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Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots

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Abstract A set of PCR primers targeted at five major phylogenetic subgroups of arbuscular mycorrhizal fungi (Glomales) was designed to facilitate specific amplification of internal transcribed spacers and 18 S rRNA gene fragments from colonized roots in the absence of spores. The subgroups include the recently discovered deeply divergent lineages of Glomales, which could not be detected by previously reported PCR primers, and the former genus *Sclerocystis*. Restriction fragment length polymorphism patterns presented allow identification of presently known members of these groups. The resulting PCR products can be used to identify the fungal symbionts at the genus or species level by restriction digests or DNA sequencing. A novel DNA extraction method allows visual control of the analyzed roots by staining procedures after analysis by PCR.

Key words DNA extraction · Glomales · Internal transcribed spacers · Molecular identification · RFLP patterns ·

Introduction

Approximately 150 species of arbuscular mycorrhizal (AM) fungi have been described (Walker and Trappe 1993) by means of morphological features of the spores. The necessity for spores in order to determine the species has seriously hampered ecological studies of AM fungi in the rhizosphere. The production of spores is not always correlated with root colonization (Clapp et al. 1995; Merryweather and Fitter 1998). When no spores are formed, the intraradical structures of AM fungi at best allow identification of the family (Merryweather and Fitter 1998). Furthermore, several newly characterized lineages do not stain at all with standard procedures (Redecker et al. 2000a). Thus, the species composition of active AM populations within roots can only be analyzed by molecular methods. For these reasons, several PCR-based detection methods have been developed in recent years and some have already been applied under field conditions (Clapp et al. 1995; Helgason et al. 1998).

The analysis of ribosomal genes allows the concurrent elucidation of phylogeny and primer design for specific phylogenetic groups of AM fungi. Simon et al. (1992) compared three Glomalean 18S subunit sequences available at that time and designed the PCR primer, VANS1. Recent studies have shown that the VANS1 annealing site is not well conserved within the Glomales (Clapp et al. 1999) and that several newly characterized ancestral lineages of the Glomales do not have this site at all (Redecker et al. 2000a). Other primers reported were specific only for single isolates or species and, therefore, are of restricted applicability (Millner et al. 1998; van Tuinen et al. 1998) or do not exclude other fungi (Helgason et al. 1998). Redecker et al. (1997) demonstrated that PCR/restriction analysis of internal transcribed spacers (ITS) of ribosomal DNA allowed species of the Glomales to be distinguished using a minute amount of fungal biomass, and that the generated fragment patterns were highly reproducible. The variation of ribosomal DNA sequences within single spores (Lloyd MacGilp et al. 1996; Sanders et al. 1995) limits the resolution of this method when closely related species or isolates are compared. However, an equally valuable molecular marker that does not show this disadvantage has not been found. Certain regions of the 28S rDNA proved useful in a Single Strand Conformation Polymorphism protocol (Kjøller and Rosendahl 2000), but only one clade of the Glomales comprising *Glomus intraradices* and *Glomus mosseae* can be analyzed at the present time.

Acaulospora gerdemannii/Glomus leptotichum, Acaulospora trappei, Glomus gerdemannii, Glomus oc-

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cultum and *Glomus brasilianum* were recently found to constitute deeply divergent lineages of the Glomales which do not fall into the previously known families (Redecker et al. 2000a; Sawaki et al. 1998). As previously published specific primers for the Glomales (Helgason et al. 1998; Simon et al. 1992) show several mismatches, it was of particular interest to establish new primers for these organisms.

A further group of AM fungi that could not be identified with molecular methods were members of the former genus *Sclerocystis*. Recently, the first ribosomal small subunit sequences from that group have become available (Redecker et al. 2000b) and show that *Sclerocystis* is situated in a monophyletic clade of *Glomus*, comprising *G. mosseae* and *G. intraradices.* In the present study, I show that ITS from *Sclerocystis* species can be amplified with the primers designed for the respective *Glomus* clade and I present restriction patterns for two species to facilitate their identification.

Materials and methods

AM taxa

In order to obtain spores for polymerase chain reaction (PCR) experiments, AM fungal inocula were obtained from the following sources:

1. International collection of vesicular-arbuscular and arbuscular mycorrhizal fungi (INVAM), Morgantown, W.V.: *Acaulospora gerdemannii/G. leptotichum* NC176 and FL130, *A. laevis* AU211-3, *A. morrowiae* BR226-1, *A. mellea* BR983-4, *A. trappei* NB112 and AU219, *G. brasilianum* WV219, *G. clarum* CL883A, *G. etunicatum* UT316, *G. occultum* HA771 *G. sinuosum* MD126
2. Centro Internacional de Agricultura Tropical, Cali, Colombia: *A. spinosa, Entrophospora colombiana* C18-3, *Gigaspora albida* BR205-1, *Gigaspora decipiens* AU102-3, *Scutellospora heterogama* BR154, *Scutellospora pellucida* C-139-3A
3. Banque Européenne de Glomales: *A. lacunosa* BEG78*, G. caledonium* BEG20, *G. claroideum* BEG88*, G. claroideum* BEG3 (=DN987-4)*, G. geosporum* BEG11
4. University of Florida, Gainesville, Fla.: *G.* sp. S329
5. Biorize, Dijon, France: *G. coremioides* (=*Sclerocystis coremioides*, = *Sclerocystis dussii*)

Voucher specimens were mounted on microscope slides in polyvinyl alcohol lactoglycerol and retained in the author's personal herbarium.

Other fungi

For specificity controls, purified genomic DNA and hyphal material of fungi other than the Glomales was used. Cultures or DNA samples of these fungi were obtained from the Microgarden fungal collection, University of California at Berkeley (*Rhizoctonia sp.*92-102*, Schizosaccharoyces pombe* 81-014, *Mortierella pusilla* C9*, Mucor rouxii* 81-064*, Chytridium confervae* 81-001). Purified DNA was obtained from the following sources at the University of California at Berkeley: the laboratory of Dr. Louise Glass (*Neurospora crassa*) and the laboratory of Dr. Thomas D. Bruns (*Lactarius xanthogalactus, Suillus sinuspaulianus, Tuber* sp./*Pinus muricata* mycorrhiza).

Plant culture

To test specific amplification from AM colonized roots, the following hosts were cultivated in combination with a single fungal species. Plants of *Plantago media* were grown for 3 months in a 8:2 mixture of sand and garden soil in SunBags (Sigma, St. Louis, Mo.). Seeds were inoculated with 10 spores of *G. clarum* or soil inoculum of *S. pellucida,* respectively, and watered with tap water through a syringe. *Sorghum bicolor* colonized by *A. gerdemannii* NC169 was cultured at INVAM (Morgantown, W.V.) under standard greenhouse conditions (Morton et al. 1993).

Samples of leaf tissue of plants for specificity testing were obtained from the Monterey Market (Berkeley, Calif.).

Root extraction and staining

Approximately 1–2 cm of colonized plant roots were chopped to 1-mm pieces with a razor blade. NaOH (20 µl of 0.25 M) was added immediately and the roots were transferred into a 0.5-ml Eppendorf tube and incubated at 90 °C for 10 min. After addition of 10 µl 0.5 M Tris HCl and 20 µl 0.25 N HCl, the extraction mixture was incubated for a further 10 min. The supernatant was separated from root fragments by centrifugation, diluted 1:100 in TE buffer and used as PCR template. Root fragments were acidified in 0.25 M HCl and stained with trypan blue using the procedure of Koske and Gemma (1989).

Polymerase chain reaction

PCR was performed as described by Redecker et al. (1997) to obtain PCR products for sequencing. $MgCl_2$ at 1.5 mM and AmpliTaq (Perkin Elmer, Foster City, Calif.) were used in the reactions. For specificity testing and amplification from roots, a two-step procedure (nested PCR) was conducted. The first amplification with the universal primers NS5 and ITS4 (White et al. 1990) was performed as described by Redecker et al. (1997) with an annealing temperature of 51 °C. Aliquots of 3 µl were run on an agarose gel to estimate the quantity of PCR product. When no PCR product was visible, the reactions were diluted 1:100. Where bands were visible, the reactions were diluted 1:1,000 or 1:10,000 for exceptionally strong bands. These dilutions were then used as template for the second PCR step.

The second step was conducted with various combinations (see below) of Glomales-specific primers, universal primers (White et al. 1990) and ITS1F, which is specific for fungi (Gardes and Bruns 1993). Annealing temperatures were 61 °C for 5 cycles, then 60 °C for 25 cycles. The PCR reactions were preheated to 61 °C during sample loading (hot start). In specificity tests, templates were tested with universal primers ITS1 and ITS4 to verify amplifiability. Specific primers (Fig. 1) were combined in two sets in the second amplification step to minimize the number of PCR reactions. Set 1 comprised the reverse specific primers (GLOM5.8R, GIGA5.8R) in combination with ITS1F. Set 2 contained the forward specific primers (ARCH1311, ACAU1660, LETC1670) combined with ITS4.

Reaction volumes of 12.5 µl were used to minimize reagent costs in this screening step. To obtain PCR products for restriction fragment length polymorphism (RFLP) analysis, positive samples were amplified in 25- or 50-µl reactions with the appropriate single specific primers. Instead of GLOM5.8R, the primer GLOM1310 was used because the PCR product of the former is too short for RFLP analysis.

Cloning and sequencing

PCR products were sequenced directly or cloned into pCR 2.1 with the Invitrogen TA Cloning Kit (Invitrogen, San Diego, Calif.) and then sequenced. Inserts were reamplified from clones using the primers of the original cloned PCR product by picking the bacterial colonies with a toothpick directly into the PCR reaction. Amplified fragments that were sequenced directly or reamplified from bacterial clones were purified with QIAquick (Quiagen, Hilden, Germany). A Prism Ready Reaction Dye Deoxy Termi-

nator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.) was used for labeling. Electrophoresis and data collection were performed on an ABI model 377 DNA Sequencer (Applied Biosystems). DNA Sequencing Analysis (version 2.01) and Sequence Navigator (version 1.01) were used for processing the raw data. Primers were designed using the sequences reported by Redecker et al. (1999, 2000a,b) and the following additional sequences: *A. laevis* (near-complete 18S subunit, accession number AJ250847), *A. morrowiae* (3′ end of 18S, AJ250848), *G. claroideum* BEG3 (3′ end of 18S, AJ250850), *Glomus* sp. S329 (3′ end of 18S, AJ250849), *G. intraradices* (ITS, T. Hurek, unpublished sequence). The origin of ITS sequences from the Glomalean genome was verified by including the 5.8S subunits into a data set comprising a wide variety of fungal taxa (Redecker et al. 1999).

Restriction analyses

Restriction analysis was performed by digesting 8 µl of PCR product in 15-µl reactions for 6 h at 37 °C. Fragment patterns were analyzed on agarose gels containing 2% NuSieve (FMC, Rockland, ME) and 1% Ultrapure Agarose (Gibco BRL, Grand Island, N.Y.) in Tris-acetate EDTA buffer at 150 mA. Gels were stained with ethidium bromide and documented with an Eagle Eye video system (Stratagene, La Jolla, Calif.). Restriction fragments were sized using the NCSA GelReader, version 2.0.5.

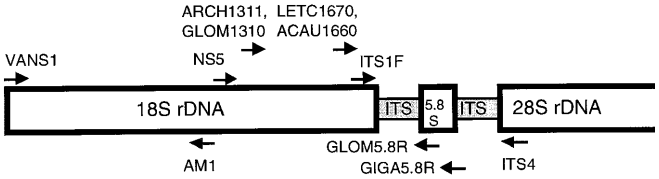

Fig. 1 Schematic representation of ribosomal RNA genes with annealing sites of primers. Diagram is not to scale

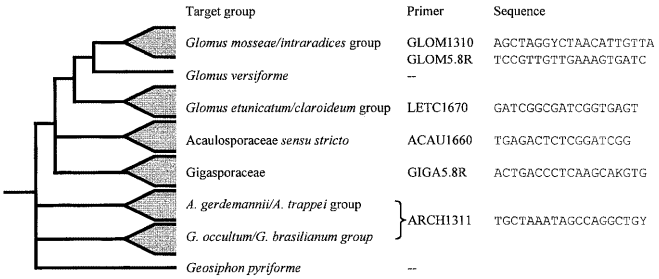

Fig. 2 The specific primers are targeted at phylogenetic groups within the Glomales. This simplified phylogenetic tree is based on studies by Redecker et al. (2000a,b)

Results

Primer design and specificity

Based on sequence data from 5.8S and 18S ribosomal RNA genes, primers were designed to specifically amplify DNA fragments including the ITS. Published sequences were included in these considerations. The relative positions of the annealing sites are depicted in Fig. 1. It was not possible to locate a single priming site from these sequences that was conserved across the Glomales and specific for these fungi.

Fig. 2 lists the primers, their sequences and the major phylogenetic groups of the Glomales at which they are targeted. Figures 3, 4 and 5 compare the sequences

Fig. 3 Priming sites of primers ARCH1311 and GLOM1310 in the 18S ribosomal subunit sequences of AM fungi and other organisms. Primers and priming sites in the target organisms are shown in boldface. Matches with the primer sequence of ARCH1311 are indicated by shading. Identity with the nine bases at the 3′ end of GLOM1310 is shown by white characters on a black background. Sequences were aligned for best display and not strictly by the criterion of homology

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ARCH1311                                  TGCTAAATAGCTAGGCTGY
Geosiphon pyriforme                       TAACCTGCTAAATAGCCAGGCCGCTTTGGCGGGTCGT
Glomus occultum HA771                     TAACCTGCTAAATAGCCAGGCTGTTCTGAACGGTCGT
Glomus occultum CL700                     TAACCTGCTAAATAGCCAGGCTGTTCTGAACGGTCGT
G. leptotichum/A. gerdemannii NC176       TAACCTGCTAAATAGCTAGGCTGCTCTGAACGGTCGT
Acaulospora trappei NB112                 TAACCTGCTAAATAGCTAGGCTGCTTCTNCGAGTGGT
Acaulospora trappei AU219                 TAACCTGCTAAATAGCTAGGCTGCTTCTNCGAGTGGT
Glomus etunicatum                         TAACCTGCTAAATAGTTAAACCTGATTTTTTATCAGG
GLOM1310                                        AGCTAGGCTTAACATTGTTA
Glomus intraradices                       TAACCTGCTAAATAGCTAGGCCTAACATTGTTA-GGT
Glomus sinuosum                           TAACCTGCTAAATAGCTAGGCCTAACATTGTTA-GGT
Glomus mosseae                            TAACCTGCTAAATAGCTAGGCTTAACATTGTTA-GGT
Glomus versiforme                         TAACCTGCTAAATAGTTAGGCGCTCCTTTCG-AGGGT
Acaulospora rugosa                        TAACCTGCTAAATAGTTAGTCCCTCTTTCCGGAGGGT
Acaulospora spinosa                       TAACCTGCTAAATAGTTAGGCACTCCTTCCGGAGGGT
Entrophospora colombiana                  TAACCTGCTAAATAGTTAGTTCCTCCTTCCGGAGGGT
Gigaspora albida                          TAACCTGCTAAATAGTCAGGCTATTCTTTTGAATGGT
Gigaspora gigantea                        TAACCTGCTAAATAGTCAGGCTATTCTTTTGAATGGT
Scutellospora dipapillosa                 TAACCTGCTAAATAGTCAGGCTATTC--TTGAATGGT
Scutellospora pellucida                   TAACCTGCTAAATAGTCAGGCTAATC--TTGAATGGT

Neurospora crassa                         TAACCTGCTAAATAGCCCGTATTGC-TTT-GGCAGTA
Schizosaccharomyces japonicus             TAACCTGCTAAATAGCTGGGTCAGC--TT--GCTGAT
Ustilago hordei                           TGACCTGCTAAATAGACGGGTTGACAATTT-GTTGGC
Russula compacta                          TAACCTGCTAAATAGCCTGGCCGGC-TCTT-GCTGGT
Mortierella polycephala                   TAACCTGCTAAATAGTTAGGCCAAC-GTTT-GTTGGT
Endogone pisiformis                       TAACCTGCTAAATAGTTAGGCCAAC-TTTG-GT-GGA
Mucor racemosus                           TATTCTGCTAAATAGGCAGGTCAACTTTTTAGTTGAT
Chytridium confervae                      TAATCTGCTAAATAGTTGCATTCAC-TTTG-GT-GGA
Homo sapiens                              TGGCATGCTAACTAGTTACGCGACCCCCGAGCGGTCG
Zea mays                                  CAGCCTGCTAACTAGCTATGCGGAGCCATCCCTCCAC
Pisum sativum                             CAGCCTGCTAAATAGCTATGTGGAGGTAACCCTCCAC
Pinus elliottii                           CAGCCTGCTAACTAGCTACGCGGAGGTTCCCCTTCGC
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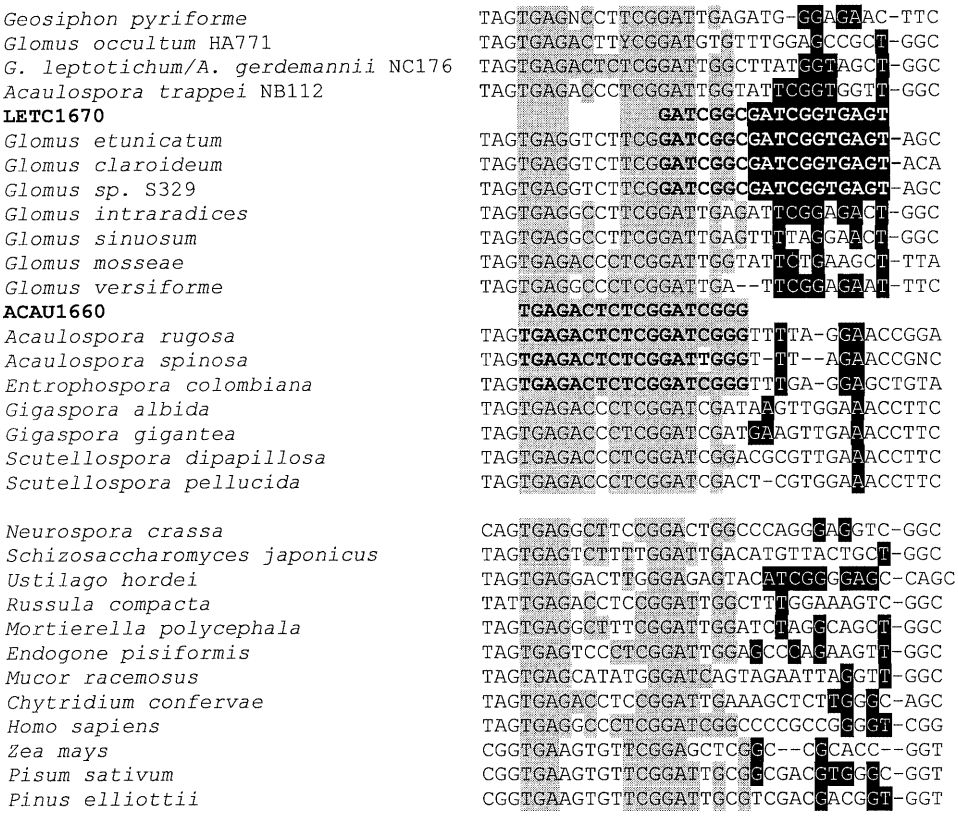

Fig. 4 Priming sites of primers LETC1670 and ACAU1660 in the 18S ribosomal subunit sequences of AM fungi and other organisms. Primers and priming sites in the target organisms are shown in boldface. Matches with ACAU1660 are indicated by shading. Identity with the 11 bases at the 3′ end of LETC1670 is shown by white characters on a black background. Sequences were aligned for best display and not strictly by the criterion of homology

of the respective priming sites from a broad range of organisms. The priming sites are well conserved within the target groups and are distinct from other groups at least at the 3′ ends of the primers. The C/T mismatch of *A. spinosa* in the ACAU1660 priming site (Fig. 4) does not prevent amplification under the conditions used (see Table 1). Neither does the mismatch of *S. pellucida* in the GIGA5.8R site (Fig. 5).

In addition to the sequences presented in Figs. 3, 4 and 5, the priming sites of the following taxa were examined and found to match the respective primer sequences: *A. laevis, A. morrowiae, E. contigua* (ACAU1660), *G. coremioides, G. clarum* (GLOM1310), *G. intraradices* (GLOM5.8R). Some taxa for which sequences were not available were tested by PCR only (Table 1). The specificity of the primers was assessed in a nested PCR system under the same conditions found useful for amplifying from roots.

The primers were found to be largely specific for their target groups (Table 1). Plant DNA is not expected to be amplified by PCR because the plant sequences are distinct at these priming sites. However, when the template concentration in the second amplification step was too high, primer specificity decreased and occasionally caused non-specific amplification. Other groups of Zygomycetes showed strongly divergent priming sites. Basidiomycetes were more similar than the Zygomycetes but could mostly be excluded by stringent amplification conditions.

Nested amplification from spores and colonized roots

A fragment (approximately 1200 bp) of the ribosomal DNA was amplified with universal primers NS5 and ITS4. In many cases, these PCR products were not visible when stained by ethidium bromide, but their concentrations were sufficient for amplification by nested PCR. In the second step, primers specific for the target organisms were used.

Combination of the primers in sets was found to be a useful approach to screen root samples for the presence of AM fungi with the minimal number of PCR reactions. In many cases, the size of the PCR product gave a first indication of the fungus present. However, when template for the primer ARCH1311 was present, additional bands were obtained with ACAU1660 and LETC1670 as a result of the high concentration of ARCH1311 product overcoming the specificity of the nested primer. Additional reactions with single primer pairs were performed to determine which of the primers showed a true positive reaction and to obtain sufficient products for subsequent restriction analysis.

In contrast to DNA extraction procedures which rely on complete destruction of the root tissue, in the protocol shown here the extracted root pieces could be recovered and stained with trypan blue (Fig. 6). This allowed visual assessment of the colonization status of the plant.

Primers ARCH1311, GLOM1310 and GIGA5.8R were tested with roots from single species cultures colo-

Table 1 Specificity of primers assayed by nested PCR. Reactions were counted positive when products of the expected size were consistently present. All forward primers were tested in combination with ITS 4, the reverse primers (R) with ITS1F (+ weak positive reactions)

	ARCH1311	ACAU1660	LETC1670	GLOM5.8R	GLOM1310	GIGA5.8R
AM fungi						
Acaulospora gerdemannii NC176	+	−	−	−	−	−
A. trappei NB112	+	−	−	−	−	−
Glomus occultum HA771	+	−	−	−	−	−
*G. brasilianum*WV219	+	−	−	−	−	−
A. laevis AU211-3	−	+	−	+	−	−
A. lacunosa BEG78	−	+	−	−	−	−
A. morrowiae BR226-1	−	+	−	−	−	−
A. mellea BR983-4	−	+	−	−	−	−
A. spinosa (CIAT)	−	+	−	−	−	−
E. colombiana C-18-3	−	+	−	+	−	−
G. etunicatum UT316	−	−	+	−	−	−
G. claroideum BEG88	−	−	+	−	−	−
G. geosporum BEG11	−	(+)	−	+	+	−
G. caledonium BEG20	-	(+)	−	+	+	−
G. clarum CL883	−	−	−	+	+	−
G. sinuosum MD126	−	−	−	+	+	−
G. coremioides (Biorize)	−	−	−	+	+	−
G. decipiens AU102-3	−	−	−	−	−	+
G. albida BR205-1	−	−	−	−	−	+
G. rosea FL105-5	−	−	−	−	−	+
S. heterogama BR154	−	−	−	−	−	+
S. pellucida C-139-3A	−	−	−	−	−	+
Controls						
Basidiomycetes:						
Lactarius xanthogalactus	(+)	−	−	−	−	−
Rhizoctonia sp.	−	−	−	−	−	−
Suillus sinuspaulianus	−	−	−	−	−	−
Ascomycetes:						
Schizosaccharomyces pombe	−	−	−	−	−	−
Neurospora crassa	(+)	−	−	−	−	−
Tuber sp./Pinus muricata	−	−	−	−	−	−
Zygomycetes:						
Mortierella pusilla	−	−	−	−	−	−
Mucor rouxii	−	−	−	−	−	−
Chytridiomycetes:						
Chytridium confervae	−	−	−	−	−	−
Dicotyledons:						
Plantago media	−	−	−	−	−	−
Eschscholzia californica	−	−	−	−	−	−
Petroselinum crispum	−	−	−	−	−	−
Glycine max	−	−	−	−	−	−
Monocotyledons:						
Hordeum vulgare	−	−	−	−	−	−
Allium cepa	−	−	−	−	−	−
Asparagus officinalis	−	−	−	−	−	−

nized by *A. gerdemannii*, *G. clarum and S. pellucida,* respectively.

Identification of AM fungi by RFLP analysis

PCR products from colonized roots or spores were analyzed by RFLP (Figs. 7, 8). Restriction fragment lengths for the AM fungi of the deeply divergent lineages are presented in Table 2. Using two restriction enzymes, the pattern variation was high enough to allow species identification and, in the case of *A. trappei,* to distinguish the two isolates tested. Restriction patterns of PCR fragments from roots colonized by *A. gerdemannii* NC169 matched the respective patterns obtained from spores. (Fig. 7).

Although GLOM5.8R can be combined conveniently with the other primers in the primer sets, the PCR product obtained with this primer was too short to allow efficient RFLP analysis. Moreover this primer showed some cross-reactivity with members of the Acaulosporaceae (Table 1). For these reasons, primer GLOM1310 was used instead to generate PCR products for restriction analysis. The RFLP patterns obtained from roots colonized by *G. clarum* CL883

Fig. 5 Annealing sites of GLOM5.8R and GIGA5.8R in the 5.8S ribosomal subunit. Matches with the primer sequences are indicated by shading. Priming sites in the target organisms and the reverse complement sequence of the primers are shown in boldface. Note that these primers amplify in the reverse direction. Their actual sequences are given in Fig. 2

matched the patterns obtained from the spores of the same isolate (Fig. 7).

Primer GIGA5.8R was successfully tested with roots of *P. media* colonized by *S. pellucida* (not shown). However, restriction fragment patterns of this PCR product were not variable enough to facilitate species or genus identification within the Gigasporaceae.

ITS from sporocarps of two species of the former genus *Sclerocystis* also can be amplified with GLOM1310. The two species were distinguished from each other with the enzyme *Dpn* II (Fig. 8). Each species from the Acaulosporaceae tested by PCR and restriction analysis from spores showed unique RFLP patterns with *Dpn* II (Fig. 8) and *Hin*f I (not shown).

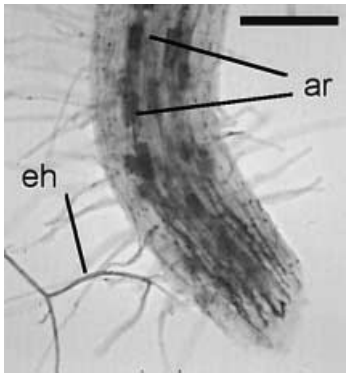

Fig. 6 Root piece of *Plantago media* colonized by *Glomus clarum* and stained with trypan blue after extraction for PCR; *bar* 0.25 mm (*ar* arbuscles, *eh* external hypha)

Discussion

The majority of DNA extracted from colonized roots is of plant origin and, therefore, specific PCR primers must be used in order to obtain fungal DNA fragments. Using universal primers that are functional with nearly

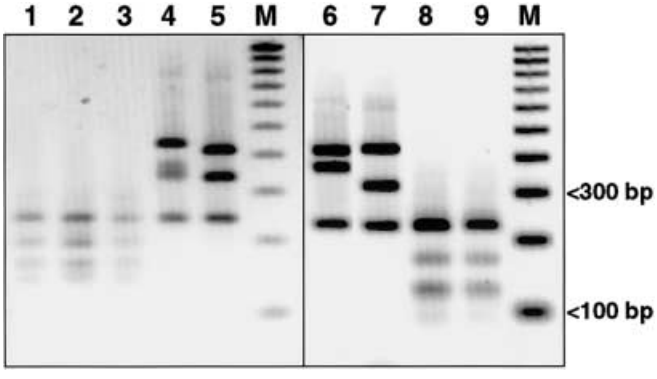

Fig. 7 Restriction patterns of PCR products from colonized roots match those obtained from spores of the respective AM fungus. Primer pairs ARCH1311/ITS4 (1–6) and GLOM1310/ITS4 (6–9) were used, respectively. *Hin*f I digests of PCR products from (1) roots colonized by *A. gerdemannii* NC169-3, (2) spore of *A. gerdemannii* NC176, (3) roots colonized by *A. gerdemannii* NC169-3 (same root sample as lane 1), (4) spores of *G. occultum*, (5) spores of *G. brasilianum*, (6) spore of *G. geosporum,* (7) sporocarp of *G. sinuosum*, (8) spore of *G. clarum*, (9) roots colonized by *G. clarum* (*M* 100-bp ladder)

Table 2 *Dpn*II and *Hin*fI restriction fragment lengths (in bp) of PCR products amplified from ITS, 5.8S subunits and 18S subunits of arbuscular mycorrhizal fungi from deeply divergent lineages. Primers ARCH1311 and ITS4 were used. *Numbers in italics* are apparent sizes derived from restriction patterns on agarose gels only; the others were confirmed from DNA sequences. Ranges of bands of similar size that could not be distinguished easily on gels are indicated by *hyphens*. Only bands larger than 80 bp are given

	*Dpn*II	*Hin*fI
Acaulospora gerdemannii/Glomus leptotichum NC176	*462, 403,* 171	*244,* 194, *165, 143,*
A. gerdemannii/G. leptotichum FL130	*460,* 419, *170*	*242,* 197, 164, 142
A. gerdemannii/G. leptotichum NC169-3(colonized roots)	*464, 417, 160*	*246, 201, 168, 143*
A. trappei NB112	457, 258, 178	240, 244, 211, 159, 145
A. trappei AU219	*463,* 409, 93	*416,* 329, *237*
G. occultum HA771	*460, 429, 157*	*442, 328–369, 241*
G. brasilianum WV219	*468, 431*	416, *331, 242*

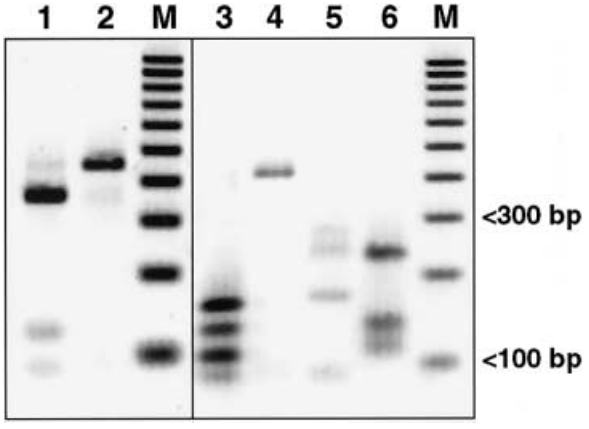

Fig. 8 Restriction patterns of species from the former genus *Sclerocystis* (lanes 1–2) and the Acaulosporaceae (lanes 3–6). Lanes 1 and 2: *G. sinuosum (Sclerocystis sinuosa*, lane 1) and *G. coremioides (Sclerocystis coremioides*, lane 2) could be distinguished by restriction analysis with *Dpn* II. Depicted are *Dpn* II digests of PCR products obtained from sporocarps with the primer pair GLOM1310/GLOM5.8R. Lanes 3–6: Species from the Acaulosporaceae tested show distinct restriction patterns. Depicted are *Dpn*II digests of PCR products obtained from spores with primers ACAU1660 and ITS4. (3) *E. colombiana,* (4) *A. morrowiae*, (5) *A. mellea*, (6) *A. laevis*(*M* 100-bp ladder)

all eukaryotes (Harney et al. 1997) does not appear to be a useful approach. I present a set of primers for five major subgroups of the Glomales that were designed by analyzing ribosomal DNA sequences under consideration of the phylogeny of AM fungal clades. The primers were shown to exclude plant DNA and the majority of other fungi. They allowed amplification of AM fungal rDNA from colonized roots and will be tested with root samples from the field in future projects.

Specific primers are also of great advantage for PCRs from spores. As the Glomales cannot be cultivated axenically, their spores harbor numerous other organisms (Clapp et al. 1999; Redecker et al. 1999; Walley and Germida 1996). My own experience shows that these frequently cause contamination in PCRs with universal or fungal-specific primers.

Plant roots or AM fungal spores may contain various compounds that inhibit PCR amplification. To avoid laborious DNA purification protocols, nested PCRs were employed successfully in a similar case (van Tuinen et al. 1998). In this approach, templates are subjected to two rounds of amplification. In the second step, the concentration of inhibitors is usually low enough not to interfere with the amplification of the minimal amounts of product obtained in the previous step. The present paper describes a DNA extraction that allows the subsequent visual examination of extracted roots fragments. Van Tuinen et al. (1998) in the reverse approach used already stained roots as PCR template to achieve a similar goal. Both protocols are useful for those members of the Glomales that stain with trypan blue.

The only known mycorrhizal clade of the Glomales not covered by the primer set presented here is *G. versiforme*. It would be possible to design a specific PCR primer for this lineage, but as no close relatives of this species are known, this does not appear to be useful at the present time.

Although different rDNA copies are indisputably present within single Glomalean spores (Lloyd MacGilp et al. 1996; Redecker et al. 1997; Sanders et al. 1995), ITS RFLP patterns are highly reproducible within one isolate. Multiple isolates of one species usually show identical or very similar patterns (Redecker et al. 1997; *A. gerdemannii* in this study). Divergent restriction patterns were found for two isolates of *A. trappei*. Whether this is due to divergent paralogues of the ITS or to unexpectedly large genetic variation within this species masked by the simple morphology needs further study. Recent claims of extremely divergent ITS within the genome of a single AM fungal isolate, which could potentially impair molecular identification (Hijri et al. 1999), could not be substantiated (Redecker et al. 1999).

The ITS of the Gigasporaceae have insufficient variability to allow identification by RFLP patterns (Redecker et al. 1997). However, DNA sequencing may help to assign samples to a genus or a species group. The diagnostic sequences for *Gigaspora* species groups reported by Bago et al. (1998) can be amplified by the specific primer GIGA5.8R in combination with the universal primer NS7. On the other hand, the ITS data

from the Acaulosporaceae presented in this study show much higher sequence variation within that group and clearly distinct RFLP patterns were obtained for the species tested.

AM fungi previously grouped in the genus *Sclerocystis* form a subgroup of the *G. mosseae/intraradices* clade (Redecker et al. 2000b). In the present study, it was shown that ITS sequences from *G. coremioides* and *G. sinuosum* can be amplified with the primers designed for that clade. Therefore, it is now possible to detect and identify fungi formerly known as "*Sclerocystis*". Ecology and symbiotic properties of these fungi have not been addressed thoroughly because of the difficulties in establishing pot cultures.

The characterization of several ancestral lineages of the Glomales (Redecker et al. 2000a) showed that previously published primer sequences for the Glomales (Helgason et al. 1998; Simon et al. 1992) are not appropriate to detect such deeply divergent clades. The intraradical structures of these fungi also do not stain with commonly used standard procedures. Thus, the occurrence of such fungi can only be elucidated with new molecular tools or novel stains. The first specific PCR primer ARCH1311 for these lineages presented in this study is of potential importance for future studies of AM populations in the field.

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