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ORIGINAL PAPER

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# Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaris* and *Lolium perenne* in a field experiment

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**Abstract** Analysis of arbuscular mycorrhizal (AM) fungal diversity through morphological characters of spores and intraradicular hyphae has suggested previously that preferential associations occur between plants and AM fungi. A field experiment was established to investigate whether AM fungal diversity is affected by different host plants in upland grasslands. Indigenous vegetation from plots in an unimproved pasture was replaced with monocultures of either *Agrostis capillaris* or *Lolium perenne*. Modification of the diversity of AM fungi in these plots was evaluated by analysis of partial sequences in the large subunit (LSU) ribosomal RNA (rDNA) genes. General primers for AM fungi were designed for the PCR amplification of partial sequences using DNA extracted from root tissues of *A. capillaris* and *L. perenne*. PCR products were used to construct LSU rDNA libraries. Sequencing of randomly selected clones indicated that plant roots were colonised by AM fungi belonging to the genera *Glomus*, *Acaulospora* and *Scutellospora*. There was a difference in the diversity of AM fungi colonising roots of *A. capillaris* and *L. perenne* that was confirmed by PCR using primers specific for each sequence group. These molecular data suggest the existence of a selection pressure of plants on AM fungal communities.

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## Introduction

The arbuscular mycorrhizal (AM) symbiosis, which appeared with the first land plants more than 400 million years ago, is still formed by the large majority of extant plant species with no host specificity (Redecker et al. 2000; Remy et al. 1994; Smith and Read 1997). Glomalean fungi provide plants with mineral nutrients in exchange for carbon compounds and protect them against diverse abiotic and biotic stresses (Newsham et al. 1995; Smith and Read 1997). It is, therefore, thought that AM fungi play an important role in most terrestrial ecosystems. Nonetheless, symbiosis efficiency depends on environmental factors as well as genetic determinants from both host plants and AM fungi (Giovannetti and Gianinazzi-Pearson 1994). Plant species vary in their responsiveness to AM fungi with respect to growth, reproduction and resistance against stresses and, in turn, AM fungi can differ in their effects on plant health (Hartnett and Wilson 1999; Streitwolf-Engel et al. 2001; Taylor and Harrier 2000; van der Heijden et al. 1998; Wilson and Hartnett 1998). The elimination of AM fungal propagules using fungicides in diverse field situations has led to either an increase or a decrease in plant diversity, depending on the ecosystems and the mycorrhizal dependency of plants in the native vegetation (Gange et al. 1993; Hartnett and Wilson 1999; O'Connor et al. 2002; Smith et al. 1999). Therefore, these studies suggest that AM fungi can influence plant community structure in natural and semi-natural ecosystems.

Moreover, increasing plant diversity in a field experiment can result in increased AM fungal sporulation and community composition (Burrows and Pfleger 2002). Some studies in the field using morphological characters of intraradicular hyphae or of spores recovered from soil samples actually indicate that preferential associations exist between plants and AM fungi (Bever et al. 1996;

Eom et al. 2000; Hetrick and Bloom 1986; Johnson et al. 1991; McGonigle and Fitter 1990; Sanders and Fitter 1992; Schenck and Kinloch 1980). This "ecological specificity" (Harley and Smith 1983), resulting in the selection of AM fungi by plants, has not been investigated extensively in natural ecosystems as few taxa can be precisely identified through the morphology of their intraradicular hyphae. Spore production in the soil can vary temporally and as a result of environmental factors, and molecular analyses have shown that this may not necessarily reflect the actual intensity of root length colonised by each AM fungal species nor their diversity (Clapp et al. 1995; Kjoller and Rosendahl 2001).

In the UK, unimproved upland grasslands that are not fertilised are usually dominated by the grass species *Agrostis capillaris* L. and *Festuca rubra* L. or *F. ovina* L. In contrast, *Lolium perenne* L. and *Trifolium repens* L. are characteristic of improved grasslands receiving regular inputs of fertilisers (Rodwell 1992). Considering the ecological importance of AM fungi, it is of interest to determine whether their diversity is affected by the plant community structure in these contrasting grassland types. Ribosomal gene polymorphism has been targeted to evaluate AM fungal diversity in plant roots from different ecosystems (Clapp et al. 1995; Kjoller and Rosendahl 2001; Sanders et al. 1995; Vandenkoornhuyse et al. 2002). Nested PCR based on the large subunit (LSU) region of ribosomal RNA genes (rDNA) has also been used successfully to detect AM fungal species colonising plant roots in microcosm experiments (Jacquot et al. 2000; Kjoller and Rosendahl 2000; van Tuinen et al. 1998), and to study diversity of AM fungi in the field (Jacquot-Plumey et al. 2001; Turnau et al. 2001). This PCR approach was extended to the analysis of AM fungal populations in a grassland ecosystem in order to investigate the influence of host species on their ecological diversity.

## Materials and methods

### Field experiment

A field experiment was carried out in an unimproved pasture at the Sourhope Research Station (Macaulay Land Use Research Institute; 55°28'30?'N; 2°14'W) at 370 m above sea level in the Scottish Borders (UK). The soil is a brown ranker derived from old red sandstone and has a pH of 4.3 (unpublished data K. Ritz et al.). Vegetation from 12 plots (6 m × 2 m each) was removed in April 1998 using a mechanical digger. The soil was mixed thoroughly with a rotovator and large stones were removed. Plastic edging was placed inside the perimeter of each plot to prevent in growth of roots from surrounding paths and soil was reintroduced. Six plots were reseeded with *A. capillaris* (var. Highland) and six with *L. perenne* (var. Parcour). The plots were kept ungrazed and checked regularly for any invading wild plant species, which were removed manually. Three cores of 6 cm in diameter and 10 cm in depth, consisting of plant roots and soil were sampled from each of the 12 plots in May 1999 when both plant species were in a vegetative state. Roots of the three cores from each plot were pooled and equally divided for analysis of the intensity of root colonisation by AM fungi and of AM fungal diversity.

### Assessment of root colonisation by AM fungi

Roots were stained with methyl blue (Grace and Stribley 1991) and the intensity of root cortex colonisation by AM fungi (M%) was determined as described by Trouvelot et al. (1986; http://www.inra.fr/Internet/Centres/Dijon/bbceipm/Mychintec/Mycocalc-prg/MYCOCALC.EXE). Results were analysed statistically by ANOVA after arcsine transformation using the Genstat 5 statistical software (Lawes Agricultural Trust, IACR Rothamsted, UK).

### DNA extraction from roots

Aliquots (1 g) of fresh roots sampled in each plot were homogenised in liquid nitrogen and then in 1 ml of extraction buffer (0.2 M Tris-HCl pH 8.0, 0.25 M NaCl, 0.025 M EDTA, 0.5% SDS, 1% PVP, 29 mM $\beta$-mercaptoethanol). The supernatant obtained after a 10-min centrifugation at 12,000 rpm at 4ºC was recovered and mixed with an equal volume of phenol. After centrifugation, one volume of chloroform was added to the aqueous phase and the samples were centrifuged again. DNA was then precipitated from the resulting aqueous phase by adding one volume of isopropanol. The pellet was rinsed with 100 µl 75% ethanol, dried and resuspended in 50 µl sterile distilled water. The quality and quantity of DNA in the samples were checked on a 0.8% agarose gel and a 1/1000 dilution was used for PCR.

Initially, aliquots of DNA extracted from roots from the six replicated plots within a treatment were pooled for the construction of LSU rDNA libraries, each representing a total of 18 cores. Afterwards, PCR using cluster-specific primers was performed on each of the six replicated DNA extracts per treatment (see below).

### Preparation of DNA extracts from spores

Spores isolated from AM fungal isolates from international collections were used for DNA extraction: *Acaulospora laevis* Gerdemann & Trappe INVAM AU211–1, *A. spinosa* Walker & Trappe INVAM NC105A-4, *Entrophospora colombiana* Spain & Schenck INVAM CL356–6, *Gigaspora gigantea* (Nicol. & Gerd.) Gerdemann & Trappe INVAM MN922A-2, *Gig. margarita* Becker & Hall INVAM WV205A-2, *Gig. rosea* Nicolson & Schenck BEG9, *Glomus caledonium* (Nicol. & Gerd.) Trappe & Gerdemann INVAM UK301–1, *G. clarum* (Schenck & Smith) INVAM BR143A-4, *G. mosseae* (Nicol. & Gerd.) Gerdemann & Trappe BEG12, *Scutellospora heterogama* (Nicol. & Gerd.) Walker & Sanders INVAM BR154–6.

Fifty spores from each isolate were collected and crushed in 25 µl of sterile distilled water and 20 µl of 20% Chelex resin were added. The extracts were incubated at 95ºC for 15 min and then briefly centrifuged. The supernatant was used in subsequent reactions at a dilution of 1/10 in sterile distilled water.

### Design of general PCR primers for AM fungi

The 5' end of LSU rDNA sequences from different AM fungi previously published in Genbank was aligned using ClustalW 1.8.1 (Thompson et al. 1994). This sequence information (Fig. 1) was used to design new primers: FLR3 (5'-TTG AAA GGG AAA CGA TTG AAG T-3') and FLR4 (5'-TAC GTC AAC ATC CTT AAC GAA-3') using Amplify (Engels 1993) for the PCR amplification of AM fungal sequences from root tissues without recognition of plant DNA. FLR3 is situated between the D1 and D2 domains of LSU rDNA whilst FLR4 is in the D2 domain.

### PCR amplification of a partial LSU rDNA region

The primers LR1 and FLR2 (Trouvelot et al. 1999; van Tuinen et al. 1998) were used for the amplification of the 5' end of LSU rDNA sequences in fungi in general. A 20-µl reaction mix

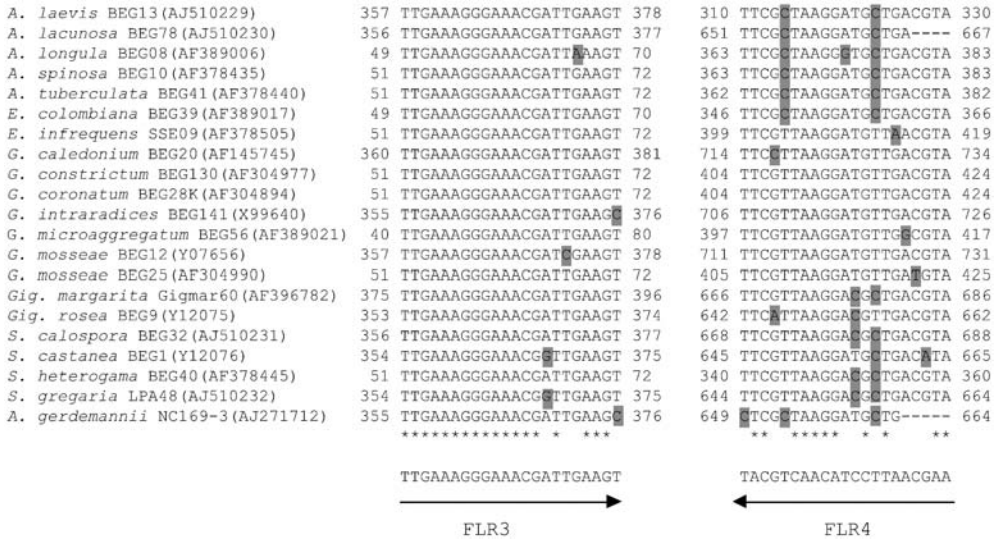


**Fig. 1** Partial alignment of large subunit (LSU) rDNA sequences from different isolates of arbuscular mycorrhizal (AM) fungi used for the design of FLR3 and FLR4 primers. Polymorphism is indicated as shaded nucleotides (*A. Acaulospora*, *E. Entrophospora*, *G. Glomus*, *Gig. Gigaspora*, *S. Scutellospora*)

**Table 1** Primers used to amplify arbuscular mycorrhizal fungal large subunit rDNA sequences from individual clusters when used in combination with the FLR4 primer

| Primer | Sequence | Expected length of PCR product (bp) |
|---|---|---|
| *Glomus mosseae* | 5'-AAAGCCTTCGGATTCGCGG-3' | 294 |
| *Glomus* 2 | 5'-CATGAGGAGGAAACCCTCG-3' | 301 |
| *Glomus* 3 | 5'-GAGCGTGAGGAGTTAAACGC-3' | 306 |
| *Glomus* 4 | 5'-TCCTTATTTGCAAATTTGTATTC-3' | 286 |
| *Glomus* 5 | 5'-GCCTTCGTTGCTTGCGTTA-3' | 289 |
| Acaulosporaceae 1 | 5'-CAACATGAGGGTTCGCTTTC-3' | 274 |
| *Acaulospora* 2 | 5'-TGTTCCCCCGGGAGCGATCT-3' | 278 |
| *Acaulospora* 3 | 5'-TTCGCTCGCGTACTTTCCGG-3' | 272 |
| *Scutellospora* 1 | 5'-GAACCTAACCTTGAAGTGCAC-3' | 269 |
| *Scutellospora* 2 | 5'-AGGGGAAACTCTGAGTGCA-3' | 264 |

contained 2 µl 10 × PCR buffer (Appligene), 200 mM dNTPs, 500 nM each primer and 0.4 U Taq polymerase (Appligene). An aliquot (1 µl) of diluted root or spore DNA extract was added to 19 µl PCR mix. The PCR program was as follows: 93ºC for 1 min, 58ºC for 1 min and 72ºC for 1 min (35 cycles), followed by 10 min at 72ºC.

PCR products were diluted 1/100 and used as templates for the second PCR with the primers FLR3 and FLR4 under the same PCR conditions. PCR products were run on a 2% agarose gel in TAE buffer and visualised under UV light after staining with ethidium bromide.

### Construction of LSU rDNA libraries and sequencing

The PCR products generated from roots of *Agrostis capillaris* and *L. perenne* using the primers FLR3 and FLR4 were cloned into the PCR 2.1 vector (Invitrogen). Inserts from 50 randomly selected clones in each LSU rDNA library were sequenced using the M13 forward (-20) primer on an ABI automated sequencer (Department of Medical Microbiology, University of Aberdeen, UK). Sequences were compared to known sequences using BLASTN (Altschul et al. 1997) and were deposited in the EMBL database (Accession numbers: AJ459321-AJ459380).

### Reconstruction of phylogenetic trees

An alignment of the sequences over 376 bp was performed using ClustalW 1.8.1 and optimised manually using the Se-Al v 2.0 software (University of Oxford). Phylogenetic analyses were performed using the neighbour joining (NJ) algorithm and using *Mortierella polycephala* as an outgroup. The reliability of the internal branches of the NJ tree was assessed using the bootstrap method with 1,000 replicates. Tree files were drawn using njplot (http://biom3.univ-lyon1.fr).

### Design of cluster-specific primers

Clusters of closely related sequences were identified through alignment and the reconstruction of a phylogenetic tree. Discriminating primers were designed for each of these clusters (see Table 1). The specificity of detection by PCR using these primers was first checked on sequenced clones from LSU rDNA libraries. After confirmation of PCR specificity, presence of each sequence cluster was analysed in DNA extracted from roots from each of the six replicated plots per plant species. PCR conditions were as described above, except that FLR3 was replaced with a discriminating primer.

## Results

### Root colonisation by AM fungi

There was no significant difference in the intensity of root colonisation by AM fungi in *Agrostis capillaris* (M%=15.4 ± 2.36; mean ± SE) or *L. perenne* (M%=14.8 ± 3.13) collected from the plots 13 months after the start of the field experiment.

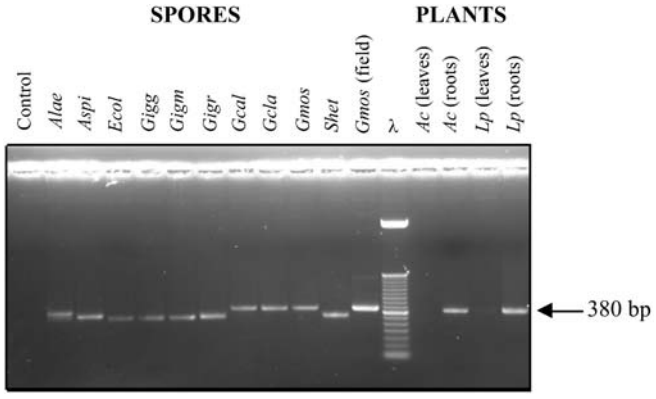


**Fig. 2** Nested PCR products obtained using DNA extracted from spores of AM fungi or from leaves or roots of the grass species *Agrostis capillaris* (*Ac*) and *Lolium perenne* (*Lp*) with LR1-FLR2 and FLR3-FLR4 primer pairs. In the control, no DNA was used in the PCR. Spores: *Alae*: *A. laevis* AU211–1, *Aspi*: *A. spinosa* NC105A-4, *Ecol*: *E. colombiana* CL356–6, *Ggig*: *Gig. gigantea* MN922A-2, *Gigm*: *Gig. margarita* WV205A-2, *Gigr*: *Gig. rosea* BEG9, *Gcal*: *G. caledonium* UK301–1, *Gcla*: *G. clarum* BR143A-4, *Gmos*: *G. mosseae* BEG12, *Shet*: *S. heterogama* BR154–6. *Gmos* (field): *G. mosseae* isolated from trap cultures (λ: 50-bp ladder)

### Amplification of AM fungal LSU rDNA sequences from spores and root tissues

Although BLASTN analysis indicated that the new FLR3 primer may bind to LSU rDNA from a few Basidiomycetes as well as from Glomales, the FLR4 primer had a perfect match only with Glomales sequences. The combination of these primers is, therefore, suitable for the preferential amplification of Glomalean LSU rDNA sequences from colonised root tissues. Length of PCR products obtained by the nested PCR protocol varied with the AM fungal genera from between 300–350 bp for *Acaulospora*, *Entrophospora*, *Gigaspora* and *Scutellospora* to about 380 bp in *Glomus*. Nested PCR on DNA from pooled roots of *Agrostis capillaris* and *L. perenne* collected in the field resulted in a main band of about 380 bp (Fig. 2). No PCR product was obtained with DNA from leaves of *A. capillaris* or *L. perenne* (Fig. 2), confirming the specificity of the PCR primers for fungal DNA in plant roots. When the sizes of the PCR products obtained from *A. capillaris* and *L. perenne* roots were compared to those from spores of the 10 different glomalean species, the results suggested that the dominant AM fungal species colonising these plants belonged to the genus *Glomus* (Fig. 2).

FLR3-FLR4 PCR products were used to construct LSU rDNA libraries from roots of *A. capillaris* and *L. perenne* and 50 randomly selected clones from each library were sequenced. Sequence analyses showed no aberrant sequence; all had a high similarity to known LSU rDNA sequences from AM fungi. Among the 100 sequences obtained in total, 60 were non-redundant (deposited sequences AJ459321-AJ459380). Despite variability in the sequences, it was possible to identify clusters of closely related sequences (Fig. 3). No new cluster was obtained after 27 and 24 clones had been sequenced from the *A. capillaris* and *L. perenne* LSU rDNA libraries, respectively. The topology of the resulting phylogenetic tree indicated the presence of AM fungi belonging to the genera *Glomus*, *Scutellospora* and *Acaulospora,* the most frequent sequences corresponding to *Glomus* species (Fig. 3).

Only sequences belonging to cluster *Glomus* 2 were found in roots of the two host plant species. In all these clusters, sequences originating from roots of *A. capillaris* were clearly separated from those of *L. perenne*. One group of sequences from *A. capillaris* roots clustered with sequences from diverse isolates of *G. mosseae*, including one isolated from the field soil by trap culture (Sourhope 2, 4; deposited sequences AJ459412, AJ459413). It can, therefore, be concluded that these sequences correspond to *G. mosseae*. Roots of *A. capillaris* were also colonised by the genotypes *Glomus* 3, *Scutellospora* 2 and *Acaulospora* 3, whilst the genotypes *Glomus* 4 and 5, *Scutellospora* 1, Acaulosporaceae 1 and 2 were only detected in *L. perenne* roots.

### PCR using specific primers

Primers specific to each cluster were designed by alignment of AM fungal sequences in the LSU rDNA D2 region (Table 1). An example of the specificity of these primers is shown for clusters *Glomus* 2 and 5 in Fig. 4A when tested against clones from the LSU rDNA libraries. The presence of each sequence group was analysed in the DNA extracts from the six replicate plots for each plant species in the field experiment (Fig. 4B, Fig. 5). Sequencing of PCR products confirmed that they belong to the corresponding cluster (results not shown). There were differences between replicate plots within each treatment, with some sequence types found in all plots and others in only a few (Fig. 5). Nonetheless, this distribution confirmed that AM fungal diversity was different in roots of *A. capillaris* and *L. perenne*, except for cluster *Glomus* 2.

## Discussion

Analysis of AM fungal diversity in natural and semi-natural ecosystems has so far been hampered by technical difficulties associated with the inability to grow these fungi in pure culture in the absence of a host plant. In addition, spores and hyphae of AM fungi are multinucleate and single spores can contain multiple sequences of the same gene (Clapp et al. 2001; Rodriguez et al. 2001; Sanders et al. 1995). Furthermore, analysis of AM fungal diversity from spores isolated from soils may not necessarily reflect the diversity of AM fungi colonising roots of individual plant species (Clapp et al. 1995; Kjoller and Rosendahl 2001). The nested PCR approach developed in the present study successfully detected LSU rDNA sequences from AM fungi belonging to different genera with no amplification of plant DNA. The FLR4 primer has a perfect match only with glomalean LSU rDNA sequences and although the FLR3 primer could


**Fig. 3** Neighbour joining tree representing AM fungal sequences isolated from roots of *A. capillaris* (ACP) and *L. perenne* (*LPP*) in comparison to known sequences. The number of redundant sequences is indicated in brackets. Bootstrap values were estimated from 1,000 replicates

recognise DNA from some Basidiomycetes as well as from Glomales, this primer pair only resulted in the amplification of Glomales sequences.

The extent of variability of the D1-D2 region of rDNA enabled not only analysis of AM fungal biodiversity by systematic sequencing but also the design of taxon-discriminating primers, which could then be used to monitor AM fungi in roots from the field. Whilst the variability observed amongst sequences was in accordance with data from Clapp et al. (2001) and Rodriguez et al. (2001), the phylogenetic tree including known sequences gave high bootstrap values and showed that 10 different clusters of sequences from AM fungi belonging to the genera *Glomus*, *Acaulospora* and *Scutellospora* were present in roots of *A. capillaris* and *L. perenne*. This is in agreement with the diversity of AM fungi expected in such a geographical location (Nicolson and Gerdemann 1968) and concords with a recent temporal analysis of AM fungi colonising roots of *A. capillaris* and *T. repens* based on 18S rDNA sequences (Vandenkoornhuyse et al. 2002). *Glomus* species dominated in both *L. perenne* and *A. capillaris* roots. Some sequences from *Scutellospora* and *Acaulospora* were identified but corresponding clones were present in low abundance in the libraries. It seems unlikely that AM fungal diversity was underestimated due to pooling of DNA extracts prior to PCR and preferential amplification of some sequences, since the low abundance of *Scutellospora* and *Acaulospora* was

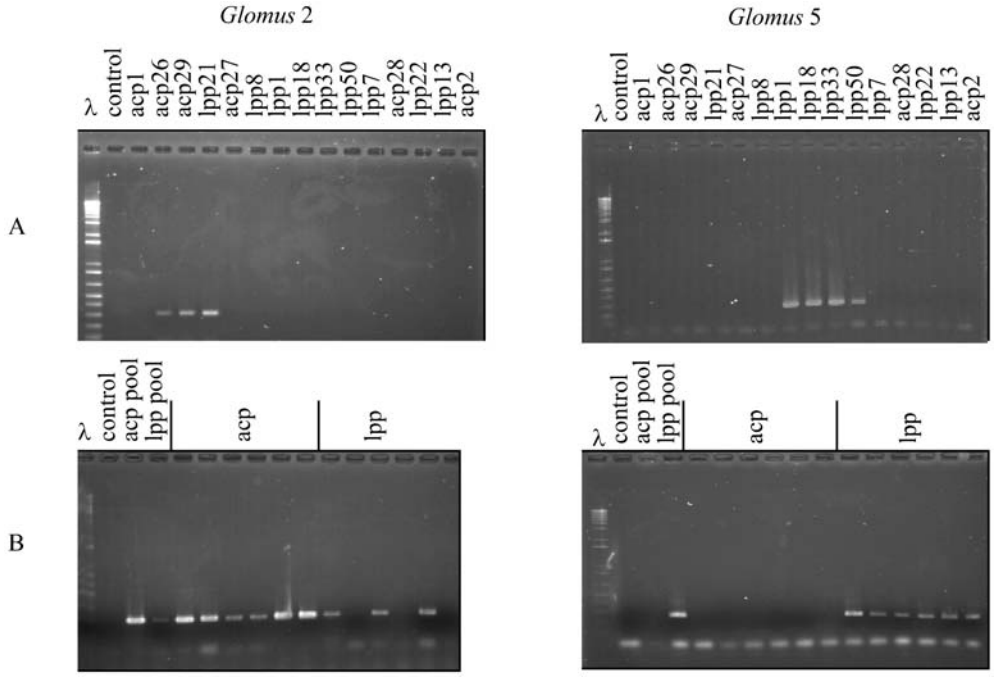


**Fig. 4A, B** Nested PCR detection of sequences from clusters *Glomus* 2 and *Glomus* 5 using a specific primer and FLR4. **A.** PCR specificity is shown using sequenced clones from the LSU rDNA libraries belonging to the different clusters: *Glomus mosseae* (acp1), *Glomus* 2 (acp26, acp29, lpp21), *Glomus* 3 (acp 27), *Glomus* 4 (lpp8), *Glomus* 5 (lpp1, lpp18, lpp33, lpp50), *Scutellospora* 1 (lpp7), *Scutellospora 2* (acp28), Acaulosporaceae 1 (lpp22), *Acaulospora* 2 (lpp13), *Acaulospora* 3 (acp2). **B.** Detection of sequence clusters in pooled aliquots of DNA extracted from roots of *A. capillaris* (acp pool) or of *L. perenne* (lpp pool), and in separate DNA samples from six replicate plots for each plant monoculture (acp, lpp). No DNA template was used in PCR controls

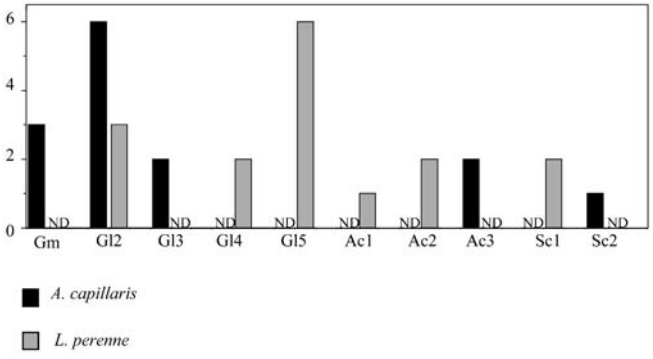


**Fig. 5** Number of DNA extracts among six replicates in which sequence clusters from AM fungi are detected by nested PCR using cluster-discriminating primers in roots of *A. capillaris* or *L. perenne* (*ND* not detected, *Gm G. mosseae*, *Gl 2 Glomus* 2, *Gl 3 Glomus* 3, *Gl 4 Glomus* 4, *Gl 5 Glomus* 5, *Ac 1* Acaulosporaceae 1, *Ac 2 Acaulospora* 2, *Ac 3 Acaulospora* 3, *Sc 1 Scutellospora* 1, *Sc 2 Scutellospora* 2)

also observed in individual root extracts from replicate plots using primers specific to the corresponding clusters. One cluster showed homology to *Entrophospora* species but it was impossible to conclude whether it corresponded to *Acaulospora* or *Entrophospora* genera because few known sequences are presently available in the databases.

The phylogenetic tree constructed from the LSU rDNA sequences obtained clearly indicates that, except for the cluster *Glomus* 2, AM fungal populations were different in roots of *L. perenne* and *A. capillaris.* This diversity can be considered significant, as each LSU rDNA library represents a total of 18 core samples per host species and results were confirmed by PCR using cluster specific primers in 12 individual root DNA extracts. *L. perenne* is characteristic of improved grasslands and does not generally grow in unimproved pastures. Therefore, AM fungi colonising *L. perenne* roots in this field experiment may be different from those naturally associated with this plant species in improved pastures. Although further temporal and spatial analyses would be required in this experiment, the present study suggests that preferential associations can occur between plant species and AM fungi. Such an ecological specificity in plant monoculture systems merits further investigation, in particular in agricultural systems where rotations are used. Previous studies based on spore morphology have indeed indicated that crop monocultures can result in the modification of AM fungal diversity, which in turn may affect growth of new crops (Johnson et al. 1991; Schenck and Kinloch 1980). In natural ecosystems where different plant species coexist, root colonisation by AM fungi may be facilitated by root-to-root contact between different plants and by mixing of root exudates, so attenuating the extent of such ecological selectivity. Molecular approaches like those developed here may provide insights into the implication of such processes in natural ecosystems.

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