*) and DIV2 (*T. zygis*) phylotypes constituted altogether less than 15% of the clones analyzed. GLO1 (21% of the total screened clones), GLO6 (14%) were also present in all the plants. However, phylotype GLO4 (17%) was detected in all the plant species except in *L. latifolia* (Fig. 4).

To examine the data in more detail, the AM fungal communities were also compared using PCA, a data analysis that plots the AM fungal community from each root sample in a multidimensional space. Thereby, the AM fungal communities of *G. cinerea*, *L. latifolia* and *T. mastichina* did not apparently differ. For *R. officinalis* and *T. zygis*, their AM fungal communities are distant from each other and from the communities found in the other three plant species, indicating that they harboured a distinct AM fungal community. Principal components 1 and 2 accounted for most of the variance (62.4 and 21.9% for PC1 and PC2, respectively) (Fig. 5).

4. Discussion

The data presented in this paper describe the AM fungal sequences found in plant roots collected in a single sampling time from a limited area of a small-shrub community from the Sierra de Baza Natural Park, Southern Spain, which it is a typical Mediterranean ecosystem. A single sequence type dominated the roots of the five plant species studied, and each of the plant species harboured a different community of AM fungi. Few sequences matched those of described species, but most of them have previously been obtained from field sites.

The absence of AM fungal sequences in the NS8-ARCH1311 PCR products indicates that fungi belonging to Paraglomeraceae and Archaeosporaceae families were absent from our samples. However, we detected some ascomycetes and other contaminant sequences, which it was not surprising because of the lack of specificity of the AM1 and ARCH1311 primers (Douhan et al., 2005; Helgason et al., 1999; Redecker et al., 2000). In agreement with previous observations by Douhan et al. (2005), who found that amplification of non-glomalean sequences with the AM1 primer depends on the plant host and ecosystem, we also observed some differences in the proportion of non-glomalean sequences among the examined plants.

All glomalean sequences collected from roots of *G. cinerea*, *L. latifolia*, *R. officinalis* and *T. mastichina* belong to one family, the Glomeraceae. Although *Glomus* species dominated in the roots of *T. zygis*, sequences belonging to the Diversisporaceae family were also found. Several studies have shown that *Glomus* species are typical of semi-arid Mediterranean ecosystems (Ferrol et al., 2004; Requena et al., 1996).

The most abundant phylotype found in our study was GLO8 (41% of the AM fungal sequences), showing 99.9% identity with sequences of different ecotypes of *G. intraradices*. Many other studies have also found a dominance of *G. intraradices* in different ecosystems (Cesaro et al., 2008; Husband et al., 2002; Scheublin et al., 2004). The rest of phylotypes belonging to the Glomeraceae family (GLO1, GLO2, GLO3, GLO4, GLO5, GLO6 and GLO7) represented undescribed *Glomus* species, similarly to other studies where many sequences related to unknown AM fungal taxa were detected (Öpik et al., 2006). Nevertheless, some of them were detected in studies from a variety of environments. Thus, GLO1 was detected in a semi-natural woodland, arable crops and contaminated soils (Daniell et al., 2001; Helgason et al., 1998; Vallino et al., 2006). Also, sequences similar to GLO2, GLO6 and GLO7 were found in an alpine ecosystem in Salzburg (Austria) (Moser and Haselwandter, unpublished), and GLO6 and GLO7 were also found in a dune grassland of Holland (Scheublin et al., 2004) and in the roots of *Taxus baccata* (Wubet et al., 2003). At the same time, GLO4 is one of the most common "unknown" sequences detected in roots collected from different places around the globe (Husband et al., 2002; Vandenkoornhuyse et al., 2002). The sequence of the phylotype DIV1 belonging to the Diversisporaceae family was previously found in the roots of a threatened conifer species in a dry forest of Ethiopia (Wubet et al., 2006). In the case of GLO3, similar sequences have been reported only in the Loess and Tibet Plateaus, in China (Liu and Feng, unpublished). On the other hand, the sequence types GLO5 and DIV2 did not present similarities higher than 97% to any sequence present in the public databases and thus they could be considered as new sequences since the examined ribosomal region is extremely conserved for this type of fungi. These data altogether suggest that within Glomeromycota, different distribution patterns may occur. Some sequence types showed broad ecological amplitude and geographical distribution (GLO4 and GLO8), likely representing generalist fungi characterized by low ecosystem/host plant specificity degree. However, most of the AM fungal sequence types found in this study were limited to determined ecosystems and host plant species (GLO1, GLO2, GLO3, GLO6, GLO7 and DIV1) or even detected for the first time (GLO5 and DIV2). This particular sequence types distribution could be due to consistent preferences to the target ecosystem and/or host plant species. Considering that the level of genetic relatedness of this type of fungi might be very high, it is tempting to speculate that adaptations to aridity might exist within species. However, further studies are needed to explore this issue.

Comparison of the sequence types identified in the roots with the sequences of the spores found in a previous study in their

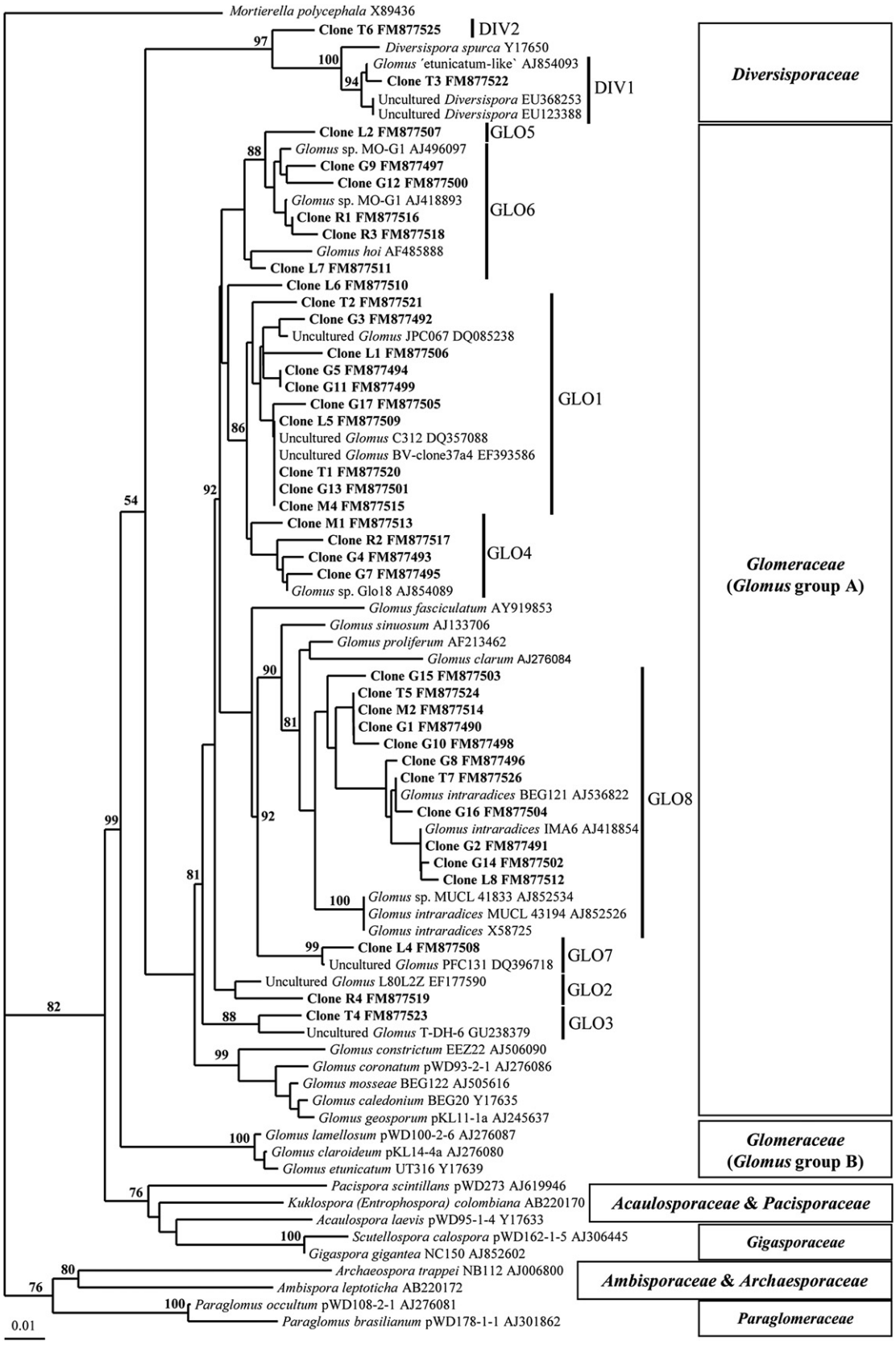

Fig. 2. Neighbour-joining tree showing relationships between the AM fungal sequences detected in this study and database sequences of known Glomeromycota and of those from environmental samples. The sequence of *Mortierella polycephala* was used as outgroup. Numbers above branches denote bootstrap values from 1000 replications. Sequences obtained in the present study are shown in boldface and labelled as described in Table 1 and with their database accession number. Lines on the right delimite the phylotypes and the boxes show the glomeromycotan subgroups. The scale bar at the bottom left is proportional to branch length.

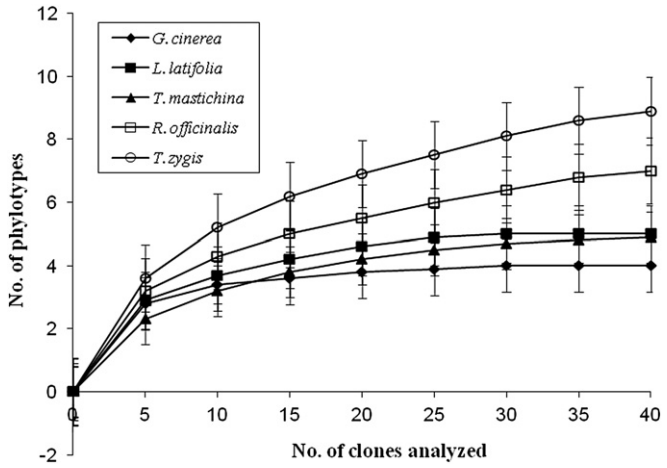

Fig. 3. Rarefaction curves of SSU rDNA libraries from roots of *G. cinerea*, *L. latifolia*, *R. officinalis*, *T. mastichina* and *T. zygis*. Higher and lower 95% confidence intervals are indicated as bars above and below the data points, respectively. Curves were obtained using the Analytical Rarefaction Program version 1.3 (http://www.uga.edu/wstrata/software/).

rhizospheric soil (Barea et al., 2007) revealed a very low correlation between the taxa present in both samples. In fact, only *G. intraradices* could be found both in the community of the functionally active AM fungi within the roots and in the community of spores in the soil. Different studies have shown a low overlap between the AM fungal communities in soils and roots (Clapp et al., 1995; Hempel et al., 2007) and a wider diversity in the spore fraction (Hempel et al., 2007; Johnson et al., 2003). Several hypothesis have been proposed to explain the differences between soil and root communities of AM fungi like that some non-sporulating species may exist (Clapp et al., 1995), that roots only recruit a fraction of the AM fungal taxa pool present as spores in soils (Johnson et al., 2003), or that AM fungi are not necessarily obligate symbionts of plants (Hempel et al., 2007). Since our data were obtained from a limited area of land, a limited mass of root and at a single time of the year, the low correlation between the root and soil communities suggests that AM fungi might have some ecological constraints to colonization of each of the plants.

The PCA plot shows how AM fungal communities within *R. officinalis* and *T. zygis* are different among them and at the same time, different from the communities detected in the other plant species analyzed in the same location. Presence of some phylotypes specifically in some of the plant species, such as GLO3, GLO5 and DIV2

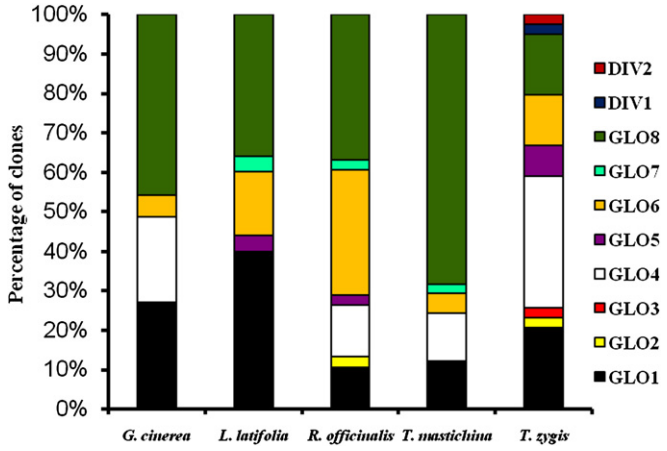

Fig. 4. AM fungal communities in the roots of *G. cinerea*, *L. latifolia*, *R. officinalis*, *T. mastichina* and *T. zygis*. The *y* axis indicates the proportion of clones assigned to each particular AM fungal phylotype.

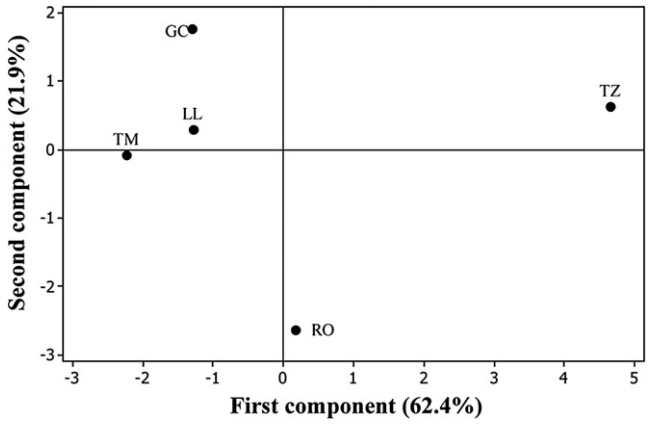

Fig. 5. PCA plot of the AM fungal communities found in the five analysed plants. GC = *G. cinerea*; LL = *L. latifolia*; RO = *R. officinalis*; TM = *T. mastichina*; TZ = *T. zygis*. Numbers in brackets represent the percentages of variance explained by the PC.

that were only found associated to *T. zygis*, and differences in the proportions of each one of these fungal sequence types among the plant species could explain this distribution. These results suggest some host preference in AM fungi. It has been widely shown that co-occurring plant species can be colonized by AM fungal communities of different composition (Sýkorová et al., 2007; Vandenkoornhuyse et al., 2002). As hypothesized by Öpik et al. (2008), differences in the number of taxa associated with host plant species could be linked to the host preferences of AM fungi, different symbiont ranges of AM plant hosts, or different sizes of fungal colonization units in roots resulting in variable fungal taxon densities.

The highest values on diversity indices were found in the roots of *R. officinalis* and *T. zygis*, the two most abundant plant species in the target ecosystem. The fact that all roots presented a similar degree of AM fungal colonization indicates that the reduced presence of different AM fungal sequence types in *G. cinerea*, *L. latifolia* and *T. mastichina* was not affected by differences in the percentage of root colonization. The diversity indices previously reported in a semi-natural broadleaved forest in England ($H' = 1.36–1.62$; Helgason et al., 1999), in a temperate grassland ($H' = 1.71$; Vandenkoornhuyse et al., 2002), in a dune grassland in the Netherlands ($H' = 1.77$; Scheublin et al., 2004) and in the roots of serpentine and non-serpentine ecotypes of *Collinsia sparsiflora* ($H' = 1.17–0.68$; Schechter and Bruns, 2008) seem to be the most similar to those found in the present study. In general, AM fungi are not especially diverse in semi-arid regions (Alguacil et al., 2009, 2011; Ferrol et al., 2004; Liu et al., 2009) and conversely, their diversity may be a lot higher in other type of ecosystems (Husband et al., 2002; Wubet et al., 2003), suggesting then lower diversities in semi-arid environments.

In summary, our data show that AM fungal communities can vary among different plant species within the same ecosystem. Even taxonomically similar plant species, such as *T. mastichina* and *T. zygis*, contained different communities of AM fungi in their roots. Moreover, three ecosystemic distribution patterns were observed in the AM fungal phylotypes detected in this study considering their geographical occurrence in other surveys around the world. Likewise, certain degree of host preference was suggested by the analysis of the different AM fungal composition in the target plant species. These findings would be of great value when trying to restore the ecosystem since they would help to understand the importance of maintaining or restoring these mycorrhizal associations in stressed semi-arid environments. Therefore, this information should be considered when designing the AM fungal inoculum composition for revegetation programs in order to maximize the potential benefits that these microorganisms can provide for the establishment of the selected

plant species to the threatened ecosystem. Further studies on the composition of the AM fungal communities present at different stages of the year are being performed in our group in order to ascertain which of the different type of propagules are responsible for the initial AM colonization of the target plants and the subsequent successional dynamics.

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