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Temporal dynamics of arbuscular mycorrhizal fungi colonizing roots of representative shrub species in a semi-arid Mediterranean ecosystem

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Abstract Arbuscular mycorrhizal (AM) symbiosis plays an important role in improving plant fitness and soil quality, particularly in fragile and stressed environments, as those in certain areas of Mediterranean ecosystems. AM fungal communities are usually affected by dynamic factors such as the plant community structure and composition, which in turn are imposed by seasonality. For this reason, a one-year-round time-course trial was performed by sampling the root system of two representative shrubland species (*Rosmarinus officinalis* and *Thymus zygis*) within a typical Mediterranean ecosystem from the Southeast of Spain. The 18S rDNA gene, of the AM fungal community in roots, was subjected to PCR–SSCP, sequencing, and phylogenetic analysis. Forty-three different AM fungal sequence types were found which clustered in 16 phylotypes: 14 belonged to the Glomeraceae and two to the Diversisporaceae. Surprisingly, only two of these phylotypes were related with sequences of morphologically defined species: *Glomus intraradices* and *Glomus constrictum*. Significant differences were detected for the relative abundance of some phylotypes while no effects were found for the calculated diversity indices. These results may help to design efficient mycorrhizal-based revegetation programs for this type of ecosystems.

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Introduction

It has been well established that arbuscular mycorrhizal (AM) associations are fundamental in optimizing plant fitness and soil quality (Smith and Read 2008). Particularly, the AM symbioses improve the resilience of natural plant communities against environment stresses (Barea et al. 2011). The universally distributed AM fungal communities are not a static element within the ecosystem since they are affected by some dynamic factors such as the plant community structure and composition (Pringle and Bever 2002). Consequently, the populations of AM fungi colonizing the roots of a particular plant species can change through space and time, and, therefore, seasonally (van der Heijden et al. 1998). Conversely, the impact of AM fungi on the growth of individual plant species varies depending on the AM fungal taxa involved (van der Heijden et al. 1998; Scheublin et al. 2007). Results from surveys analyzing the temporal dynamics of AM fungal communities are rather contradictory. For example, no significant seasonal changes were observed in a semi-natural grassland in central Sweden (Santos-González et al. 2007) or in the roots of *Hieracium pilosella* in an undisturbed coastal grassland on the north of Denmark (Rosendahl and Stukenbrock 2004). On the other hand, Vandenkoornhuyse et al. (2002) found seasonal changes in the AM fungal community of a semi-natural grassland ecosystem in Scotland that correlated with functional aspects. In a different scale, Husband et al. (2002) demonstrated a persistent

pattern of replacement in the AM community present in a tropical forest in Panama over 3 years. In the same way, a significant change in AM fungal colonization rates and community composition over an annual cycle was observed in a chronosequence of 5- to 42-year-old *Caragana korshinskii* plantations in a semi-arid region in China (Liu et al. 2009).

As far as we know, no information is available concerning the temporal dynamics of AM fungi associated to plant communities in arid or semi-arid environments. This is the case with Mediterranean ecosystems which are developed under characteristic climatic conditions including long dry and hot summers and low winter temperatures, with erratic, sometimes torrential, rainfalls (López-Bermúdez et al. 1990; Vallejo et al. 1999). These hydrothermal conditions generate aridity and other associated environmental stresses including nutrient deficiency, drought, and soil disturbance, which may cause soil erosion and alterations in the characteristic plant cover of these ecosystems (Barea et al. 2011). This, in turn, affects negatively the diversity and activity of AM fungal propagules (Requena et al. 1996; Azcón-Aguilar et al. 2003; Caravaca et al. 2003; Ferrol et al. 2004). Diminution of AM propagules is a critical ecological constraint because the AM symbiosis is fundamental for plants to thrive in arid and semi-arid conditions (Caravaca et al. 2003; Allen 2007; Dag et al. 2009). This special AM role is exerted by helping the supply of nutrients such as P and N when their mobility is lowered by water scarcity (Allen 2007), by improving soil aggregation in these eroded soils (Requena et al. 2001; Piotrowski et al. 2004), and by reducing plant water stress (Aroca et al. 2007; Ruíz-Lozano et al. 2008).

As recently reviewed by Barea et al. (2011), some analyses of the AM fungi actually colonizing the plant roots in Mediterranean ecosystems from Southeast Spain have been carried out by using molecular methodologies. These studies include that by Sánchez-Castro et al. (2008) on a rosemary grove, those involving gypsophyllic plant communities (Alguacil et al. 2009a, b), and those by Martinez-García et al. (2011) and Alguacil et al. (2011) concerning degraded semi-arid plant communities. However, none of these studies analyzed the temporal dynamics of these endophytic microbial communities.

Identification of efficient AM fungi under prevalent stress conditions during the different seasons of the year in these Mediterranean ecosystems is essential to understand the population dynamics of AM fungi in the target environment and to select the adequate fungal ecotypes for producing the appropriate inocula for mycorrhizal-based revegetation programs. This strategy, based on the management of native ecotypes of AM fungi, might be an effective strategy in recuperation of Mediterranean degraded areas (Requena et al. 2001; Jeffries et al. 2002; Hart et al. 2003; Ouahmane et al. 2007) helping to the promotion of seedling establishment by integrating emerging plantlets into extensive hyphal networks increasing nutrient supply (van der Heijden et al. 2004).

Accordingly, the present research was aimed to study the seasonal dynamics of the symbiotically active AM fungal communities in the roots of two representative shrub species (*Rosmarinus officinalis* and *Thymus zygis*) in a semi-arid Mediterranean ecosystem from Southeast Spain, using a one-year-round time-course sampling design.

Materials and methods

Field site and sampling

This study was conducted in a representative well-preserved area of 100 m^2 within a semi-arid Mediterranean ecosystem at Sierra de Baza Natural Park, Granada, Spain (2° 50′ W, 37° 24′ N). The climatic conditions are based on low-irregular rainfall patterns, with approximately 385 mm annual mean precipitation and a mean temperature of 6°C in winter and 25°C in summer. Soils are calcaric cambisols, with sparse and patchy plant cover. Two representative slow-growing shrubs widely distributed in the target ecosystem were selected for this study: *R. officinalis* and *T. zygis*. A representative part of the root system of five randomly selected individuals, located between 1 and 2 m from one another, were collected up to a depth of 20 cm, for each one of the plant species within the target area on May (spring), September (summer-autumn), and January (winter). All samples were placed in plastic bags and immediately transported to the laboratory. Roots of each plant species and sampling time were washed under running tap water, dried on paper, frozen with liquid nitrogen, and stored at −80°C until further processing. Before freezing, a portion of each root system was collected and the percentage of root colonized by AM fungi was estimated using the gridline intersect method (Giovannetti and Mosse 1980) after trypan blue staining (Phillips and Hayman 1970).

DNA extraction and polymerase chain reaction

Genomic DNA was extracted from 125 to 150 mg of frozen roots or leaves using the DNeasy Plant Mini Extraction Kit (Qiagen Inc., Mississauga, ON, Canada), following manufacturer's instructions. Depending on the thickness of the roots, this root weight represents 3–6 m of root. A nested PCR approach was used to amplify the AM fungal DNA from the root samples. The first PCR was performed with the universal eukaryotic primers NS31 and NS41 (Simon et al. 1992). Amplification products were then diluted (1:10)

and 1 μl was used as template for the second PCR. The second round of amplification was performed with two different primer sets, NS8 and ARCH1311 (White et al. 1990; Redecker 2000) for Paraglomeraceae and Archaeosporaceae families, and NS31 and AM1 (Helgason et al. 1998) for the rest of Glomeromycotan families. PCR amplifications were conducted in 25-μl reactions using 1 μM of forward and reverse primers and a Pure-Taq Ready-To-Go PCR bead (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 denaturation at 94°C (30 s), annealing at 50°C (45 s), and elongation at 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).

Cloning of the PCR products

Amplicons resulting from the second PCR of each sample were separated electrophoretically on 1.2% agarose gels, stained with ethidium bromide, and visualized by UV illumination. The expected bands were excised with a scalpel and DNA isolated from the gel and purified using the QIAEX Gel Extraction Kit (Qiagen), following the manufacturer's protocol. The five different PCR products corresponding to each plant species and sampling time were pooled to yield one composite sample per plant species and sampling time (Renker et al. 2006). The mixed PCR products were 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). Within each resulting SSU rDNA gene library, putative transformants were screened by PCR using the primer set corresponding to the second PCR with the same conditions described above.

Selection and sequencing of the clones

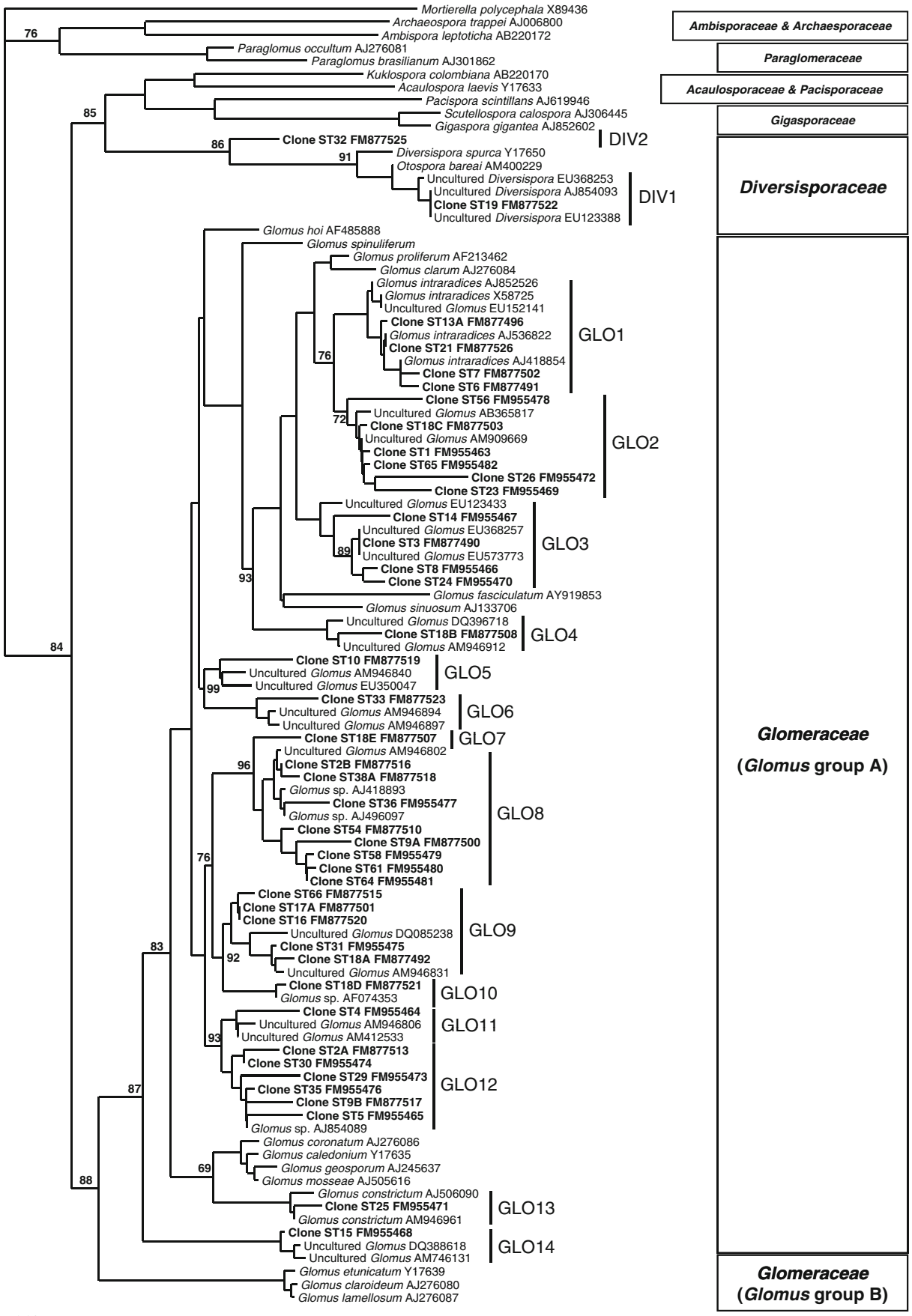
Fifty positive clones from each SSU rDNA gene library were screened by single-strand conformation polymorphism (SSCP). Prior to SSCP analysis, 4 μl of denaturing loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to 4 μl of the PCR amplification product of each clone. Samples were incubated at 95°C for 5 min and immediately cooled on ice. After 3 min at room temperature, samples were loaded on 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) (Sambrook et al. 1989). The gels, 20 cm×20 cm and 0.5-mm thick, were casted vertically and polymerized with 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). DNA in the gels was visualized by silver staining with the Bio-Rad Silver Stain Kit (Bio-Rad Laboratories) according to the manufacturer's protocol.

Comparisons for grouping the different clone types in the different SSCP gels were performed using InfoQuest FP software v.4.50 (Bio-Rad Laboratories). The PCR product of at least one clone from each SSCP profile was purified using the Montage® PCR Centrifugal Filter Devices (Millipore Corporation, Billerica, MA, USA), according to the instructions of the manufacturer, and determined by using *Taq* polymerase cycle sequencing and an automated DNA sequencer (Perkin-Elmer ABI Prism 373). DNA fragments were sequenced in both strands.

Phylogenetic analysis and definition of sequence phylotypes

Sequences were edited with Sequence Scanner software (Applied Biosystems, Foster City, CA, USA), manually proofread, corrected if necessary, and checked for the presence of chimeras by using the RDP Chimera Check program (http://rdp8.cmu.mse.edu/html/analyses.html) (Maidak et al. 2001). Sequence data were compared to gene libraries (EMBL Data Library and GenBank) using BLAST program (Altschul et al. 1990). The sequences were deposited in the EMBL Nucleotide Sequence Database, under the accession numbers (available online) indicated in the phylogenetic tree (see Fig. 1), and aligned to the 18S rRNA corresponding region of other AM fungi available in the public databases. Multiple sequence alignments of gene sequences were carried out using the program CLUSTALW (Thompson et al. 1997). The Kimura two-parameter method was used to estimate distances and the phylogenetic analysis was performed by the neighbor-joining method by using PHYLIP package V3.2 (Felstein 1993). The 18S rDNA sequence of *Mortierella polycephala* was used as outgroup. The relative support of the different groups was determined based upon 1,000 bootstrap trees. Results were verified by performing parsimony analyses by using PHYLIP (data not shown). Phylogenetic trees were drawn using TREEVIEW (Page 1996).

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 a level of pairwise similarity higher than 97%. The pairwise analysis within clusters was carried out using BioEdit


Mortierella polycephala X89436
Archaeospora trappei AJ006800
Ambispora leptoticha AB220172
Paraglomus occultum AJ276081
Paraglomus brasilianum AJ301862
Kuklospora colombiana AB220170
Acaulospora laevis Y17633
Pacispora scintillans AJ619946
Scutellospora calospora AJ306445
Gigaspora gigantea AJ852602
Clone ST32 FM877525
Diversispora spurca Y17650
Otospora bareai AM400229
Uncultured Diversispora EU368253
Uncultured Diversispora AJ854093
Clone ST19 FM877522
Uncultured Diversispora EU123388
DIV2
DIV1
Glomus hoi AF485888
Glomus spinuliferum
Glomus proliferum AF213462
Glomus clarum AJ276084
Glomus intraradices AJ852526
Glomus intraradices X58725
Uncultured Glomus EU152141
Clone ST13A FM877496
Glomus intraradices AJ536822
Clone ST21 FM877526
Glomus intraradices AJ418854
Clone ST7 FM877502
Clone ST6 FM877491
GLO1
Clone ST56 FM955478
Uncultured Glomus AB365817
Clone ST18C FM877503
Uncultured Glomus AM909669
Clone ST1 FM955463
Clone ST65 FM955482
Clone ST26 FM955472
Clone ST23 FM955469
GLO2
Uncultured Glomus EU123433
Clone ST14 FM955467
Uncultured Glomus EU368257
Clone ST3 FM877490
Uncultured Glomus EU573773
Clone ST8 FM955466
Clone ST24 FM955470
GLO3
Glomus fasciculatum AY919853
Glomus sinuosum AJ133706
Uncultured Glomus DQ396718
Clone ST18B FM877508
Uncultured Glomus AM946912
GLO4
Clone ST10 FM877519
Uncultured Glomus AM946840
Uncultured Glomus EU350047
GLO5
Clone ST33 FM877523
Uncultured Glomus AM946894
Uncultured Glomus AM946897
GLO6
Clone ST18E FM877507
Uncultured Glomus AM946802
Clone ST2B FM877516
Clone ST38A FM877518
Glomus sp. AJ418893
Clone ST36 FM955477
Glomus sp. AJ496097
Clone ST54 FM877510
Clone ST9A FM877500
Clone ST58 FM955479
Clone ST61 FM955480
Clone ST64 FM955481
GLO7
GLO8
Clone ST66 FM877515
Clone ST17A FM877501
Clone ST16 FM877520
Uncultured Glomus DQ085238
Clone ST31 FM955475
Clone ST18A FM877492
Uncultured Glomus AM946831
GLO9
Clone ST18D FM877521
Glomus sp. AF074353
GLO10
Clone ST4 FM955464
Uncultured Glomus AM946806
Uncultured Glomus AM412533
GLO11
Clone ST2A FM877513
Clone ST30 FM955474
Clone ST29 FM955473
Clone ST35 FM955476
Clone ST9B FM877517
Clone ST5 FM955465
Glomus sp. AJ854089
GLO12
Glomus coronatum AJ276086
Glomus caledonium Y17635
Glomus geosporum AJ245637
Glomus mosseae AJ505616
Glomus constrictum AJ506090
Clone ST25 FM955471
Glomus constrictum AM946961
GLO13
Clone ST15 FM955468
Uncultured Glomus DQ388618
Uncultured Glomus AM746131
GLO14
Glomus etunicatum Y17639
Glomus claroideum AJ276080
Glomus lamellosum AJ276087
0.01
Ambisporaceae & Archaesporaceae
Paraglomeraceae
Acaulosporaceae & Pacisporaceae
Gigasporaceae
Diversisporaceae
Glomeraceae
(Glomus group A)
Glomeraceae
(Glomus group B)

◀ **Fig. 1** Neighbor-joining tree showing phylogenetic relationships of the AM fungal sequences obtained 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 1,000 replications. Sequences obtained in the present study are shown in *boldface* and with their database accession number. *Lines* on the right delimit the phylotypes and the *boxes* show the glomeromycotan subgroups. The *scale bar* at the *bottom left* is proportional to branch length

software (Hall 1999). Splitting the lineages was avoided unless there was positive evidence for doing so.

Calculation of rarefaction and diversity indices

To determine whether the number of clones analyzed was enough to represent the diversity of AM fungi in the different samples, rarefaction curves were constructed, based on number of AM fungal sequences analyzed, using the analytical approximation algorithm (Hurlbert 1971) 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 $H' = -\sum (n_i/N)\ln(n_i/N)$, where n_i represents the number of sequences belonging to each phylotype and N the total number of phylotypes (Shannon and Weaver 1963). The phylotypes' specific richness (S) was also calculated for each plant species and sampling time. The abundance of each sequence type in the different plant species at each sampling time were coded as relative abundance in relation to the total number of AM fungal clones detected in each clone library. It is assumed that each AM fungal type is amplified and cloned proportionally, being the numbers of each phylotype considered as an approximate estimation of their proportion in the root. Clones of non-AM fungal origin were excluded from the analysis.

Statistical analysis

Differences in the relative abundance of AM fungi among plant species and sampling times were analyzed using analysis of variance (ANOVA). To analyze the similarity of the AM fungal communities into roots based on the relative abundance of the different phylotypes obtained in each plant species and sampling time, a principal components (PC) analysis was performed, followed by a hierarchical cluster analysis using Ward's minimum variance method based on the χ^2 distance as agglomerative method. Relationships between the relative abundance of a particular AM fungal phylotype with respect to the other AM fungal phylotypes, the diversity indexes, and the extracted PC were studied by means of a bivariate regression analysis. In all cases, the statistical analyses were performed with the SPSS software v. 14.0. (SPSS, Inc., Chicago, IL, USA).

Results

PCR–SSCP and phylogenetic analysis

The nested PCR approach showed a single band of the expected size for both primer sets. Additionally, DNA extracted from leaves of the plant species analyzed was used to confirm the specificity of the different primers for fungal DNA, not producing any amplification with the nested PCR approach used. Analysis of the SSCP patterns of the clones of the six SSU rDNA gene libraries revealed 63 different band profiles (data not shown). Blast analyses of the corresponding sequences revealed that 43 of them had a high similarity (96–100% identity) to AM fungal sequences and 20 to sequences of ascomycetes (*Tetracladium* sp., *Helminthosporium solani*, and *Phoma* sp.) and other organisms (data not shown). All sequences derived from NS8-ARCH1311 primer set resulted to be no AM fungal sequences.

Out of the 43 AM fungal sequences, 41 closely matched to Glomeraceae family and two to Diversisporaceae family. No chimeras were detected within these sequences. The phylogenetic relationships among the sequences clearly revealed discrete sequence groups and enabled identification of 16 AM fungal phylotypes: 14 belonged to the Glomeraceae (named GLO1 to GLO14) and two to the Diversisporaceae (DIV1 and DIV2). Two of these phylotypes clustered with sequences of morphologically characterized species: GLO1 corresponded to *Glomus intraradices* and GLO13 to *Glomus constrictum*. The other phylotypes were related to environmental samples-derived sequences in GenBank not taxonomically characterized, except phylotypes GLO7 and DIV2 which do not fit with any previously sequenced AM fungi (Fig. 1).

AM fungal diversity and community structure

The rarefaction curves, including the numbers of clones analyzed and the cumulative numbers of phylotypes, indicated that, in all cases, the curves reached a plateau before 40–45 screened clones per sample (Fig. 2).

Significant differences due to the host plant species and sampling times were found for the relative abundance of some phylotypes. While the relative abundance of phylotypes GLO1, GLO4, and GLO12 was significantly affected by the host plant, the relative abundance of phylotypes GLO5 and GLO14 was affected by the sampling time (Table 1).

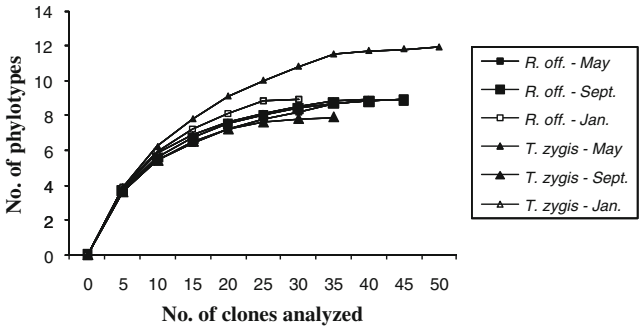


Fig. 2 Rarefaction curves of SSU rDNA libraries from glomeromycotan fungal phylotypes obtained from roots of *Rosmarinus officinalis* (*R. off.*) and *Thymus zygis* (*T. zygis*) at three sampling times (May, September, and January). Curves were obtained using the Analytical Rarefaction Program version 1.3 (http://www.uga.edu/~strata/software/)

Comparison of the AM fungal communities colonizing the two target plant species revealed that GLO1 and GLO8 were the most common phylotypes colonizing the roots of *R. officinalis*, representing 27% and 18% of all obtained sequences, respectively (Fig. 3). On the other hand, GLO12 (30%) and GLO3 (16%) were the most common phylotypes found in *T. zygis* roots. In addition, GLO1, GLO3, GLO8, and GLO12 were detected in roots of both plant species at all sampling times. On the contrary, eight phylotypes (GLO4, GLO5, GLO6, GLO10, GLO11, GLO13, DIV1, and DIV2) represented less than 10% of all the sequenced clones in both plant species and at all sampling times. Only one phylotype (GLO4) was found exclusively in *R. officinalis*, and phylotypes GLO6, GLO10, GLO13, DIV1, and DIV2 were exclusively associated to *T. zygis* roots. Depending on the sampling time, the AM fungal community composition was also different for both plant species (Fig. 4). In May and January, the most common sequence type in both plant species was GLO12 (23%), while GLO1 was the most dominant (24%) in September. Diversisporaceae phylotypes (DIV1 and DIV2), GLO6, and GLO10 were detected only in the May samples of *T. zygis*, while phylotype GLO13 was exclusively detected in January in *T. zygis*. On the other hand, GLO11 and GLO14 were not found in the May samples, and GLO5 was not detected in the September ones.

Based on the number of phylotypes, *R. officinalis* presented an AM fungal *S* value of 9 in all the sampling times evaluated. In the case of *T. zygis*, *S* was also 9 in the samples collected in January, 12 in those of May, and 8 in September. In the same way, the *H′* values for *R. officinalis* were 1.86 in May, 1.93 in September, and 1.98 in January, while for *T. zygis* values were 2.12, 1.84, and 1.98, respectively. No significant differences were found for the diversity indices used (Table 1).

The correlation analysis demonstrated significant positive relationships between the relative abundance of phylotypes GLO2 and GLO3 (Fig. 4, Table 2). Moreover, significant negative correlations were detected between the

Table 1 *F* values and significance for the main effects of relative abundance of glomeromycotan fungal phylotypes and diversity indices by means of a two-way ANOVA, considering two shrubs representative of semiarid Mediterranean ecosystems from Southeast Spain analyzed at three sampling times

Phylotypes and diversity indices[a]	Plant		Sampling time	
	F	*P* value	*F*	*P* value
GLO1	19.9	0.01	0.25	0.80
GLO2	3.16	0.10	0.41	0.69
GLO3	1.54	0.28	2.93	0.20
GLO4	100	0.00	0.03	0.97
GLO5	0.05	0.84	43.0	0.00
GLO6	1.00	0.37	1.00	0.47
GLO7	0.17	0.70	0.48	0.66
GLO8	2.18	0.21	1.24	0.40
GLO9	1.16	0.34	1.98	0.28
GLO10	1.00	0.37	1.00	0.47
GLO11	0.50	0.52	3.00	0.19
GLO12	27.6	0.00	0.00	0.99
GLO13	1.00	0.37	1.00	0.47
GLO14	0.18	0.69	21.5	0.02
DIV1	1.00	0.37	1.00	0.47
DIV2	1.00	0.37	1.00	0.47
Richness (*S*)	0.31	0.60	1.30	0.39
Evenness (*H′*)	0.42	0.56	0.53	0.63

[a]Glomeromycota fungal phylotypes were analyzed as the relative abundance of each one relative to the total AM fungal phylotypes obtained

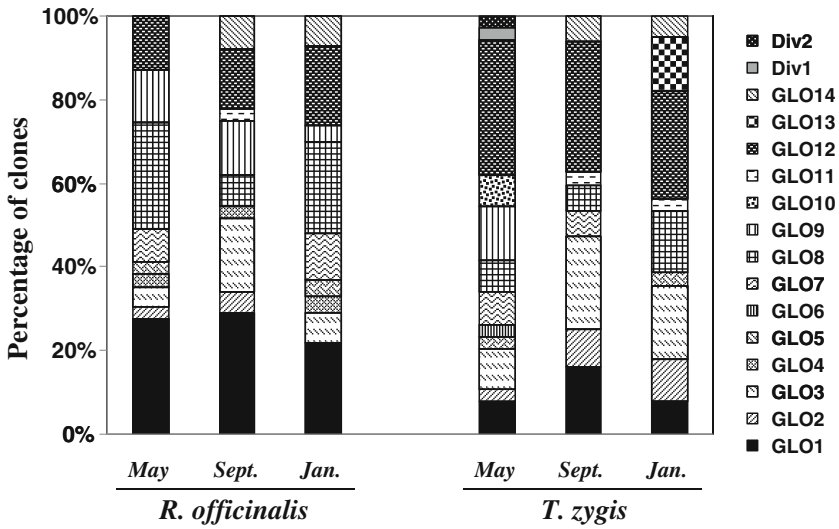


Fig. 3 Global proportional distribution of the glomeromycotan phylotypes obtained from roots of *Rosmarinus officinalis* and *Thymus zygis* at three sampling times (May, September, and January). The *y* axis indicates the proportion of clones assigned to each particular sequence type

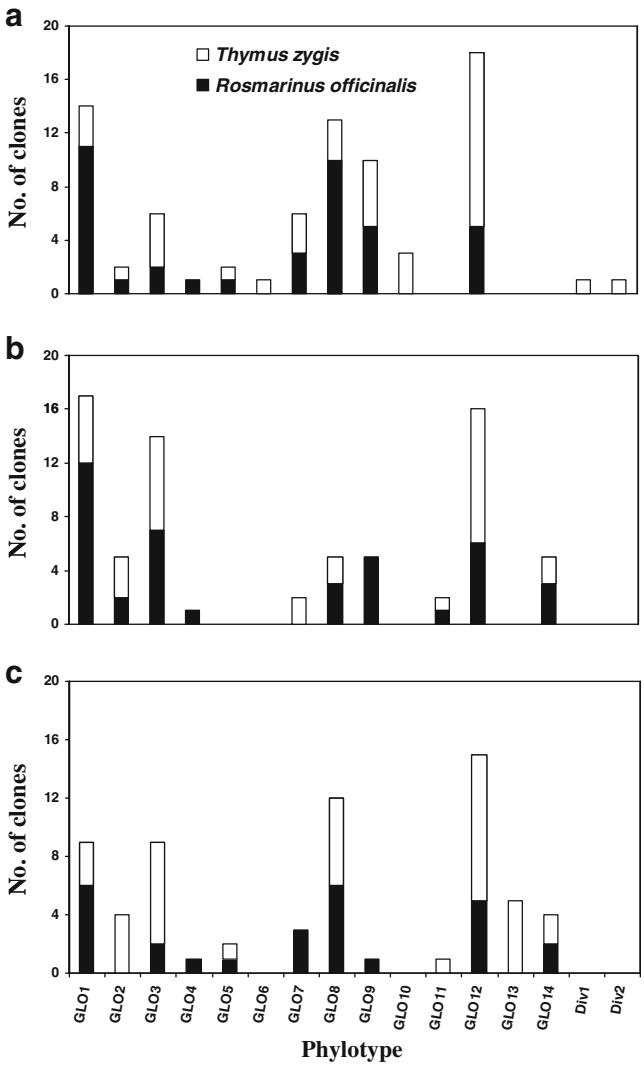


Fig. 4 Frequency diagram of glomeromycotan sequence types obtained from roots of *Rosmarinus officinalis* and *Thymus zygis* at three sampling times. **a** May sampling time, **b** September sampling time, and **c** January sampling time

relative abundance of the GLO12 in respect to GLO1 and GLO4 sequence types (Table 2). PC1 was significant and highly correlated with both diversity indices, S and *H*′, while PC2 was directly correlated with the relative abundance of GLO2 and GLO3. A hierarchical cluster analysis (Fig. 5) showed the highest similarity in AM fungal phylotypes colonizing roots of *R. officinalis* in May and January, and in root samples of *T. zygis* in January and September. On the other hand, AM fungal phylotypes and their relative abundance in the September *R. officinalis* root samples were more similar to samples obtained in September and January in *T. zygis* root samples than any other *R. officinalis* sample. *T. zygis* roots sampled in May differed from all the other analyzed samples.

Discussion

Results showed that the presence of certain AM fungal phylotypes colonizing the roots of the plant species here analyzed changes at different seasons throughout the year, but the predominant fungal types (as GLO1, GLO3, GLO8, and GLO12) are always present in both shrubs. Then, the major changes registered in the AM fungal community composition are based only on the presence or absence of some types that occur in some sampling times in both plant species (GLO5 and GLO14, Table 1), or as DIV1 and DIV2, present only in *T. zygis* at the first sampling time. Consequently, root samples taken at a single sampling time should not be enough for a proper characterization of AM fungal diversity in ecosystems like the one studied here, with strong seasonal weather changes, but could be a useful approach to rapidly know the predominant AM fungal types of communities colonizing roots of both Mediterranean representative shrubs since these types seem to be present over time.

Table 2 Correlation matrix of some Glomeromycota fungal phylotypes[a] found in roots of *Rosmarinus officinalis* and *Thymus zygis* with the principal components (PC)[b] obtained

	GLO2	GLO3	GLO4	GLO5	GLO7	GLO8	GLO9	GLO11	GLO12	GLO14	S	*H'*	PC1	PC2
GLO1	−0.42	−0.25	0.84*	−0.27	0.05	0.36	0.46	−0.08	−0.89*	0.27	−0.44	−0.58	−0.47	−0.72
GLO2		0.85*	−0.73	−0.51	−0.72	−0.50	−0.57	0.85*	0.42	0.21	−0.38	−0.31	−0.46	0.82*
GLO3			−0.56	−0.78	−0.71	−0.78	−0.51	0.95**	0.42	0.56	−0.40	−0.26	−0.50	0.83*
GLO4				0.21	0.30	0.63	0.37	−0.39	−0.87*	0.27	−0.26	−0.24	−0.26	−0.90*
GLO5					0.52	0.71	0.02	−0.74	−0.07	−0.44	0.40	0.49	0.48	−0.44
GLO7						0.44	0.10	−0.84*	0.05	−0.38	0.23	0.11	0.46	−0.57
GLO8							0.09	−0.59	−0.65	−0.27	−0.18	−0.20	−0.08	−0.81
GLO9								−0.47	−0.41	−0.47	0.55	0.25	0.47	−0.45
GLO11									0.15	0.63	−0.56	−0.38	−0.68	0.69
GLO12										−0.17	0.39	0.44	0.45	0.78
GLO14											−0.61	−0.28	−0.70	0.15
S												0.89*	0.96**	0.09
H'													0.82*	0.19
PC1														0.00

*$P<0.05$; **$P>0.01$, significance conventions

[a] The Glomeromycota fungal phylotypes were analyzed as the relative abundance of each one relative to the total Glomeromycota fungal phylotypes obtained

[b] The PC1 and PC2 explain the 76% of the total experimental variance

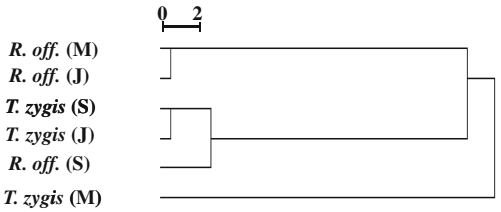


Fig. 5 Hierarchical cluster analysis of the presence and relative abundance of glomeromycotan fungi phylotypes obtained from roots of *Rosmarinus officinalis* and *Thymus zygis* at three sampling times (May, September, and January). The agglomerative method used was Ward's minimum variance method. The corresponding units (dimensionless) were calculated on the basis of the principal components obtained from a factorial analysis considering the relative abundance of the different phylotypes in the roots of each plant at three sampling times. *R. off.*—*Rosmarinus officinalis*, *T. zygis*—*Thymus zygis*, *M*—May, *S*—September, *J*—January

To optimize the cloning reaction and reduce the possible cloning bias, a strategy similar to that described by Renker et al. (2006) was used. Several DNA extractions from different individuals were performed, and the extracted DNA was amplified individually and combined before cloning. This sampling design has been used lately in different studies with satisfactory results (Cesaro et al. 2008; Yang et al. 2010). In addition, we found a high and constant AM fungal colonization in both plant species at any sampling time, between 50% and 66% (data not shown), suggesting a high mycorrhizal dependence of the selected plant species along the annual growth cycle.

The AM fungal sequences detected in the roots of *R. officinalis* and *T. zygis* were grouped in several sequence types, most of them belonging to *Glomus* group A. This reinforces the findings from a previous study, based on spore characterization and performed in rhizospheric soil samples obtained in the same target ecosystem, in which the genus *Glomus* resulted to be the prevalent spore type (Barea et al. 2007). In this sense, the absence of AM fungal sequences in the NS8-ARCH1311 PCR products probably indicates that fungi from Paraglomeraceae and Archaeosporaceae families were present in an extremely low proportion, or even not present in our samples, supporting the idea of a high prevalence of *Glomus* species in this kind of ecosystems. In addition, two sequence types of genus *Diversispora* were also obtained, but in a very low proportion and exclusively in the May samples of *T. zygis*, which could denote a preferential association between both symbionts at this season.

The two sequence types corresponding to previously morphologically characterized AM fungi (GLO1 and GLO13) have been found elsewhere in molecular studies based on root analysis. Sequence type GLO1, corresponding to *G. intraradices* and representing the 18% of the total AM fungal sequences obtained, has been

reported in a wide range of ecosystems (Husband et al. 2002; Öpik et al. 2003; Cesaro et al. 2008; Liu et al. 2009; Sonjak et al. 2009), and the sequence type GLO13, related to *Glomus constrictum*, was previously found in a boreal herb-rich coniferous forest (Öpik et al. 2008). Most of the other phylotypes have been reported in other studies in a wide range of environments. Phylotype GLO12, the most common sequence type found in this study (23% of the AM fungal sequences), has been reported in different ecosystems in relative high proportions (Öpik et al. 2003; Scheublin et al. 2004; Santos-González et al. 2006). Similar sequences to GLO4, GLO5, GLO8, and GLO11 were found in an alpine ecosystem in Salzburg, Austria (Moser and Haselwandter, unpublished sequences from NCBI database). DIV1, belonging to the Diversisporaceae family, was detected in the roots of threatened conifer species in a dry forest of Ethiopia (Wubet et al. 2006). Phylotype GLO8 was previously reported in grassland soils in Estonia (Öpik et al. 2003) and in a dune grassland in Holland (Scheublin et al. 2004). Besides, the phylotype GLO10 resulted to be commonly detected in different environments, such as a semi-natural woodland (Helgason et al. 1998), some arable crops (Daniell et al. 2001), and heavy-metal-polluted soils (Vallino et al. 2006). Analysis of the associations between AM fungi and different ecotypes of *Collinsia sparsiflora* revealed the presence of GLO2 and GLO3 sequence types colonizing this plant species (Schechter and Bruns 2008), while GLO9 was detected in the sporophyte of *Botrychium virginianum*, a fern species (Kovacs et al. 2007), and GLO14 in soil samples of a red cedar forest (Liang et al. 2008). Finally, some sequences closely similar to GLO6 were found in diversity studies performed in the Loess Plateau of China (Wu et al. 2006). The sequence types GLO7 and DIV2 are here described for the first time since no references of these sequences in previous studies have been found. The high percentage of unknown sequences usually obtained in the different molecular studies agrees with previous predictions that the 230 AM fungal morphospecies described so far, according to Oehl et al. (2011), represent only a small fraction of the diversity of the phylum Glomeromycota (Öpik et al. 2006).

Our results reveal moderate changes in the AM fungal phylotypes colonizing roots through time, in particular the decrease of some dominant AM fungal types which seem to be partially replaced by others, as well as other rare phylotypes with a scarce presence that occur just at determined sampling times. In this sense, a moderate increase in *S* value (not significant) was found during spring in the roots of *T. zygis*, which is consistent with previous studies by Santos-González et al. (2006, 2007), who showed that the number of AM fungal phylotypes detected in roots increased during the plant growing season (spring). Apparently, the moderate intra-annual variations here reported cannot be directly related to factors that vary consistently across different times in a year and suggest that AM fungal associations could be more dynamic than commonly considered, mainly in the case of rare AM fungal types.

Some previous studies related to the dynamics of AM fungal communities have reported seasonal changes in *Gigaspora gigantea* spore populations (Lee and Koske 1994) and root colonization patterns (Merryweather and Fitter 1998), results that were supported in a subsequent season by DNA sequencing (Helgason et al. 1999). Similar patterns, in soil and/or root, were obtained in other studies with different target ecosystems like arable crops (Daniell et al. 2001; Hijri et al. 2006; Oehl et al. 2009), grasslands (Vandenkoornhuyse et al. 2002; Santos-González et al. 2007), and a tropical forest (Husband et al. 2002). Distribution of the different phylotypes through time in this study could suggest the existence of certain AM fungal functional diversity that varies in function of seasonal fluctuations of abiotic environmental factors (Bever et al. 2001) and changes in host phenology (Merryweather and Fitter 1998; Eom et al. 2000) to satisfy plant requirements at different stages of plant growth. However, this variation could be presumably of limited impact based on the low number and scarce presence of the AM phylotypes that change throughout the year.

The influence of the host species in the dynamics of the AM fungal communities colonizing the roots of the target plants suggests the existence of a high degree of compatibility or preference of some AM fungal isolates for both host plants, as previously suggested (Vandenkoornhuyse et al. 2002). Furthermore, significant positive and negative correlations in the relative abundance of some AM fungal phylotypes were detected, especially in the case of phylotypes with a low or temporal presence, suggesting a moderate functional complementarity between determined phylotypes, as well as a temporal competence between other scarcely represented phylotypes, that cannot co-occur in the same root system at the same time (Table 2). This complementarity between different phylotypes (here assumed as distinct glomeromycotan species), and especially the preference of the different fungal species for a host plant, must be an aspect to consider when designing inoculants to be used in plants with the aim of re-establish the plant cover in semi-arid environments since some of them can act synergistically or, on the contrary, competitively, which may eventually reduce the presence of the dominant types.

In conclusion, this study shows that there are moderate but significant intra-annual differences in the composition of AM fungal communities colonizing two different plant species co-occurring in a semi-arid Mediterranean ecosys-

tem from Southeast Spain. This, together with the detected host preferences in some rare AM fungi colonizing plant roots, can help to select the preferable AM fungal inoculum composition for implementing rehabilitation programs according to the target plant species involved. Research is being done in our laboratory for assessing the existing functional diversity and the possible relationship between the phylotypes detected in the roots with those existing as propagules (spores) in soil, which are those usually used for the production of mycorrhizal plants to be established in the field.

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