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Complexity of Semiarid Gypsophilous Shrub Communities Mediates the AMF Biodiversity at the Plant Species Level

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Abstract The community composition of arbuscular mycorrhizal fungi (AMF) was analyzed in roots of *Gypsophila struthium* growing in gypsum soils under semiarid conditions. In order to investigate the effect of plant community degradation on the AMF biodiversity at the single species level, on the basis of the plant community complexity level, we selected four areas affected by degradation and shrub species spatial heterogeneity. The AM fungal community colonizing *G. struthium* was investigated from the morphological and molecular points of view. All plants were well colonized and showed a high level of infective AM propagules. Roots were analyzed by polymerase chain reaction, restriction fragment length polymorphism screening, and sequence analyses of the ribosomal DNA small subunit region. Four AM fungal types were identified and clustered into the AM fungal family: Glomeraceae, *Glomus* being the only taxon present. One fungal type was present in all the selected areas. Two fungal types are distinct from any previously published sequences and could be specific to gypsum soils. The chemical–physical properties of the soil were not correlated with the AMF diversity in roots. Our data show vegetation cover complexity-dependent differences in the AM fungal community composition.

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Introduction

The Iberian Peninsula possesses the largest area of gypsum outcrops in Europe [4, 47]. These particular ecosystems occur in arid and semiarid areas, are very restrictive habitats for vegetation, and support strict gypsophile vegetation. Together with the arid conditions, gypsum ($CaSO_4$ $2H_2O$) soils have particularly stressful physical and chemical properties for plant life (presence of a hard soil surface crust, low porosity, low nutrient availability, high concentration of sulfate ions, etc.). For these reasons, gypsophytes are one of the most specialized arid-endemic groups of plants [16, 33] and are very often rare and threatened species [34]. The Iberian gypsum vegetation is considered by the European Community as a priority habitat type for conservation purposes (*Gypsophiletalia*. Natura Code 2000:1520) [18]. The Nature 2000 Network provides a good opportunity to promote the preservation of these gypsum outcrops, which, due to their geographically punctuated distribution, have an extraordinarily varied flora [34]. The ecology of gypsophilous plant communities has only recently received attention [17, 41], and it is not clear which soil processes allow gypsophytes to survive on soils rich in gypsum and which processes impede non-gypsophytes [17, 40].

The gypsophilous plant community is characterized by spatial heterogeneity, is dominated by shrub species, and has cover values of 20–40% [11]. This spatial heterogeneity usually involves facilitative interactions among plants [6, 45] through an environmental amelioration which, in gypsum soils, could include the thinning of the gypsum

crust [15]. This positive interaction between plants seems to be quite common in hydric-stressed or saline environments [45], being one of the main processes that affects the structure and composition of the community; therefore, the presence of certain shrubs enhances the development of many other species.

Gypsophila struthium Loefl. (*Caryophyllaceae*), is an endemic shrub species which grows exclusively on Gypsisols. Several plant species belonging to this family have been reported as nonmycorrhizal [22, 61]; however, we have observed mycorrhizal occurrence in *G. struthium* roots showing an important degree of arbuscular mycorrhizal (AM) fungal colonization. *G. struthium* is one of the main primo-colonizers (high growth rates, greater dispersion abilities, rapid germination, high seed and seedling production, greater tolerance of nutrient-poor soils, etc.) in these gypsum environments [34]—although it is also present in the late stages of succession.

In many plant species, the presence of an AM symbiosis confers ecophysiological benefits, improving plant resistance to drought stress—particularly in saline soils with low levels of available nutrients. The contribution of AM fungi to enhancement of nutrient uptake and plant growth in saline conditions is well known [3, 8, 10]. Populations of AM fungi in saline–alkaline soils are variable and affected by many factors [26], but little is known about the presence of these fungal symbionts in gypsum soils. Landwehr et al. [30] found a variable number of spores in samples of European saline, sodic, and gypsum soils, with *Glomus geosporum* as the dominant species. It is evident that AM fungi (AMF) are a major factor contributing to the maintenance of ecosystem functioning and resilience. It was shown that plant diversity and productivity in ecosystems might significantly influence AM fungal diversity in the soil [60]. Therefore, information regarding the AMF detectable in plant roots, and as spores in the rhizosphere of plant communities, is crucial for the effective management and preservation of AMF diversity in ecological field studies.

Identification of AM fungi has relied extensively on the morphology of spores and related structures. But, spore populations isolated from the soil and identified may not necessarily be closely correlated with the AMF species communities colonizing plant roots [24, 51]. Also, an accurate identification of spores extracted from field samples may be hampered because spores can be unrecognizable due to degradation. Advances in molecular techniques over recent years have made possible the application of polymerase chain reaction (PCR)-based molecular methods to identify and to analyze the diversity of AM fungi colonizing roots in the field [46, 32]. Both Helgason et al. [23] and Redecker [46] designed specific PCR primers for the small subunit (SSU) ribosomal DNA (rDNA) that would amplify all-known glomalean fungi (AM1 and ARCH1311, respectively), excluding plant DNA. The primer AM1 is more or less specific for species of Glomerales and Diversisporales but does not amplify DNA of the Archaeosporales and Paraglomerales. Through the use of the two primer pairs species of all four AM fungi orders can be detected and that of in total about known 200 AM fungi species about 180–190 AM fungi species might be detected by the AM1 primer, while the species of Archaeosporales and Paraglomerales (<20 species) are detectable by the ARCH1311 primer [46].

We hypothesized that plant community complexity influences the AMF biodiversity, not only at the community level but also at the single-species level. In order to verify this hypothesis, we studied the diversity of AMF associated with *G. struthium* in four areas affected by degradation and with shrub species spatial heterogeneity. The selected areas are gypsum soils under Mediterranean semiarid conditions. Our experiment was carried out to identify AM fungal taxa present in roots and as spores in the rhizosphere soils on the basis of the SSU rDNA sequences subjected to restriction fragment length polymorphism (RFLP) and phylogenetic analyses.

Materials and Methods

Study Site and Sampling

The study area is located in "Paraje de los Cabecicos" close to Villena (Alicante), in Southeastern Spain (38°39′ N, 0° 54′ W, 515 m altitude). The climate is semiarid, with an evapotranspiration of 743 mm, an annual average rainfall of 370 mm, mostly concentrated in autumn and spring, and a mean annual temperature of 13.7°C. The soils are classified as Petrogypsic Gypsiorthid [56] and Gypsisol [19] with a gypsic and petrogypsic horizon within 100 cm from the surface, developed on gypsum parental rocks. Four areas numbered 1 to 4 according to the increasing complexity of the gypsophilus plant community were selected from a heterogeneous area measuring approximately 2500 m^2. Some characteristics of the four areas of the soil are shown in Table 2.

All samples were collected in the second half of May 2007 (late spring), when the highest fungal activity could be expected in Mediterranean semiarid soils [49]. *G. struthium* was present in all the four chosen areas and involved in AM symbiosis. Within each area, three 10-m^2 plots were established. Three plants from each plot were sampled (a total of 36 plants). Plants, including parts of their root systems, were collected along with a 10-cm deep soil core of 7×7 cm. Soil cores were placed in plastic bags and immediately transported to the laboratory, where the

samples were carefully washed to separate fine roots from the soil. Roots were then briefly rinsed, quickly dried on paper, and used partly for morphological and partly for molecular analysis.

Mycorrhizal Determinations

The percentage of mycorrhizal root infection was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (*v*/*v*), according to Phillips and Hayman [43]. The extent of mycorrhizal colonization was calculated according to the gridline intersect method [21].

Mycorrhizal potential in soil was measured by a dilution technique [54] that allows calculation of the most probable number of mycorrhizal propagules able to develop colonization units on the root of a test plant.

AMF spores coming from the rhizospheric soil samples of each area were extracted by wet sieving and sucrose density gradient centrifugation [13]. They were grouped and used for DNA extraction, PCR reactions, and cloning of PCR products.

Plant Diversity and Cover Percentage

Three 100-m^2 quadrats were randomly established within each point. The number of species and their individual and total coverage (visually estimated) were recorded at each plot for the perennial gypsophilous woody species (restricted gypsophytes and nonresticted gypsophytes). The relation between gypsophytes cover percentage and total plant cover percentage of the community was calculated.

Chemical and Physical Analysis of the Soil

Soil pH and electrical conductivity were measured in a 1:5 (*w*/*v*) aqueous solution. The total N was determined with the Kjeldahl method, consisting of titration after distillation and sample digestion. Available P, extracted with 0.5 M $NaHCO_3$, was determined by colorimetry according to Murphy and Riley [35]. Extractable potassium (K, extracted with ammonium acetate) was measured by atomic adsorption spectroscopy on a UNICAM 969 AA-Spectrometer.

The percentage of stable aggregates was determined by the method described in Lax et al. [31]. Bulk density was determined by the paraffin method described by Barahona and Santos [5] after maintaining soil moisture at 60% of field capacity for 1 month.

The percentage of gypsum was determined by Thermogravimetric analysis, which is based on the loss of weight when a sample containing gypsum is heated according to Porta [44].

Soil organic carbon was determined by potassium dichromate oxidation [36].

DNA Extraction and PCR

Total DNA was extracted from four pieces (2 cm each) of the root samples for each plant and from 100 spores isolated from soils of each plot using DNeasy plant mini Kit following the manufacturer's recommendations (Qiagen). The spore and root samples were placed into a 2-ml screw-cap propylene tube, and the DNA extracts were obtained by disrupting spores or roots with a sterile disposable micropestle in liquid nitrogen. The DNA was resuspended in 20 μl of water. Several dilutions of extracted DNA (1/10, 1/50, 1/100) were prepared. Partial ribosomal SSU DNA fragments were amplified using two different sets of primers. One set consisted in a universal eukaryotic primer NS31 [55] and the primer AM1, designed to amplify AM fungal SSU sequences but not plant sequences [23]. The second set of amplification was conducted with the specific primer ARCH1311 [46] in combination with NS8 [62]. ARCH1311 was used to detect the AM fungi species of Archaeosporales and Paraglomerales which cannot be detected by the AM1 primer.

PCR reactions were carried out in a final volume of 25 μl using the "ready to go" PCR beads (Amershan Pharmacia Biotech), 0.2 μM dNTPs, and 0.5 μM of each primer (PCR conditions: 95°C for 3 min, then 35 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, then 72°C for 10 min). All the PCR reactions were run on a Perkin Elmer Cetus DNA Thermal Cycler.

Cloning and Sequencing

The PCR products were purified using a Gel extraction Kit (Qiagen) cloned into pGEM-T Easy (Promega) and transformed into *Escherichia coli* (Xl1 blue). Putative positive transformants were screened in each resulting SSU ribosomal RNA (rRNA) gene library, using 0.7 unit of RedTaq DNA polymerase (Sigma) and a second NS31/AM1 or ARCH1311/NS8 amplification with the same conditions described above. Positive clones from each sample were tested for RFLP by independent digestion with HinfI and Hsp92II, according to the manufacturer's instructions (Promega). Finally, they were analyzed on 2.5% agarose (in TBE) gel electrophoresis. Examples of each RFLP type were chosen for sequencing. They were grown in liquid culture and the plasmid extracted using the QIAprep Spin Miniprep Kit (Qiagen). The sequencing was done by Molecular Biology Laboratory (SACE-Murcia University, Spain) using the universal primers, SP6 and T7.

Sequence editing was done using the program Sequencher version 4.1.4 (Gene Codes Corporation). The sequences have been deposited at the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov) with accession numbers AM909662-AM909684.

Phylogenetical Analysis

Sequence similarities were determined using the Basic Local Alignment Search Tool (BLASTn) sequence similarity search tool [2] provided by GenBank. Phylogenetic analysis was carried out on the sequences obtained in this study and those corresponding to the closest matches from GenBank. Sequences were aligned with other published glomeralean sequences using the program ClustalX [58], and the alignment was adjusted manually in GeneDoc [37]. Neighbor-joining (NJ) and maximum parsimony phylogenetic analyses [50] were performed with the program PAUP4.08b [57] and using the default parameters. The putative choanozoan *Corallochytrium limacisporum*, a close relative of fungi [9] was chosen as outgroup and used to root the trees on the basis of the widely accepted phylogenetic hypothesis that the fungal radiation is contemporary with the choanozoan radiation [59].

Diversity of AM Fungal Community

The Shannon–Weaver (H') index was calculated as an additional measure of diversity, as it combines two components of diversity, i.e., species richness and evenness. It is calculated from the equation $H' = -\sum pi(\ln pi)$, where *pi* is the proportion of individuals found in the *i*th species (in a sample, the true value of *pi* is unknown but is estimated as *ni*/*N*, [here and throughout, *ni* is the number of individuals (clones) in the *i*th species]).

Statistical Analysis

Treatment effects on measured variables (mycorrhizal determinations and soil properties) were tested by analysis of variance, and comparisons among treatment means were made using a least significant difference test calculated at $P<0.05$. Statistical procedures were carried out with the software package SPSS 14.0 for Windows [20].

Results

Morphological Analyses

The degree of mycorrhization of plant roots in the different areas did not differ significantly (Table 1). The colonization percentage ranged from 30% to 46% of the root length. The lowest mycorrhizal colonization rates were found in the samples of area 1.

Soils from all four areas contained AMF spores (Table 1). The distribution of spores in these areas was patchy. Areas 3 and 2 showed significantly higher numbers of spores (on average 85 spores/100 g dry soil) than areas 1 and 4. The lowest number of spores was found in area 1.

The mycorrhizal potential of area 4 was significantly higher than in the other areas (Table 1), while area 1 had the lowest mycorrhizal potential [43 (20–92) infective propagules per 100-g dry soil].

Vegetation Cover Percentage

Table 2 shows the percentage vegetation cover and the gypsophytes cover of the four areas considered in this study. Significant differences were observed in total vegetal cover, being highest in area 2 (70%) and considerably lower in area 3 (34%). Gypsophytes cover percentage, in relation to the total cover percentage of the community, was highest in area 4 (75%) and lowest in areas 2 and 1, with 22.86% and 21.05%, respectively.

Chemical and Physical Analyses of the Soil

The results of the chemical and physical analyses of the soils are shown in Table 2. The fertility levels were similar

Table 1 Degree of AM fungal colonization in roots of *G. struthium*, number of the spores isolated from the soil adhering to the roots and arbuscular mycorrhizal propagules in soil for the four areas of study ($n=3$)

Location	Colonized root length (%)	Average number of spores (100 g^{-1} dry soil)	MPN of AM propagules (100 g^{-1} dry soil)
Area 1	29.85±2.8a	15.7±2.0b	43(20–92)
Area 2	46.34±3.4a	81.1±4.1a	477(223–1019)
Area 3	46.05±3.7a	92.7±4.5a	120(56–257)
Area 4	45.06±3.3a	24.9±2.2b	1548(724–3308)

Values in columns followed by the same letter do not differ significantly ($P<0.05$) as determined by the LSD test. In parenthesis, lower and upper limit of confidence at 95% probability
MPN Most probable number [54]

Table 2 Some characteristics of soil and plant community in the four studied areas (n=3)

	Area 1	Area 2	Area 3	Area 4
Total N (g kg^{-1})	0.34±0.02a	1.08±0.04b	0.61±0.03ab	0.48±0.02a
Available P (μg g^{-1})	9.1±0.4b	7.5±0.3b	4.5±0.2a	8.3±0.3b
Available K (μg g^{-1})	196±47a	199±49a	236±51a	194±46a
pH (H_2O)	8.2±0.1b	7.9±0.0a	7.9±0.0a	8.1±0.1b
EC (1:5, μS cm^{-1})	249±2b	252±3b	237±2a	251±2b
Bulk density (g cm^{-3})	1.58±0.05c	1.40±0.02b	1.08±0.01a	1.12±0.01a
Aggregate stability (%)	66.2±3.0ab	62.2±2.0a	70.9±2.0ab	77.5±3.0c
Gypsum (%)	83.4±0.2a	73.5±0.2a	82.0±0.1a	80.4±0.2a
Organic C (g kg^{-1})	0.9.4±0.6a	1.3.9±0.8b	6.2.1±0.9c	1.8.1±0.8b
Total vegetal cover (%)	57±1b	70±3c	34±1a	50±1b
Gypsophytes cover/total vegetal cover (%)	21.05±0.1a	22.86±0.1a	60±0.3b	75±0.3c

Values in columns followed by the same letter do not differ significantly (P<0.05) as determined by the LSD test

in all four areas, with no significant differences in the content of extractable K. The soils of the four areas had similar contents of gypsum (around 80%), while the values of electrical conductivity, bulk density, and percentage organic matter measured in area 3 were significantly different from those obtained in the other areas. Regarding aggregate stability, the significantly highest percentage was in area 4 (77.5%).

Molecular Analysis of *G. struthium* Roots and Spores

The template DNA extracted from a pool of 100 spores for each plot either was not amplified or did not yield enough PCR product. Therefore, we used only DNA obtained from plant roots. Template DNA extracted from the pooled roots of three plants from each area was amplified successfully with the primers pair AM1/NS31, giving the expected band of about 550 bp. The generated PCR products were used for cloning and creating a clone library. Overall, 192 clones were screened by PCR; out of these, 148 contained the SSU rRNA gene fragment. In our previous study [1], 30 clones/sample were sufficient to detect the AM fungal biodiversity present in the roots. Therefore, all 148 clones were analyzed according to RFLP patterns with two restriction enzymes, HinfI and Hsp92II. Two to five clones of each sample for each RFLP type were sequenced, for a total of 64 sequences; the other 84 sequences were classified by RFLP typing. After preliminary BLASTn searches, 32% of the clones were represented by sequences belonging to AM fungi (47 sequences), providing seven different patterns or AM fungal types (Fig. 1A). The remaining clones were identified as non-AMF sequences (101 sequences; data not shown). Amplification of non-AMF rDNA with these primers was reported recently also by Douhan et al. [14] and in our previous investigation studying the impact of tillage practices on AMF biodiversity [1]. In all cases, the non-AMF sequences belonged to Ascomycetes.

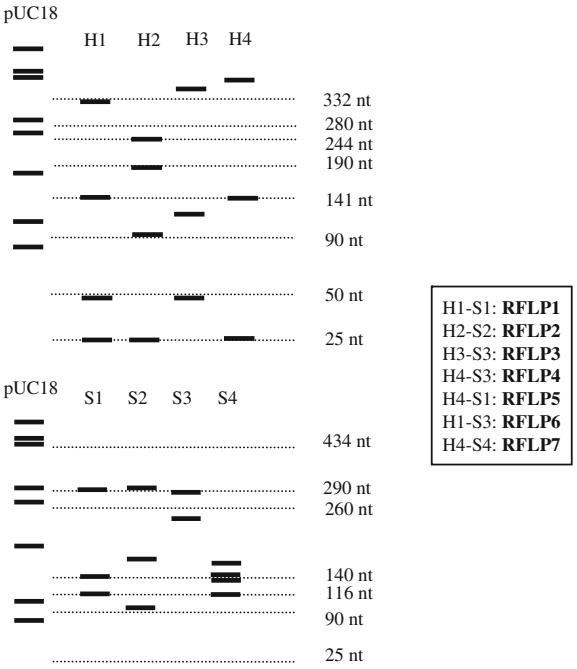
Of the seven RFLP patterns detected, RFLP1 was found in all areas studied (1, 2, 3, and 4). RFLP2 and RFLP5 were present in areas 3 and 4 and represented 13% and 48% of the analyzed clones, respectively. The remaining patterns seem to be specific for one single area: RFLP3 and RFLP6 for area 3 and RFLP4 and RFLP7 for area 4. In area 3, five different RFLP patterns were present, RFLP1 being predominant. Area 4 showed the highest number of clones, with five different AM fungal types. Areas 2 and 1 had only the RFLP1 pattern (Fig. 1B).

In order to investigate whether AMF belonging to the *Paraglomeraceae* and *Archaeosporaceae* were present inside the roots analyzed in our experiment, the same DNA samples used for AM1/NS31 amplification were used in PCR reactions with ARCH1311 and NS8 primers. None of the samples analyzed gave a positive result.

Identification of AM Fungal Groups

BLAST analysis of the sequences obtained in this study with the GenBank database showed that all sequences belong to members of the *Glomeromycota* phylum and have high similarity (98–100% identity) with AM fungal sequences obtained from other geographic regions and hosts. Two different types of phylogenetic analysis were carried out with them (neighbor joining and maximum parsimony) to construct a tree showing the position of AM fungal phylotypes. This analysis allowed us to separate the phylotypes of AM fungi colonizing the roots of *G. struthium* into four main groups or taxonomic units (TUs), grouped in the *Glomeraceae*, exclusively in the GlGrA (Fig. 2). All sequence clusters were supported by a bootstrap >75%. Three of them (Glo1, Glo2, and Glo3) clustered into the previously separated subgroup GlGrAb (according to Schwarzott et al. [53]). Clade Glo4 formed a distinct group with moderate support, without affinity to the subgroups of GlGrA. In the cluster Glo2, diverse sequence

A. NS31/AM1 amplified fragments



B.

	RFLP1	RFLP2	RFLP3	RFLP4	RFLP5	RFLP6	RFLP7
AREA 1	1						
AREA 2	1						
AREA 3	7	2	1		1	1	
AREA 4	2	4		5	21		1
Total	11	6	1	5	22	1	1

Figure 1 **A** RFLP patterns of SSU rDNA PCR products obtained from *G. struthium* roots. AM1/NS31 amplification. *H1–H4* restriction pattern obtained with enzyme *Hinf*I. *S1–S4* restriction pattern obtained with enzyme *Hsp*92II. **B** Distribution and frequencies in the four areas of the seven RFLP patterns obtained after H*inf*I and H*sp92*II digestions of NS31/AM1 amplified clones

types appear to exit, forming ambiguous branching, but they were not well supported by the bootstrap value: for this reason, they were classified as a single type. Clade Glo2 grouped together with sequences of known taxa and also with sequences of uncultured *Glomus* spp. originating from plant studies of different hosts carried out in different geographic regions, even including different continents.

Clades Glo3 and Glo4 did not cluster closely with any sequences of AM fungi maintained as reference AMF isolates in international culture collections. These newly obtained AM fungal sequences might be unique to this environment or host plant; moreover, they were present principally in area 4.

Clade Glo1 includes sequences (the only two detected in areas 2 and 1) with 99% identity to the sequences of *Glomus intraradices* BEG121.

AMF Diversity

The Shannon diversity index calculated for the different areas sampled was 1.23 in the case of area 3, 1.11 for area 4, and 0.01 for both areas 2 and 1. The Shannon index calculated for the whole study was 0.60.

Discussion

To our knowledge, this is the first molecular diversity study of AMF in gypsum soils under Mediterranean semiarid conditions. Two primer combinations, NS31-AM1 and ARCH1311-NS8, were used in this study to detect and identify all AM fungi belonging to the Glomeromycota phylum and actually present in *G. struthium* roots. No result was obtained after amplification with the ARCH1311 and NS8 primer combination; therefore, sequences from the orders *Archaeosporales* and *Paraglomerales* were not detected in plant roots growing in the gypsum soils. With the NS31-AM1 primer, we amplified sequences related to fungi exclusively belonging to the order Glomerales. BLAST searches with closely related AM fungal sequences in GenBank and thorough phylogenetic analysis allowed us to assign these sequences obtained from *G. struthium* roots to four groups or fungal types belong to the genus *Glomus* group A [53].

The fungal type Glo 1, which can be assigned to *G. intraradices*, was found in all four areas investigated, suggesting that this fungi was the most frequent in the studied area. The AM mycelium developing around the root system is known to play an important ecological role in connecting the root systems of plants growing near to each other, being the main source of inoculum in semiarid and arid ecosystems [42, 7]. Öpik et al. [39] proposed that some AMF species occur globally, showing high local abundance and low host specificity. *G. intraradices* clearly falls into this category as a generalist because it has been found in a surprisingly broad range of environments. Sequences similar to *G. intraradices* were found also by Landwehr et al. [30]; moreover, it is a surprise that the position of their sequences in the phylogenetic tree based on the 5.8S rDNA region matched with the one we constructed from the 18S rDNA region. By contrast, we never found sequences closely related to *G. geosporum,* which is dominant for saline, sodic, and gypsum soils in Germany, according to these authors.

Sequences grouped in the fungal type Glo 2 were closely related to sequences of uncultured *Glomus* spp. originating from different hosts and geographic regions in previous studies [29, 48, 63]. The sequences clustered in the Glo 3 and Glo 4 fungal types were detected for the first time, i.e., no sequences belonging to these groups were

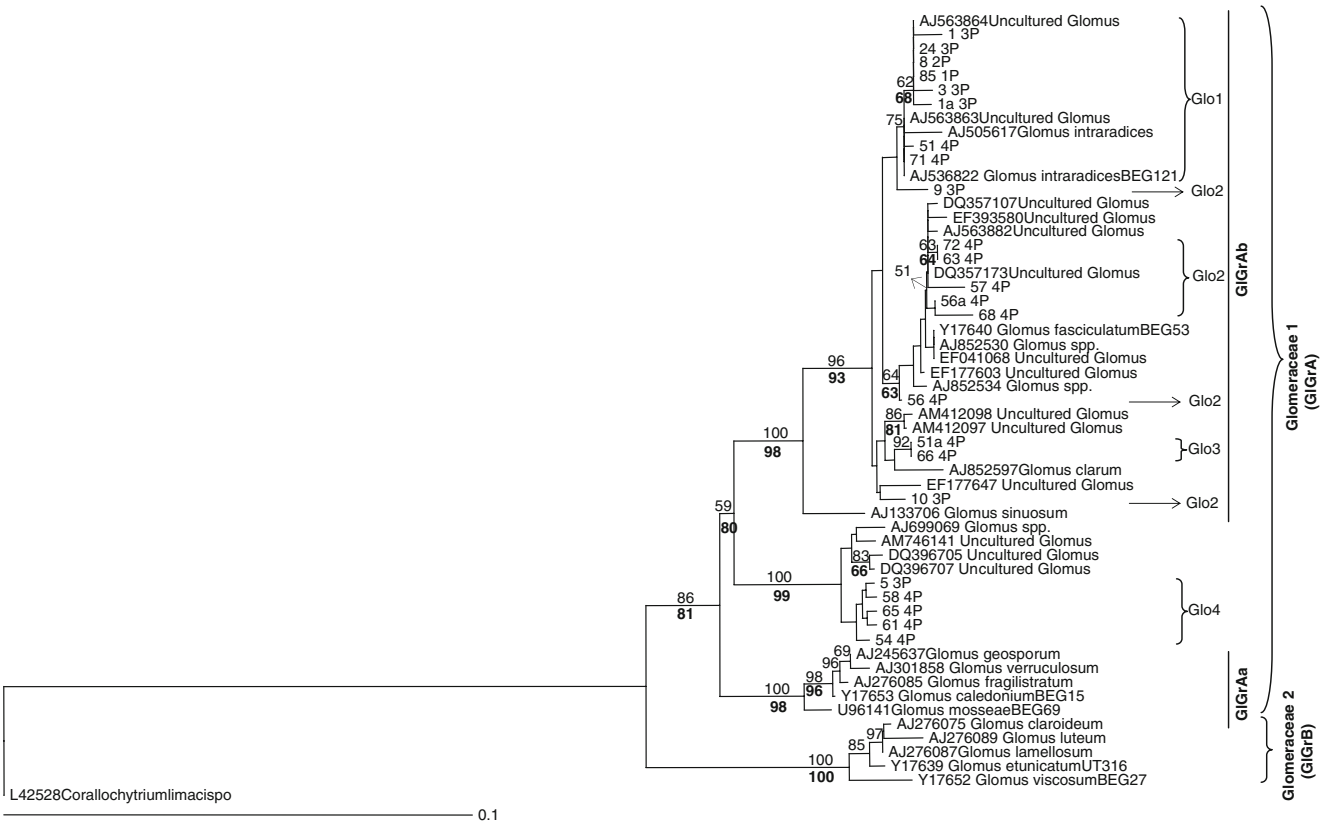


Figure 2 NJ phylogenetic tree showing AM fungal sequences isolated from roots of *G.* and reference sequences from GeneBank. Numbers above branches indicate the bootstrap values (above 50%, 100 replicates) of the NJ analysis; *numbers below branches* indicate the bootstrap values of the Maximum Parsimony analysis. Clone identifiers relate to site (*1P*, *2P*, *3P*, *4P*) and clone number (first number). Group identifiers (for example Glo1) are AM fungal sequences types found in our study. *C. limacisporum*, a putative choanoflagellate [9], was used as outgroup

found in the EMBL database; therefore, they could be assigned to *Glomus* species unique to this environment or not described until now. Interestingly, these new fungal types were found mainly in area 4; therefore, they could possess some functional traits or mechanisms which give them the capacity to proliferate under the stress conditions present in this area.

The predominance of the genus *Glomus* has been reported in other places, such as tropical forest [27, 28, 64], agricultural sites [12, 25], wetland soil [63], and gypsum soil [30]. *Glomus* species could dominate gypsum soils because they seem to be better adapted to the peculiar saline conditions in such soils. One possible reason why *Glomus* species are dominant in these soils may be found in their high sporulation capacity and ability to colonize via fragments of mycelium, which may enable them to survive and propagate more readily [38].

The isolation of four fungal types or TUs from the *G. struthium* roots indicates a low diversity of AM fungi (H= 0.6) in gypsum soils under semiarid conditions, compared with other ecosystems, such as dry forest (H=2.58 from one host species; [64]), tropical forest (H=2.33 from two host species; [28]), or wetland grass (H=2.4 from one host species; [63]). However, if we compare our data with the six fungal types detected by Helgason et al. [23], who analyzed the AM fungal community in agricultural sites, from four different plant species, or with our previous study [1], where ten fungal types colonized the roots of three hosts, we can see that the richness of the AM fungi was not so low. Moreover, it is necessary to consider that only one AM species was present in areas 2 and 1, which reduced the overall Shannon–Weaver diversity index. Also, Landwehr et al. [30] found low species diversity in gypsum soils; however, these data are not comparable with ours because their study was based on spores isolated from the soil—which may not necessarily correlate with the diversity of species colonizing plant roots [24, 51].

As suggested by Douhan et al. [14], care should be taken when using the primer pair AM1-NS31 for the analysis of AMF communities of field samples, especially if sequencing is not involved in the study. In fact, in our experiment two clones which showed similar RFLP patterns to AMF clones were non-AMF sequences. Problems of specificity with this set of primers were encountered also in our

previous study [1]. In all cases, the non-AM fungal sequences found were from members of the Ascomycetes.

Of all the spore samples analyzed, only DNA from two samples was amplified. One sample did not yield enough PCR product for cloning and the other gave PCR products of two sizes. The rest of the samples did not show any PCR product. The absence of PCR product from the spores may have manifold explanations (nonvital spores, degraded DNA, salt load interfering with the DNA amplification, etc.).

Our data indicate that some AMF phylotypes (Glo3 and Glo4) seem to show strong preference for area 4, since they were found mainly in this area. In other studies, environmental factors, such as field site [27], sampling season and soil nitrogen content [52], or age classes of seedlings [28], influenced the composition of the AMF community in the roots of some plant species.

Our data show no relationships between physical–chemical soil properties and the diversity of AMF. Even the results obtained for numbers of spores, percentage of root colonized, and numbers of propagules indicate that there is no correlation with AMF diversity or with percentage of gypsophilous cover. Therefore, we thought that the fungal composition might depend on complex physiological and ecological parameters of the plants. In fact, the vegetation composition differed between areas.

Area 4 showed 75% gypsophyte cover with respect to total cover percentage and also the highest AM fungal diversity whereas areas 2 and 1 had just one AM fungal species and the lowest gypsophyte cover percentage (Table 2). The plant community in area 4 represents a late state of succession in gypsum soils and, in consequence, the number of propagules and diversity of AMF are greater. Area 2 represents a 30-year-old *Pinus halepensis* restoration, where the soil profile was altered by plowing techniques. Restoration by man has a negative effect on the abundance of gypsophytes, especially for the more-restricted endemic taxa [34]; thus, a negative effect on the AMF population was observed. *G. struthium* is the most abundant species in area 1. It is considered a common species throughout the initial stages of succession and shows a marked preference for disturbed gypsum soils [34]. This information and the low gypsophilous cover percentage in area 1 (21.05%) indicate a low complexity level of the community and some degree of degradation. Degradation of this area could be the cause of the low presence of AMF.

These results allowed us to reveal the selective pressure of the plant species (*G. struthium*) on representative AMF species and diversity. An ecological specialization of AMF would explain the differences observed among areas. Gypsophilous community differences imply differences in AMF diversity, even if we are taking into account only a single species. Despite the relatively low numbers of AMF species found, the diversity was high in area 4—where the gypsophilous community was more complex. Therefore, the diversity of AM fungal species was related closely with gypsophyte cover percentage in each area. These results suggest that the complexity level in the gypsophilous community affects the diversity of AMF. Knowledge of the fungal diversity associated with *G. struthium* may be useful for the conservation and restoration of gypsum soils under Mediterranean semiarid conditions.

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