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Assessing the diversity of AM fungi in arid gypsophilous plant communities

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Summary

In the present study, we used PCR-Single-Stranded Conformation Polymorphism (SSCP) techniques to analyse arbuscular mycorrhizal fungi (AMF) communities in four sites within a 10 km^2 gypsum area in Southern Spain. Four common plant species from these ecosystems were selected. The AM fungal small-subunit (SSU) rRNA genes were subjected to PCR, cloning, SSCP analysis, sequencing and phylogenetic analyses. A total of 1443 SSU rRNA sequences were analysed, for 21 AM fungal types: 19 belonged to the genus *Glomus*, 1 to the genus *Diversispora* and 1 to the *Scutellospora.* Four sequence groups were identified, which showed high similarity to sequences of known glomalean species or isolates: Glo G18 to *Glomus constrictum*, Glo G1 to *Glomus intraradices*, Glo G16 to *Glomus clarum*, Scut to *Scutellospora dipurpurescens* and Div to one new genus in the family *Diversisporaceae* identified recently as *Otospora bareai*. There were three sequence groups that received strong support in the phylogenetic analysis, and did not seem to be related to any sequences of AM fungi in culture or previously found in the database; thus, they could be novel taxa within the genus *Glomus*: Glo G4, Glo G2 and Glo G14. We have detected the presence of both generalist and potential specialist AMF in gypsum ecosystems. The AMF communities were different in the plant studied suggesting some degree of preference in the interactions between these symbionts.

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Introduction

Gypsum outcrops are very restrictive habitats for vegetation. Their presence inhibits dense vegetation cover and a restricted vascular gypsophyte flora inhabits them in arid regions. Gypsum soils support strict gypsophilous vegetation mainly dominated by well-adapted shrubs species called gypsophytes. Together with the arid conditions and the long drought periods in Mediterranean areas, gypsum soils have particularly stressful physical and chemical properties for plant life (presence of hard soil surface crust, low porosity, low nutrients availability, high concentration of sulfate ions etc.). For these reasons gypsophytes are one of the most conspicuous specialist arid-endemic groups of plants, and are very often rare and threatened species (Mota *et al*., 2004).

The richness and rarity of the vascular flora colonizing these gypsum deposits strongly justify the official conservation of these habitats (indeed, the EU Habitats Directive gives priority to their conservation). In this regard, Mediterranean gypsum ecosystems are considered one of the most threatened habitats in the Mediterranean Basin (European Community, 1992).

The ecology of gypsophilous plant communities has only recently received attention (Escudero *et al*., 2000; Palacio *et al*., 2007) and the soil and biological processes that allow gypsophytes to survive on soils rich in gypsum, and impede non-gypsophytes, are not clear (Escudero *et al*., 2000; Oyonarte *et al*., 2002).

Arbuscular mycorrhizal fungi (AMF) form associations with the majority of terrestrial plant species and have been shown to improve the growth and nutrition of individual plants (Smith and Read, 1997; Wang and Qiu, 2006). Until recently, AMF species were generally assumed to be functionally similar, so there was little focus on AMF diversity in natural habitats. However, recent work has shown that plant diversity (Van der Heijden *et al*., 1998) and ecosystem variability and productivity (Hart and Klironomos, 2002) are directly influenced by AMF diversity, making an accurate assessment of species richness and community composition crucial to understanding the role of AMF in ecosystem functioning. The growing evidence of the multifunctionality and importance of AMF diversity for ecosystem functioning has led to a great effort to identify the species that colonize plants in natural ecosystems. Gypsum soils are harsh environments where AMF

may play an important role in promoting plant establishment and growth. If environmental conditions favour certain symbiont combinations, then specialized AMF communities could be expected. Previous studies have shown AMF colonizing the roots of *Gypsophila struthium*, a specialized gypsophyte from gypsum soils in Spain, and used molecular techniques to assess AMF diversity in the roots (Alguacil *et al*., 2009). But biodiversity and ecological data on the structure of AMF communities in gypsophilous plants are still scarce in comparison with other ecosystems.

Molecular techniques that allow identification of AMF within roots have been developed during the last decade, targeting the nuclear-encoded ribosomal genes (Redecker, 2002). Field studies based on molecular markers have provided numerous new insights into the dynamics of AMF communities and have presented evidence for host preference and host specificity (Helgason *et al*., 2002), effects of season and host plant development stage (Husband *et al*., 2002), as well as an influence of agriculture and ecosystem type (Hijri *et al*., 2006). These studies have also shown that while some AMF species appear to be rather limited in their distribution, others are generalists and surprisingly widespread (Öpik *et al*., 2006; Appoloni *et al*., 2008).

In the present study, we used PCR-Single-Stranded Conformation Polymorphism (SSCP) techniques to analyse AMF communities in an extensive gypsum area in Southern Spain. Four common plant species from these ecosystems have been selected: *G. struthium* L., *Teucrium libanitis* Schreber, *Ononis tridentata* L. and *Helianthemum squamatum* (L.) Dum.Cours, all of them specialized gypsophytes. Only *G. struthium* have been previously reported as mycorrhizal (Alguacil *et al*., 2009).

The primary questions addressed in this study were: (i) Do the host plants influence the AMF associated with their roots? (ii) Are there mostly AMF phylotypes specialized in gypsum environments or are AMF phylotypes with wide tolerance the most frequents? (iii) Is there spatial variation in the composition of AM fungal community in the roots of the gypsophilous plant community?

Results

Morphological analyses

The analyses were carried out with six plants belonging to each of the four selected species for each area (a total of 96 plants).

Regarding the percentages of mycorrhization obtained for plant roots, *H. squamatum* showed the lowest values (23.37–31.46%) with no significant differences among the four areas (Table 1). *Gypsophila struthium*, *T. libanitis* and *O. tridentata* showed higher mean values around 50% and no significant differences between areas were observed, except for *T. libanitis* that showed higher values (around 80%) in areas 1 and 2.

The number of AMF spores in the rhizosphere of all studied plant was very irregular, ranging from 8 to 153.7 spores per 100 gram dry soil. The number of spores was the lowest in *H. squamatum*, similar to what was found regarding the degree of fungal colonization (Table 1).

Most probable number (MPN) of AMF propagules varies among plant species from 5 (propagules per 100 gram dry soil) for *T. libanitis* in area 4 to 1548 (propagules per

Table 1. Degree of AM fungal colonization in roots of the four species studied, number of the spores isolated from the soil adhering to the roots and arbuscular mycorrhizal propagules in soil for the four areas of study ($n = 6$).

Species	Location	Colonized root length (%)	Average number of spores (per 100 gram dry soil)	MPN of AM propagules (per 100 gram dry soil)
Gypsophila struthium	Area 1	39.37a	89.8b	96 (43–165)
	Area 2	41.02a	92.0b	178 (59–293)
	Area 3	40.79a	19.7a	623 (210–901)
	Area 4	37.33a	26.0a	36 (15–79)
Teucrium libanitis	Area 1	80.96b	57.0b	74 (37–151)
	Area 2	79.77b	111.7c	20 (8–51)
	Area 3	58.05a	41.3b	35 (19–81)
	Area 4	65.36ab	8.0a	5 (4–13)
Ononis tridentata	Area 1	61.61b	66.3a	46 (19–103)
	Area 2	55.60ab	40.0a	513 (213–1201)
	Area 3	39.94a	62.3a	92 (48–189)
	Area 4	43.00ab	52.0a	106 (59–242)
Helianthemum squamatum	Area 1	26.85a	18.0a	184 (87–425)
	Area 2	31.46a	48.0a	158 (69–294)
	Area 3	26.03a	49.0a	362 (190–799)
	Area 4	23.37a	153.7b	168 (63–301)

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

Table 2. Some characteristics of soil in the four studied areas ($n = 6$).

	Area 1	Area 2	Area 3	Area 4
Total N (g kg^{-1})	1.91a	1.38a	1.50a	0.88a
Available P (μg g^{-1})	5.2a	6.8b	8.1b	7.8b
Extractable k (μg g^{-1})	237a	292a	171a	293a
pH (H_2O)	7.8a	7.9a	8.0a	8.0a
EC 1:5, μS cm^{-1}	263a	248a	261a	266a
Bulk density (g cm^{-3})	1.25a	1.28a	1.20a	1.20a
Aggregate stability (%)	64.6a	66.4a	64.1a	71.9a
Gypsum (%)	83.4a	73.5a	82.0a	80.4a
Organic matter (%)	0.38a	2.12b	1.78b	1.36ab
Total vegetal cover (%)	34a	70c	50b	57b

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

100 gram dry soil) for *G. struthium* in area 3 (Table 1). It also varied within species showing a patchy distribution.

Physical and chemical analyses of the soil

Results of physical and chemical analyses on soil are shown in Table 2. Soils of all areas had a similar content with respect to the assayed nutrients (N, P and extractable K), except that P content measured in area 1 was significantly lower than that obtained in the other areas.

Regarding pH and electrical conductivity, there were no differences among the areas – the values being homogeneous in the studied site. The soil gypsum content was very high in all sampled areas (around 80%). No differences in the physical properties (bulk density and aggregate stability) were found among the sampled areas. Measurement of soil organic matter gave very similar data in areas 2, 3 and 4 while area 1 showed the lowest value.

The vegetation coverage was also measured and significant differences were observed between areas. Area 2 showed the highest values (70%) and area 3 the lowest (39%).

The few differences observed between the data showed a significant consistency in all areas sampled.

PCR and SSCP type analysis

All DNA preparations consisted of pooled roots of individual plants. DNA extraction from 96 root samples was conducted (six root samples for each plant species and area). Template DNA was amplified successfully with the combination of primers AM1, AM2 and AM3/NS31. The NS31 universal eukaryotic primer was used as forward primer. The AM1 primer amplifies DNA from taxa belonging to *Glomus* group A, *Acaulosporaceae* and *Gigasporaceae* while the primers AM2 and AM3 are modifications of the primer AM1 and amplify DNA from taxa belonging to *Glomus* group B and *Glomus* group C (*Diversisporaceae*) respectively. All samples generated PCR products of the expected band of 550 bp, which were used for cloning and creating a clone library. From the 96 clone libraries, a total of 3072 clones were screened by PCR (on average, 32 clones were analysed per sample); out of these, 2482 contained the small-subunit (SSU) rRNA gene fragment (see Table S1). All of these 2482 clones were subjected to SSCP analysis. Representative clones of each sample for each SSCP pattern were sequenced, for a total of 650 sequences, while the remaining 1832 clones were classified by SSCP typing. The BLAST search in the GenBank sequence database revealed that 1443 clones (58.14%) resulted to correspond to AM fungi sequences showing a high degree of similarity to sequences from taxa belonging to the phylum *Glomeromycota* (Table 3). The 1039 remaining clones (41.86%) were identified as non-AMF since they showed high similarity to sequences of basidiomycetes, ascomycetes or hyphomycetes. Sequences belonging to the non-AMF were detected in 88 clone libraries. No chimeric sequences were detected in this study. The band patterns that produced sequences of non-glomalean origin in the SSCP gels were easily distinguished, as they migrated further in the gel and showed migration patterns different from those of glomalean bands. Since the aim of this study was to analyse the AMF diversity, we did not consider the presence of non-AMF for this purpose.

With respect to the other set of primers used, ARCH1311/NS8, the aim of which was to analyse whether AMF belonging to the *Paraglomeraceae* or *Archaeosporaceae* were present inside the roots of each plant species in the four areas investigated, no PCR products or only non-AMF amplicons were obtained – probably because of the absence of these fungi in our samples (data not shown).

Phylogenetic analysis and identification of AM fungal groups

After Neighbour-joining (NJ) and maximum parsimony (MP) analyses of 111 different glomalean SSU sequences obtained in this study with those corresponding to the closest matches from GenBank, 21 discrete sequence groups or phylotypes could be distinguished on the basis of bootstrap values ≥ 75% (Fig. S1). Examples of clones that produced the same sequence were represented just once in the alignment for clarity (see *Supporting information* for a detailed description of the clone groups with identical sequences). The sequence identity within each group ranged from 98% to 100%. Of the 21 sequence groups which were mainly consistent with SSCP patterns, 19 belonged to the genus *Glomus*, 1 to the genus *Diversispora* and 1 to the genus *Scutellospora*. *Glomus* group A was the most frequently represented group with 18 sequence types belonging to this group, while *Glomus*

Table 3. Number of clones of each SSCP pattern obtained for each plant species in the four areas ($n = 6$).

	Gypsophila struthium				*Teucrium libanitis*				*Ononis tridentata*				*Helianthemum squamatum*				Total	
	A1	A2	A3	A4	A1	A2	A3	A4	A1	A2	A3	A4	A1	A2	A3	A4	*n*	%
Glo G4	14	22	44	76	54	92	62	30	28	32	24	36	29	68	30	44	685	47.47
Glo G1	26	–	28	34	48	–	32	38	54	–	6	2	23	–	24	8	323	22.38
Glo G3	18	–	–	14	18	22	24	20	6	72	4	–	5	4	4	–	211	14.62
Glo G14	–	–	–	–	–	3	20	–	–	–	16	–	–	–	–	–	39	2.70
Glo G2	2	–	1	–	2	2	1	–	4	2	–	3	–	2	3	2	24	1.66
Glo G18	–	–	12	5	2	2	–	–	–	–	–	–	–	–	–	–	21	1.46
Glo G9	–	–	10	–	–	1	–	2	–	4	–	–	–	–	–	–	17	1.18
Glo G5	7	3	–	–	–	–	–	–	–	6	–	–	–	–	–	–	16	1.11
Glo G15	–	–	–	–	–	–	6	–	–	2	6	–	–	2	–	–	16	1.11
Glo G16	–	–	–	–	–	–	–	8	–	–	4	–	–	–	2	1	15	1.04
Glo G8	–	2	–	–	–	2	8	–	–	–	2	–	–	–	–	–	14	0.97
Glo G19	–	–	–	–	–	–	–	–	–	–	12	–	–	–	–	–	12	0.83
Glo G10	–	–	9	–	–	–	–	–	–	–	–	–	–	–	–	–	9	0.62
Glo G11	–	–	4	4	–	–	–	–	–	–	–	–	–	–	–	–	8	0.55
Glo G21	–	–	–	–	–	2	–	–	–	6	–	–	–	–	–	–	8	0.55
Division	–	–	–	–	–	–	–	–	–	6	–	–	–	–	–	–	6	0.42
Glo G20	–	–	–	–	–	–	–	–	–	–	5	–	–	–	–	–	5	0.35
Glo G12	–	–	–	3	–	–	–	–	–	2	–	–	–	–	–	–	5	0.35
Glo G6	–	–	–	–	–	–	–	–	–	–	–	–	–	4	–	–	4	0.28
Glo G7	1	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	3	0.21
Scut	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	2	0.14
Total	68	27	108	136	124	126	153	98	92	134	81	41	57	80	63	55	1443	100

group B was represented by only one type. The majority of AM fungal types showed high similarity to previously described, root-derived sequences in GenBank belonging to uncultured glomalean species and are therefore not taxonomically characterized. Four sequence groups identified in this study showed high similarity to respective sequences of known glomalean species or isolates: Glo G18 to *Glomus constrictum* (bootstrap value 100%), Glo G1 to *Glomus intraradices* BEG121 and BEG123 (90%), Scut to *Scutellospora dipurpurescens* (98%) and Div to one new genus in the family *Diversisporaceae* identified recently as *Otospora bareai*, although with low support (57%). The sequence type Glo G16 showed high similarity to *Glomus clarum*, but this relationship was not well supported in the analysis. There were three sequence groups that received strong support in the phylogenetic analysis, and did not seem to be related to any sequences of AM fungi in culture or previously found in the database; thus, they could be novel taxa within the genus *Glomus*: Glo G4 (85%), Glo G2 (98%) and Glo G14 (83%) (Fig. S1).

The distribution of AM fungal communities

The three most common sequence types (Glo G4, Glo G1 and Glo G3) accounted for 84.5% of the AMF clones analysed and occurred in all four plant species (Table 3). The sequence type Glo G4 was the most widespread; it was present in all the 96 roots samples analysed, therefore in all four plant species and four areas and accounted for 47.5% of the AMF clones. Interestingly, the other most common sequence type (Glo G1) was present in three areas (areas 1, 3 and 4), with the exception of area 2 for the four plant species. The Glo G2 fungal type, although accounting only for 1.7% of the clones, was found also in all four species. The rest of the fungal types were more uncommon and their representation (contribution) varied between 0.14% and 1.5% of the analysed AMF clones. Glo G14 and Glo G21 were found exclusively in *T. libanitis* and *O. tridentata* roots. Glo G5, Glo G12 and Glo G7 represented 1.7% of the clones and appeared only in *G. struthium* and *O. tridentata* roots. There were sequence types occurring exclusively in the plant species analysed; for example, four rare sequence types (Scut, Div, Glo G20 and Glo G19) appeared exclusively in *O. tridentata* roots. Glo G10 and Glo G11 were found only in *G. struthium* roots and Glo G6 only in *H. squamatum* roots. No fungal types specific to *T. libanitis* were found in the present study. The remaining AM fungal types appeared to be distributed haphazardly in the different host plant species and areas (Table 3).

To determine whether the number of analysed root samples was sufficient to detect the majority of sequence types present in the roots of the four plant species roots, sampling effort curves were constructed (Fig. 1). The data showed that for all four plant species the number of analysed root samples was sufficient to detect the majority of sequence types present in their roots. In fact, all the curves almost reached a plateau.

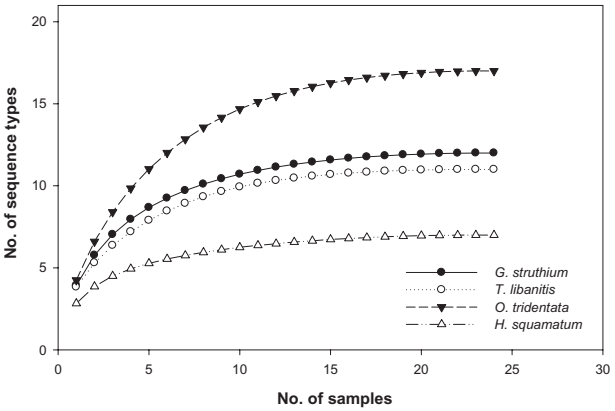

Fig. 1. Sampling effort curves for *G. struthium*, *T. libanitis*, *O. tridentata* and *H. squamatum*. The sample order was randomized by 100 replications in EstimateS, version 8.0 (Colwell, 2005).

Analysis of AM fungal communities in different host plant species and areas

The relationship between the host plant species and the areas (explanatory variables) regarding the distribution of AMF sequence types was studied using log-linear modelling. The results show that the variable host plant had a highly significant effect on the AMF community (Table 4, model 1b). In fact, *O. tridentata* had a richer AMF community than the rest of plant species (Shannon diversity index $H' = 1.24$), hosting 17 of the 21 fungal types found in this study (Table 3, Fig. S1). *Helianthemum squamatum* had the least varying AMF community (seven fungal types), and exhibited also the lowest Shannon diversity index ($H' = 0.84$). The AM fungal communities in the roots of *G. struthium* and *T. libanitis* were very similar hosting 12 and 11 fungal types respectively. Also, the Shannon diversity index was similar: 1.20 in the case of *G. struthium* and 1.24 for *T. libanitis*. The Shannon index calculated for the whole study was 1.13. The Euclidean-based multidimensional scaling (MDS) analysis (Fig. S2) also demonstrated these results, with the exception of some outliers; the AM communities in *O. tridentata* roots are distant from each other and from those of the other plant species, which indicated that they harboured distinct AMF types; however, the AM communities in the roots of *G. struthium*, *T. libanitis* and *H. squamatum* were more similar and they were all distributed towards the top of the diagram. Moreover in the log-linear analysis we could observe that the difference between the four areas was found to be not significant when of all the samples are considered together (Table 4, model 1c); however, analysis of the samples separately, by plant species, shows that, for *G. struthium* samples, area 2 was significantly different from areas 1, 3 and 4 ($P = 0.039$) and, for *O. tridentata* samples, areas 1 and 4 differed significantly from areas 2 and 3 ($P = 0.01$), as Table 3 also shows. The MDS analysis showed these results (Fig. S2), since the AMF communities on the four areas were not clearly separated by the first two dimensions of the diagram with the exception of the area 2, whose half of outliers were very distant from the rest. This effect could be related to the variation in the fungal diversity found in this area. On the other hand, a comparison of likelihood ratios in the log-linear analysis indicated that the area–host interaction had a greater influence on the AM fungal community than either host or area alone (Table 4, model 1a), which implies that within a determined area, the four hosts could be colonized by significantly different AM fungal communities (Table 3).

Table 4. Likelihood ratios (LR) produced by general log-linear analysis.

	Model	LR	d.f.	*P*
1a	Area*host interaction	129	9	< 0.001
1b	Host main effect	92	3	< 0.001
1c	Area main effect	6	3	0.119

The table shows the LR produced by the model when the specified term is removed. Higher LR values indicate greater deviation from the saturated model.

Discussion

Despite the fact that gypsum soils are extremely adverse habitats for plant life, they give rise to one of the most particular plant communities in arid and semi-arid regions. Gypsophytes are considered as edaphic endemics that occur in these soils because they are stress-tolerant species specifically adapted to the atypical soils in which they grow (Palacio *et al*., 2007). The chemical and physical characteristics of gypsum soils may play an important role in the distribution of gypsophytes, but little is known about their mycorrhizal associations and the influence of these particular conditions in AM fungal diversity. To our knowledge, there is only a previous study on semi-arid gypsum soils (Alguacil *et al*., 2009), but they conducted their research in areas affected by degradation process. Therefore, this is the first survey and most extensive of the molecular diversity of AMF in a mature and well-preserved community of gypsophilous endemic plants in semi-arid soils from the Mediterranean area and possibly worldwide.

The diversity of AM fungi colonizing roots of the four plant species studied was found to be remarkably rich. We detected 21 AM fungal types, which is comparable to the fungal richness reported for other natural ecosystems with a much higher plant densities, such as semi-natural grassland (Vandenkoornhuyse *et al*., 2002), boreal forest (Öpik *et al*., 2003), tropical forest (Husband *et al*., 2002), wetland soils (Wirsel, 2004), temperate

grassland (Santos-González *et al*., 2007) and serpentine soils (Schechter and Bruns, 2008). Other studies reported a low AM fungal diversity characteristic of regions under semi-arid conditions (Ferrol *et al*., 2004; Liu *et al*., 2009).

The composition of the AMF community colonizing the roots of *G. struthium*, *T. libanitis*, *H. squamatum* and *O. tridentata* was dominated by sequence types belonging to *Glomus* group A. Of the remaining fungal sequence types found in this study, one corresponded to *Glomus* group B, one to *Scutellospora* and the other to *Diversispora*. The three most common fungi in the studied ecosystem, unless there is strong PCR amplification bias, representing the 85% of the AMF clones detected, were Glo G4, Glo G1 and Glo G3. Glo G1, the second most abundant in our study, was related to the *G. intraradices* species group, which seem to represent a ubiquitous generalist fungus, since this is the most common taxon that has been detected from many worldwide locations in very different both ecosystems and host species (Öpik *et al*., 2006), even in an environment as extreme as thermal soils (Appoloni *et al*., 2008). Glo G3 matched with a sequence group that was previously collected at the same location by ourselves (Alguacil *et al*., 2009) and that seems to be characteristic of gypsum soils. In this way, the sequence types Glo G3 together with Glo G4, Glo G2 and Glo G14 (detected in this study for the first time) could be novel AM fungal groups, specific to these soils presumably adapted to proliferate in gypsophilous habitat.

It is interesting to note that the fungal type identified as 'Div' corresponded to *O. bareai*, a new fungal species in the family *Diversisporaceae* that was isolated recently by Palenzuela and colleagues (2008) from the rhizosphere of *Pterocephalus spathulatus*, a rare endemic plant growing on dolomite in the Sierra de Baza (Granada, Southern Spain). The rest of fungal types are related to sequences from the GenBank database detected in different host plant roots and ecosystems, suggesting a certain adaptation of specific ecotypes of these species to a wide range of habitats.

Our results clearly show that the AM fungal community composition is host plant-dependent in gypsum soils, in accordance with several previous studies developed in very different habitats (Helgason *et al*., 2002; Vandenkoornhuyse *et al*., 2002; 2003; Santos-González *et al*., 2007). This fact can be partly explained by different characteristics of the plant species considered. *Ononis tridentata* had the richest AMF community of the studied plants, hosting 17 of the 21 fungal types detected. This could be explained considering that *O. tridentata* belongs to the family *Fabaceae* (legumes), one of the typical mycorrhizal-dependent families; indeed, it is the major group of arbuscular mycorrhizal land plants, as reported by Wang and Qiu (2006) in their extensive survey of mycorrhizal occurrence in land plants. *Ononis tridentata* represent the largest shrub species (reaching a height of 100–110 cm) in the gypsophilous community; that means a suitable habitat for the establishment of other species under their canopy. It is known that facilitation increases with increasing environmental stress and particularly when aridity intensifies (Li *et al*., 2007). *Ononis tridentata* shrubs act as nurse for many annual herbs, perennial grasses and juvenile small shrub species that grows under their canopy sharing AM fungal species and then creating the so-called fertility islands (Callaway, 1997). As Azcón-Aguilar and colleagues (2003) found in Mediterranean shrublands, plants that create fertility islands develop an AM mycelial network that connects the root system of plants growing nearby and allows the exchange of nutrients between them.

Helianthemum squamatum showed the lowest AMF diversity hosting seven fungal types. This species is a small shrub (10–30 cm) that usually grows alone or under an *O. tridentata* canopy. The *Cistaceae* has also been reported as ectomycorrhizal- or ectomycorrhizal-derived (ectendomycorrhizal) species. The genus *Helianthemum* is considered mainly to be ecto- or ectendomycorrhizal (Gutierrez *et al*., 2003) and just two species (*H. apeninum* and *H. canum*) have been cited as AM symbionts (Harley and Harley, 1987). In a previous survey we detected AM and ectendomycorrhizal structures in *H. squamatum* unstained roots (our unpublished data). Therefore, we consider that *H. squamatum* root system are colonized by both AMF and ectendomycorrhizal fungi and we suggest that *H. squamatum* form dual mycorrhizal symbioses, hence there are more fungal symbionts and more competition within the same ecological niche by carbon compounds produced by the host plant and probably this leads to a less diverse AM fungi community. In fact, the proportion of non-AMF sequences to the total number of sequences analysed for *H. squamatum* was higher than in the rest of studied plant species (Table S1).

The number of fungal types was similar for *G. struthium* and *T. libanitis* plants, although these are two different shrub species with distinct ecological behaviour. *Gypsophila struthium* is the only gypsophyte that grows in both mature and altered gypsum ecosystems and tends to increase its coverage in degraded areas acting as a pioneer (Mota *et al*., 2004). Traditionally pioneers or primo-colonizers species have been considered as non-mycorrhizal plants (Harley and Harley, 1987). The *Caryophyllaceae* are reported as being non-mycorrhizal species (Wang and Qiu, 2006) and specifically genus *Gypsophila* was determined for the first time as mycorrhizal by Alguacil and colleagues (2009). *Teucrium libanitis* is the smallest shrub species in the present study, usually grows alone and rarely appears under the canopies of large shrubs.

In a previous study in gypsum soils Alguacil and colleagues (2009) found a considerably lower AM fungi diversity in the roots of *G. struthium* (Shanon index of 0.6 versus 1.2 in the present study). This difference could be related with the number of screened clones, since in the previous study 192 clones were screened by PCR while now a number of 768 clones were examined. According to Öpik and colleagues (2008), when comparing AM fungal diversity data the results can be different depending on the number of samples screened/sequenced. Also the RFLP analysis used in the previous study versus the SSCP used now could have underestimated the AMF diversity.

We also studied whether the AMF community changed spatially. We did not find a significant spatial change in the composition of the AMF community as a whole; however, there were some differences between the areas in two plant species when observed independently. The area 2 showed significantly lower AMF richness than areas 1, 3 and 4 in *G. struthium* and for the case of *O. tridentata* the areas 1 and 4 had significantly lower number of AMF species than areas 2 and 3. It has been proposed that soil factors such as pH, nutrient content etc. could influence the AMF community distribution, since these factors influence the distributions of the spores (Cuenca and Meneses, 1996; Husband *et al*., 2002). In our case the soil characteristics in the four areas were not distinct enough to allow us to draw conclusions (Table 2), therefore we think that the differences in the AMF association may be due to space-dependent host preference or geographic range and dispersal barriers for fungi.

In summary, we have detected the presence of both generalist and potential specialist AMF in gypsum ecosystems. The AMF communities were different in the plants studied suggesting some degree of preference in the interactions between these symbionts. Knowledge of the phenomena responsible for this preference would help to solve unresolved questions on the function of AMF and their interactions with plant communities.

Experimental procedures

Study site and sampling

The studied area is located close to Villena (Alicante), in south-eastern Spain (38°39′N, 0°54′W, 515 m above sea level). The climate is semi-arid, with an evapotranspiration (ETP) 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 (SSS, 1999) and Gypsisol (FAO, 1998) with a gypsic and petrogypsic horizon within 100 cm from the surface, developed on gypsum parental rocks. Four 100 m^2 areas (numbered 1–4) were selected for sampling from an heterogeneous area measuring approximately 10 km^2. Some characteristics of the four areas are shown in Table 1.

The field sampling was conducted using a factorial design with two factors and sixfold replication. The first factor included four areas. The second factor involved four plant species. Within each area, six plants belonging to each of the four selected species were sampled (a total of 96 plants). The following plant species were selected: *G. struthium* L., *T. libanitis* Schreber, *O. tridentata* L. and *H. squamatum* (L.) Dum.Cours.

All samples were collected in the second half of May 2008 (late spring), when the highest AM fungal activity could be expected in Mediterranean semi-arid soils (Roldán and Albaladejo, 1993). Plants, including root systems, were collected and placed in polyethylene bags for transport to the laboratory, where fine roots were separated 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 (1970). The extent of AM mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980).

Mycorrhizal potential in soil was measured by a dilution technique (Sieverding, 1991; modified by Azcón-Aguilar *et al*., 2003) that allows calculation of the MPN of mycorrhizal propagules able to develop colonization units on the root of a test plant.

Arbuscular mycorrhizal fungi spores coming from the rhizospheric soil samples of each area were extracted by wet sieving and sucrose density gradient centrifugation (Daniels and Skipper, 1982). They were counted.

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 (1962). Extractable (with ammonium acetate) K 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 and colleagues (1994). Bulk density was determined by the paraffin method described by Barahona and Santos (1981) after maintaining soil moisture at 60% of field capacity for 1 month.

The percentage of gypsum was determined by thermogravimetric analysis (DTA), which is based on the loss of weight when a sample containing gypsum is heated according to Porta (1998).

Soil organic carbon (SOC) was determined by potassium dichromate oxidation (Nelson and Sommers, 1982).

DNA extraction and PCR

For each sample, total DNA was extracted from root material (representing approximately 5–8 cm root length) using a DNeasy plant mini Kit following the manufacturer's recommendations (Qiagen). The roots samples were placed into a 2 ml screw-cap propylene tube and the DNA extracts were obtained by disrupting roots with a sterile disposable micropestle in liquid nitrogen. The DNA was re-suspended 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 (Simon *et al*., 1992) used as forward primer and a mixture of equal amounts of the AM1 (Helgason *et al*., 1998), AM2 and AM3 (Santos-González *et al*., 2007) primers used as the reverse primer combination. 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 30 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min and 30 s; and 72°C for 8 min). As a template, 2 μl of extracted DNA was used in all reactions. Positive and negative controls using PCR-positive products and sterile water, respectively, were also included in all amplifications. DNA extracts were stored at –20°C.

Concerning the other primer set, a two-step procedure (nested PCR) was conducted. The first amplification with the primers NS31 and NS41 (Simon *et al*., 1992) was performed as described above but with an annealing temperature of 50°C. Aliquots of 5 μl were run on agarose gel to estimate the quantity of PCR product. Several dilutions (1/10, 1/20, 1/50 and 1/100) were used as template for the second PCR step. The second step was conducted with the specific primer ARCH1311 (Redecker, 2000) in combination with NS8 (White *et al*., 1990). Reactions were performed in the same conditions as described above for AM1–3/NS31.

All the PCR reactions were run on a Perkin Elmer Cetus DNA Thermal Cycler. Reactions yields were estimated by using a 1.2% agarose gel containing ethidium bromide.

Cloning, generation of SSCPs 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). Thirty-two putative positive transformants were screened in each resulting SSU rRNA gene library, using 0.7 unit of RedTaq DNA polymerase (Sigma) and a re-amplification with NS31/AM1, AM2, AM3 or ARCH1311/NS8 primers with the same conditions described above.

Positive clones from each sample were analysed by SSCP. Samples for SSCP were prepared by mixing 5 μl of the clone PCR product with 5 μl of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 0.5 M EDTA). The samples then were denatured at 95°C for 5 min, placed on ice to stabilize single strands and then loaded on a 20 × 20 cm × 0.75 mm gel containing 0.6× MDE gel (BMA, Rockland, ME, USA), 10% ammonium persulfate (APS), 10× TBE buffer and 1% *N*-Tetramethylethylenediamine (TEMED). Electrophoresis was performed in 0.6× TBE buffer and run at 20°C at constant 150 V for 16 h in a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Bands were visualized by silver staining using a DNA Silver Staining Kit provided by Bio-Rad.

The SSCP banding patterns obtained from different root samples were compared by eye, and the bands were grouped according to similar mobility. Representatives of each SSCP pattern were chosen for sequencing. They were grown in liquid culture and the plasmid extracted using the QIAprep Spin Miniprep Kit (Qiagen). The sequencing was performed by Molecular Biology Laboratory (SACE – Murcia University, Spain) using the universal primers SP6 and T7. Sequence editing was performed using the program Sequencher version 4.1.4 (Gene Codes Corporation). Representative sequences of the clones generated in this study for each fungal type have been deposited at the National Centre for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov) under the Accession No. FN263078–FN263187.

A search for chimeric sequences was performed using the program CHIMERA_Check 2.7 of the Ribosomal Database Project (http://rdp8.cme.msu.edu/html/analyses.html) (Maidak *et al*., 2001).

Phylogenetical analysis

Sequence similarities were determined using the Basic Local Alignment Search Tool (BLASTN) sequence similarity search tool (Altschul *et al*., 1997) 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 (Thompson *et al*., 1997) and the alignment was adjusted manually in GeneDoc (Nicholas and Nicholas, 1997). Neighbour-joining and MP phylogenetic analyses (Saitou and Nei, 1987) were performed with the program PAUP4.08b (Swofford, 2002) and using the default parameters. *Paraglomus occultum* and *Paraglomus brasilianum*, ancient taxa of the phylum *Glomeromycota* were used as the out-group.

Statistical analysis

Treatment effects on measured variables (mycorrhizal determinations and soil properties) were tested by analysis of variance, and comparisons among means were made using a Least Significant Difference (LSD) test calculated at $P < 0.05$. All statistical procedures were carried out with the software package SPSS 15.0 for Windows (Ferrán Aranaz, 1996).

The experimental design allowed us to compare changes in the composition of an AM fungal community among plants of a given species, plant species and areas. General log-linear modelling and MDS were carried out using SPSS. A log-linear saturated model that contained all the independent variables as well as combinations of the variables was constructed to examine the relationships among the variables. The saturated model reproduced the observed data exactly. The relative importance of a given interaction term was estimated after having removed this term from the saturated

model. The overall goodness-of-fit statistics were calculated using likelihood radio chi-square to determine the significance levels and deduce whether this new unsaturated model fits the data. Here, the host plant, areas and the AM fungal types (i.e. SSCP pattern) counts were included in the model. The nine less frequent AM fungal types were not taken into account in this analysis in order to limit a possible bias in likelihood ratio estimates. Multidimensional scaling indicates the degree of dissimilarity of the fungal communities colonizing each root, based on pairwise comparisons. To model dissimilarities, Euclidean distances were computed.

The presence or absence of AMF phylotypes in each root sample was used to construct the sampling effort curves (with 95% confidence intervals) using the software EstimateS 8.00 (Colwell, 2005). The sample order was randomized by 100 replications.

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 $H' = -\Sigma pi(\ln pi)$, 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 in the *i*th species)].

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Neighbour-joining (NJ) phylogenetic tree showing examples of the AM fungal sequences types isolated from roots of *G. struthium*, *T. libanitis*, *O. tridentata* and *H. squamatum* in four different areas and reference sequences from GenBank. Numbers above branches indicate the bootstrap values (above 75%, 100 replicates) of the NJ analysis; numbers below branches indicate the bootstrap values of the maximum parsimony analysis. Sequences obtained in the present study are shown in bold type. They are labelled with the clone identity number, host (Gs, *G. struthium*; Tl, *T. libanitis*; Ot, *O. tridentata*; Hs, *H. squamatum*) and the area from which they were obtained (A1, A2, A3 and A4). Identical sequences are grouped, followed by the number of clones, in parentheses, having that particular sequence. Group identifiers (e.g. Glo G1) are AM fungal sequences types found in our study. *Paraglomus occultum* and *Paraglomus brasilianum* were used as out-groups.

Fig. S2. Euclidean-based multidimensional scaling (MDS) analysis emphasizing changes in the patterns of AM fungal community composition. Each point represents an AM fungal root community, with communities of similar composition being located close together (black symbols = *G. struthium*; grey symbols = *T. libanitis*; open symbols = *O. tridentata*; light grey symbols = *H. squamatum*; triangles = area 1; square = area 2; circles = area 3;, rhombuses = area 4).

Table S1. Root samples used for each plant species and area and number of clones analysed in the analysis of AM fungal diversity ($n = 6$).

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