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Combining nested PCR and restriction digest of the internal transcribed spacer region to characterize arbuscular mycorrhizal fungi on roots from the field

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Abstract Identification of arbuscular mycorrhizal fungi (AMF) on roots is almost impossible with morphological methods and, due to the presence of contaminating fungi, it is also difficult with molecular biological techniques. To allow broad investigation of the population structure of AMF in the field, we have established a new method to selectively amplify the internal transcribed spacer (ITS) region of most AMF with a unique primer set. Based on available sequences of the rDNA, one primer pair specific for AMF and a few other fungal groups was designed and combined in a nested PCR with the already established primer pair ITS5/ITS4. Amplification from contaminating organisms was reduced by an *Alu*I restriction after the first reaction of the nested PCR. The method was assessed at five different field sites representing different types of habitats. Members of all major groups within the Glomeromycota (except Archaeosporaceae) were detected at the different sites. Gigasporaceae also proved detectable with the method based on cultivated strains.

Keywords *Alu*I restriction · Arbuscular mycorrhizal fungi · Internal transcribed spacer · Monitoring of arbuscular mycorrhiza · Nested PCR

Introduction

The fine roots of about 90% of the terrestrial plant species form mycorrhizas with soil fungi. These symbioses, which enhance both mobilization of water and nutrients and plant resistance to a broad range of stresses in ecosystems, are considered as ecologically obligatory (Smith and Read 1997). Of the different types of mycorrhizas, the arbuscular mycorrhiza (AM) is the most widespread. AM probably co-evolved with the first land plants (Heckman et al. 2001) and is formed by about 80% of all mycorrhizal plant species (Harley and Harley 1987). The mycobionts (fungal partners) of AM belong to approximately 150 taxa that were initially all placed in a specific order (Glomales) within the Zygomycota. A classification based on the morphology of asexual spores is classically used for their identification (Morton and Benny 1990). Attempts to infer their phylogeny and natural classification have been made using different molecular biological methods, and an improved system based on sequence analysis of the small subunit (SSU) in the rRNA genes has been proposed recently (Redecker et al. 2000; Schüßler et al. 2001; Schwarzott et al. 2001). According to this new phylogenetic research, the Glomales do not belong to the Zygomycota and can be unequivocally separated from all other major fungal taxa. To give these fungi their proper stage, a new phylum was proposed, the Glomeromycota (Schüßler et al. 2001).

Direct identification of AM mycobionts in the field is an important challenge for ecologists. There is experimental evidence that plant biodiversity and productivity in ecosystems are significantly influenced by AM fungal (AMF) diversity (van der Heijden et al. 1998). However, no easy and fully reliable method exists for the identification of AMF in field-collected roots up to now.

Unlike spores, AM themselves do not provide reliable typological criteria for identification at the species level due to their homogenous morphology and anatomy. However, spores only partially reflect the diversity of AMF involved in mycorrhization (Clapp et al. 1995; Merryweather and Fitter 1998). Although many single and nested PCR techniques have been developed to make AM identification easier (see Sanders et al. 1996; van Tuinen et al. 1998b), the low quantity of mycobiont DNA and the presence of contaminating microorganisms in AM structures do not usually allow the use of fungal-specific

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primers. In the genus *Scutellospora*, PCR with broad-spectrum fungal primers first led to the conclusion that the rDNA had an unusual polymorphism (Hijri et al. 1999; Hosny et al. 1999). Detailed analyses, however, revealed that the sequences originated from ascomycetes contaminating the spore material (Redecker et al. 1999; Schüßler 1999). Therefore, besides some attempts with broad-spectrum primers (see Discussion), most PCR-based identification techniques use more or less taxon-specific primers (Helgason et al. 1999; Jacquot et al. 2000; Redecker 2000; Turnau et al. 2001; van Tuinen et al. 1998a).

The present paper has its origin in attempts to monitor AMF diversity in the field. Preliminary investigations of the polymorphism of the internal transcribed spacer (ITS) in AM and in spores revealed high levels of contaminants in the PCR products. We optimized a method involving a nested PCR with a new primer pair specific for AMF and a few other fungal groups together with an intermediary restriction digest, in order to limit the amplification of contaminants from AM roots. A first assessment of this new technique at five different field sites is presented.

Material and methods

Field sites

AM roots or spores from the field used in this work all originated from fresh soil samples. All field sites are situated within Thuringia (Germany). Field site no. 1 is a grassland surrounding a former fertilizer factory located in the Central Saale Valley, 13 km north of Jena (11°40'51"E/51°00'46"N, elevation 150–180 m above sea level). The phosphorus content of this soil is high (between 26 and 121 g kg^{-1}; detailed description in Langer and Günther 2001). A calcareous forest, also situated in the Central Saale Valley 10 km north of Jena (11°39'25"E/50°59'31"N, elevation 150 m above sea level), was chosen as field site no. 2. Two mountain meadows in the Thüringer Schiefergebirge, one intensively and the other extensively farmed, were taken as field site no. 3 (intensively used meadow near Schlegel: 11°37'31"E/50°24'32"N, elevation 640 m above sea level) and 4 (extensively used meadow near Grumbach: 11°30'48"E/50°25'22"N, elevation 710 m above sea level). Site no. 5 was a fresh meadow near a stream in Friedmannsdorf (12°13'33"E/50°45'25"N, elevation 300 m above sea level). pH-values ranged from 5.5 at site no. 4 to 7.7 at site no. 1, i.e. acidic to alkaline conditions.

DNA extraction

Total plant root systems were removed from the field and fine roots were chosen randomly for DNA extraction as described by Redecker (2000). Single spores were separated in a drop of sterile water. The water was removed before spores were crushed, suspended in 8 µl of the PCR-mix (see below) and used directly for PCR. Material from cultures of *Glomus intraradices* (BEG 75, BEG 140) and *Gigaspora rosea* (BEG 9) was used as references.

PCR conditions

Amplification of the ITS region by PCR was performed on a Hybaid Ltd. OmniGene TR3 CM220 Thermo Cycler (MWG-Biotech, Ebersberg, Germany) in a total volume of 50 µl containing 2 U Taq DNA polymerase (Promega, Heidelberg, Germany), 5 µl of 10× Taq polymerase reaction buffer (Promega), 4 µl 25 mM $MgCl_2$, 10 nmol of each dNTP (MBI-Fermentas, St. Leon-Rot, Germany), 50 pmol of each of the two primers and 100–500 ng of the genomic DNA. The reactions were performed as hot start PCR with 10 min initial denaturation at 94°C before adding the Taq polymerase at 80°C. The PCR program comprised 40 cycles (40 s at 94°C, 30 s at 54°C, 40 s at 72°C) when working with single spores, and 32 cycles otherwise. A final elongation of 10 min at 72°C followed the last cycle.

In the first reaction of nested PCR, the newly designed primer pair SSU-Glom1/LSU-Glom1 (see Results) was used, while the second step of the PCR was performed with the primers ITS5/ITS4 (White et al. 1990).

Restriction digest between the two reactions of the nested PCR

In order to avoid amplification of ITS regions containing the *Alu*I restriction site, 5 µl of the PCR products obtained after the first reaction of the nested PCR were digested for 1 h in a total volume of 20 µl with 5 U of *Alu*I (MBI Fermentas, St. Leon-Rot, Germany). DNA was precipitated with 50 µl of 100% ethanol and stored on ice for a minimum of 1 h. Samples were centrifuged at 20,000 *g* for 10 min. The supernatant was discarded, the pellets were dried for 30 min at 50°C and redissolved in 10 µl of demineralized water. A 1 µl aliquot of this DNA template was used for the second reaction of the nested PCR.

Cloning, sequencing and sequence analyses

PCR products were cloned into the pCR4-Topo Vector following the manufacturer's protocol of the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany) and transformed into TOP10 Chemically Competent *Escherichia coli*. Sequencing was done on a LI-COR DNA Sequencer Long Reader 4200 using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, UK).

DNA sequences of the full nrITS sequences were submitted to the EMBL database under the accession numbers given in Fig. 1 as bold type. An attempt to align the ITS1 and ITS2 sequences of all these data was unsuccessful due to the high variation within the dataset. In accordance, only the 5.8S subunit genes embedded between the ITS1 and ITS2 regions were aligned by hand. Sequences from all families within the Glomeromycota taken from GenBank were also included to show the systematic position of the new sequences. Thus, the 5.8S dataset we used consisted of sequences of 4 Basidiomycota and 62 glomalean fungi (32 sequences obtained in this study and 30 sequences published previously by others) with 158 sites. Trees were rooted with sequences of *Endogone pisiformis* and *Mortierella alpina*, members of the Zygomycota.

Phylogenetic trees (Fig. 1) were inferred using distance criteria as implemented in PAUP* 4.0b8 (Swofford 2000). The confidence of branching was assessed using 1000 bootstrap resamplings in distance analysis (neighbor joining method).

Results

Preliminary assays and selection of the primer pair ITS5/ITS4

In preliminary assays, the primer pairs ITS1/ITS4 and ITS5/ITS4 were assessed in single PCRs on material from cultures or field site no. 1. ITS1/ITS4 amplified DNA from only 45% of the tested single spores from cultures and 36% of single spores from the field, while corre-

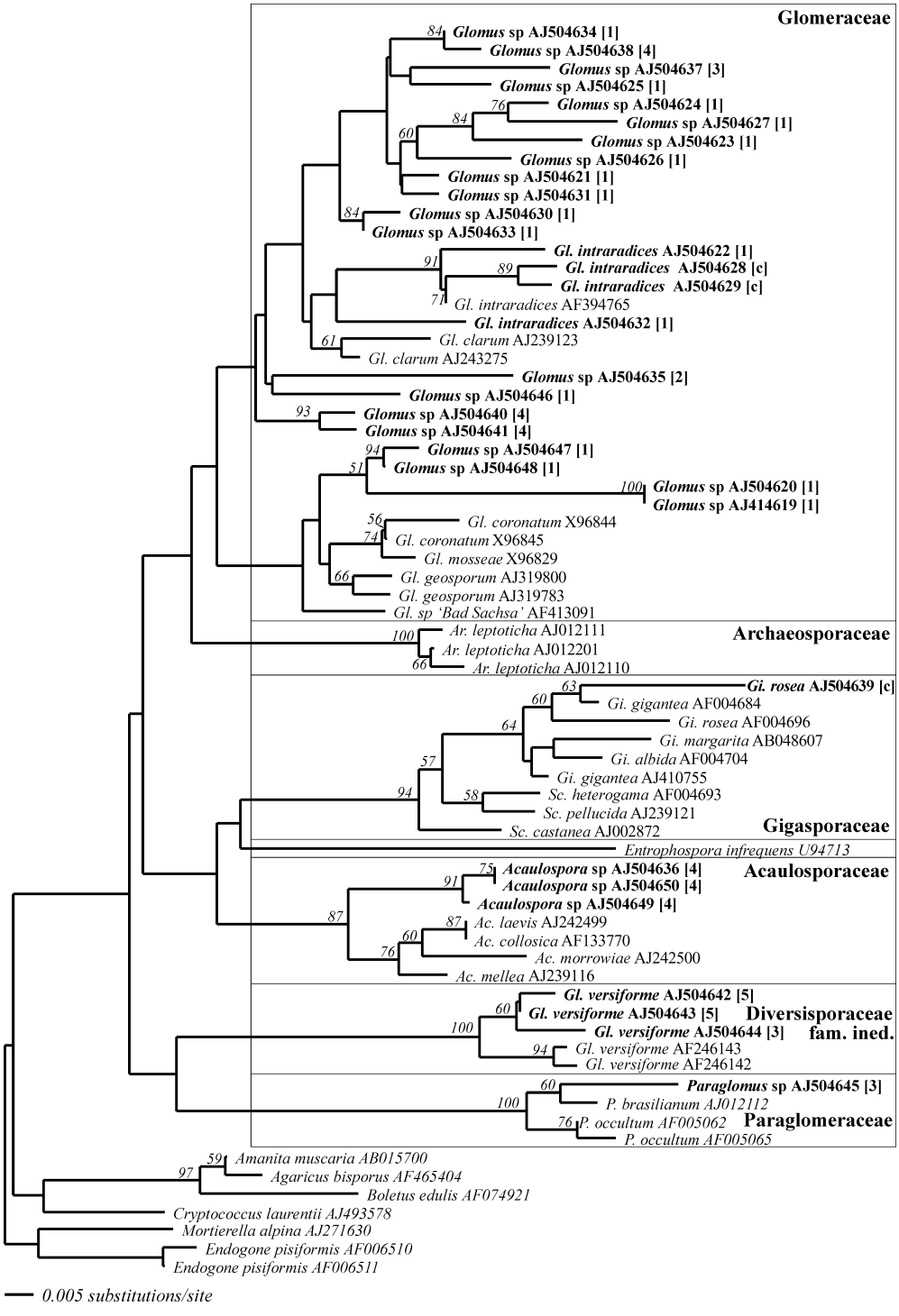

Fig. 1 Phylogenetic tree of 5.8S rDNA sequences obtained by distance analysis with the neighbor joining method. *Endogone pisiformis* and *Mortierella alpina* were used as outgroups. Bootstrap values (>50%) were determined for neighbor joining (1000 resamplings). Sequences that were not previously published are given in *bold*. The *numbers in brackets* refer to the field sites, which are all located within Thuringia (Germany). [1]: Phosphorus-contaminated grassland in the Central Saale Valley 13 km north of Jena, [2]: Calcareous forest in the Central Saale Valley 10 km north of Jena, [3]: Intensively farmed mountain meadow in the "Thüringer Schiefergebirge", [4]: Extensively farmed mountain meadow in the "Thüringer Schiefergebirge", [5]: Fresh meadow near a stream in Friedmannsdorf, [c]: Material taken from reference cultures

sponding success rates of 83% and 70% were obtained with ITS5/ITS4. Additionally, about 90% of the amplification products with both primer pairs represented AMF in the case of spores from cultures, but the respective proportions with spores from the field were only 53% with ITS5/ITS4 and 23% with ITS1/ITS4. Single PCRs on roots with both primer sets were not successful. Thus, ITS5/ITS4 appeared convenient to routinely identify spores from the field and was selected as one primer pair for a nested PCR to be optimized for roots.

Development of the primer pair SSU-Glom1/LSU-Glom1 for the first reaction of the nested PCR

For the nested PCR, the primers of the second pair had to anneal outside the highly variable ITS in order to be

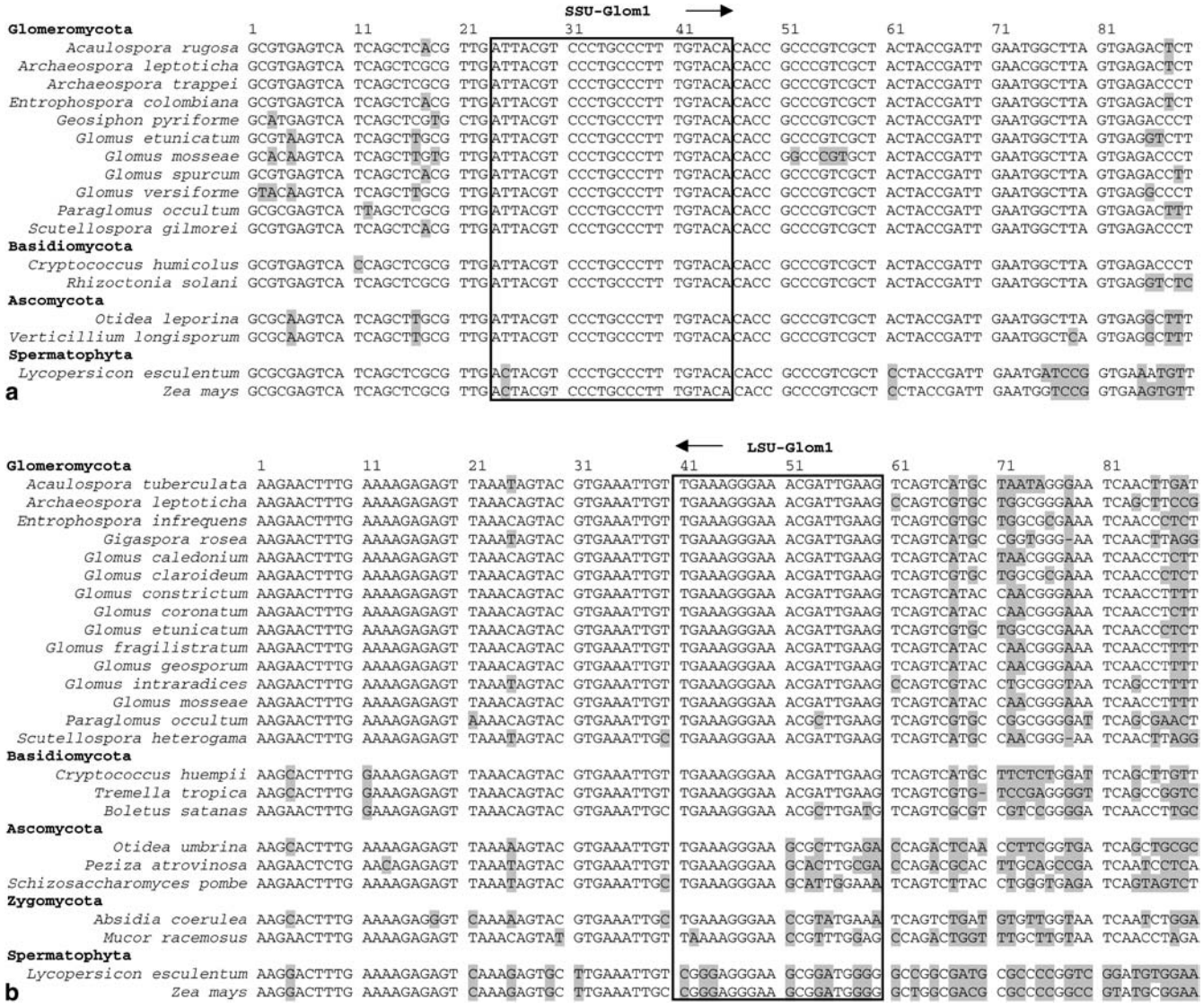

Fig. 2. a Alignment of a part of the 18S ribosomal DNA sequences of AM and other fungi. Priming site of the primer SSU-Glom1 given in the *box*. *Grey shaded letters* mark the deviations from the consensus. Sequence data derived from GenBank, representing parts of sequences given under the following accession numbers: *Ac. rugosa* (Z14005), *Ar. leptoticha* (AJ012201), *Ar. trappei* (AJ243420), *E. colombiana* (Z14006), *Ge. pyriforme* (X86686), *G. etunicatum* (Z14008), *G. mosseae* (Z14007), *G. spurcum* (Y17650), *G. versiforme* (X86687), *P. occultum* (AJ276082), *S. gilmorei* (AJ276094), *C. humicolus* (AB035584), *R. solani* (D85643), *O. leporina* (U53381), *V. longisporum* (AF153421), *L. esculentum* (X51576, X07889, X13828), *Z. mays* (AF168884). **b** Alignment of a part of the 28S ribosomal DNA sequences of AM and other fungi. Priming site of the primer LSU-Glom1 given in the *box*. *Grey shaded letters* mark the deviations from the consensus. Sequence data derived from GenBank, representing parts of sequences given under the following accession numbers: *Ac. tuberculata* (AF378440), *Ar. leptoticha* (AJ271712), *E. infrequens* (AF378506), *Gi. rosea* (AF378454), *G. caledonium* (AF145746), *G. claroideum* (AJ271928), *G. constrictum* (AF304977), *G. coronatum* (AF304871), *G. etunicatum* (AF145749), *G. fragilistratum* (AF145747), *G. geosporum* (AF305000), *G. intraradices* (X99640), *G. mosseae* (AF145735), *P. occultum* (AJ271713), *S. heterogama* (AF378448), *C. huempii* (AF189844), *T. tropica* (AF042251), *B. satanas* (AF071528), *O. umbrina* (AF086584), *P. atrovinosa* (U40613), *Sch. pombe* (Z19578), *Ab. coerulea* (AF113443), *M. racemosus* (AJ271061), *L. esculentum* (X13557), *Z. mays* (AJ309824)

compatible with all Glomeromycota. Therefore, they were designed within the genes encoding for the ribosomal small and large subunits (SSU and LSU). A further prerequisite was that at least one primer of this pair should be as specific as possible for AMF.

Sequence data from the SSU and LSU genes of different taxa in the Glomeromycota and other fungal groups were aligned. Fig. 2 gives a representative selection from these alignments. Two entirely conserved regions of about 30 bases each were found in the SSU region of the Glomeromycota (Fig. 2a). The primer SSU-Glom1 (ATT ACG TCC CTG CCC TTT GTA CA) was designed according to one of these highly conserved regions to amplify toward the 3' sense. Its 3' end is located 112 bp (tested with *Glomus versiforme* X86687) to 114 bp (tested with *Archaeospora leptoticha* AJ012201 and *Geosiphon*

Table 1 Representative species of different non-glomalean fungal orders containing the SSU-Glom1 primer site

Species	Order	Accession
Zygomycota		
Basidiobolus haptosporus	Entomophthorales	AF113413
Endogone pisiformis	Endogonales	X58724
Mortierella alpina	Mucorales	AJ271630
Umbelopsis isabellina	Mucorales	AF157166
Ascomycota		
Cyttaria darwinii	Cyttariales	U53369
Coccodinium bartschii	Dothideales	U77668
Sphaerotheca cucurbitae	Erysiphales	AB033482
Bulgaria inquinans	Helotiales	AJ224362
Hypogymnia physodes	Lecanorales	AF117984
Pseudogymnoascus roseus	Onygenales	AB015778
Lepolichen coccophorus	Pertusariales	AF274110
Morchella esculenta	Pezizales	U42642
Otidea leporina	Pezizales	U53381
Protomyces macrosporus	Protomycetales	D85143
Taphrina deformans	Taphrinales	U00971
Umbilicaria subglabra	Umbilicariales	AF088253
Saitoella complicata	Mitosporic Ascomycota	D12530
Basidiomycota		
Heliocybe sulcata	Aphyllophorales	AF334915
Agaricus bisporus	Agaricales	L36658
Lepiota procera	Agaricales	L36659
Hobsonia mirabilis	Atractiellales	AF289663
Boletus satanas	Boletales	M94337
Clavariadelphus pistillaris	Cantharellales	AF026639
Heterotextus alpinus	Dacrymycetales	L22259
Tilletiopsis lilacina	Entylomatales	AB023414
Dentipellis separans	Hericiales	AF334911
Bovista pusilla	Lycoperdales	AJ237858
Crucibulum laeve	Nidulariales	AF026624
Gloeophyllum sepiarium	Poriales	AF026608
Scleroderma citrinum	Sclerodermatales	AF026621
Cymatoderma caperatum	Stereales	AF082849
Asterotremella parasitica	Tremellales	AF231709
Chytridiomycota		
Monoblepharis hypogyna	Monoblepharidales	AF164334
Neocallimastix frontalis	Neocallimasticales	X80341
Olpidium brassicae	Spizellomycetales	Y12830

Table 2 Representative species of different non-glomalean fungal orders with (+) or without (-) the LSU-Glom1 primer site

Species	Order	Accession	LSU site
Zygomycota			
Basidiobolus ranarum	Entomophthorales	AF113452	–
Genistelloides hibernus	Harpellales	AF031062	–
Spiromyces minutus	Kickxellales	AF031070	–
Cunninghamella elegans	Mucorales	AF113460	–
Ascomycota			
Aspergillus avenaceus	Mitosporic Ascomycota	U15500	–
Botryotinia convoluta	Leotiales	Z81401	–
Cladobotryum apiculatum	Hypocreales	AF213025	–
Trapeliopsis percrenata	Lecanorales	AF279302	–
Amauroascus albicans	Onygenales	U17914	–
Stictis radiata	Ostropales	AF356663	–
Morchella elata	Pezizales	U42667	–
Chaetomnium piluliferum	Sordariales	AF286406	–
Taphrina deformans	Taphrinales	AJ276064	–
Basidiomycota			
Leucoagaricus naucinus	Agaricales	U11921	–
Bolbitius vitellinus	Agaricales	U11913	–
Agaricus arvensis	Agaricales	U11910	–
Datronia mollis	Aphyllophorales	AF393052	+
Exidia saccharina	Auriculariales	AF291323	+
Hygrophoropsis aurantiaca	Boletales	AF042007	–
Boletus satanas	Boletales	AF071528	–
Cystofilobasidium infirmo-miniatum	Cystofilobasidiales	AF075505	+
Melanogaster broomeianus	Melanogastrales	AF098383	-
Merulius tremellosus	Stereales	AF291346	+
Tremella moriformis	Tremellales	AF291374	+
Cryptococcus curvatus	Trichosporonales	AF374618	+
Chytridiomycota			
Blastcladiella emersonii	Blastocladiales	X90411	–

pyriforme AJ276074) upstream of the 5' end of the ITS5 primer site. A comparison with available sequences of the SSU genes from representative fungi of different orders suggested that this primer is almost universal for fungi (Table 1). Even the rRNA genes of most Spermatophyta, and those of the AM model plants *Lycopersicon esculentum*, *Medigaco truncatula* and *Zea mays*, only differ in the second position from the SSU1 primer sequence, where thymine is replaced by cytosine (Fig. 2a). Although this high sequence identity could cause problems in the PCR of AM roots, no band of plant origin was obtained with SSU1, because it was combined in the nested PCR with a second primer with narrower specificity.

Between 355 bp (*Glomus intraradices* AJ416418) and 359 bp (*Glomus geosporum* AF145743) from the 3' end of the ITS4 primer annealing site, a well-conserved region of 65 bp was found within the LSU (Fig. 2b). The LSU-Glom1 primer (CTT CAA TCG TTT CCC TTT CA) was designed for this region, in order to be as specific as possible for Glomeromycota and to amplify in the 5' sense. Screening of available sequences of different fungal orders indicated that this primer is also compatible with some basal groups of Basidiomycota, including the Aphyllophorales, Auriculariales, Stereales, Tremellales and Trichosporonales, but should not amplify most higher Basidiomycota, like Agaricales, Boletales, or most Asco- and Zygomycota (Table 2).

Selection of a restriction enzyme specifically not cutting the ITS region of the Glomeromycota

In order to reduce the amplification of DNA from fungi contaminating AM roots, a restriction enzyme cutting the ITS of many fungi but not of Glomeromycota was selected. The intention was to eliminate most PCR products of contaminants obtained after the first reaction of the nested PCR despite the selectivity of the LSU-

Table 3 Presence (+) and absence (-) of *Alu*I restriction sites in the internal transcribed spacer (ITS) region of members of the Glomeromycota (data taken from GenBank)

Species	Accession	*Alu*I site + / –
Acaulospora collosica	AF320635	0 / 1
Acaulospora denticulata	AJ239115	0 / 1
Acaulospora laevis	AJ242499	0 / 1
Acaulospora mellea	AJ239116	0 / 1
Acaulospora morrowiae	AJ242500	0 / 1
Archaeospora gerdemannii	AJ012111	0 / 1
Archaeospora leptoticha	AB048630, AB048681, AB048663, AJ012201 AB048632, AB048673, AJ271712, AJ012109	6 / 2
Archaeospora trappei	AJ243419, AJ243420	0 / 2
Entrophospora colombiana	AJ239117	0 / 1
Entrophospora infrequens	U94713	0 / 1
Gigaspora albida	AF004702, AJ239118	0 / 2
Gigaspora decipiens	AJ239119	0 / 1
Gigaspora gigantea	AF004685	0 / 1
Gigaspora margarita	AF162453-AF162471, AB048607	1 / 9
Gigaspora rosea	AF004696, AF004700	0 / 2
Glomus claroideum	AF004687, U94715	0 / 2
Glomus clarum	AJ239123	0 / 1
Glomus coronatum	X96844	0 / 1
Glomus dimorphicum	X96838	0 / 1
Glomus etunicatum	AF004680, AJ239125, AF004682, U94711, U94712	0 / 5
Glomus fasciculatum	X96842	0 / 1
Glomus fistulosum	AJ239126	0 / 1
Glomus geosporum	AF197918, AJ239122, AF231469	1 / 2
Glomus intraradices	AF185648, AF185652, AF185659, AF185661 AF185663, AF185669, AF185674, AF185675 AF197917, AF197919	2 / 8
Glomus monosporum	AF125195, AF004689	0 / 2
Glomus mosseae	AF161043-AF161064,AF166276, X84232 X96829, X96830, X96833, X96836	0 / 28
Paraglomus brasilianum	AF165918, AF165922, AJ012112	3 / 0
Paraglomus occultum	AF004675, AF004677, AF005062, AF005065 AF165918, AF165920, AJ012113, U81987	8 / 0
Scutellospora castanea	AJ002872	0 / 1
Scutellospora cerradensis	AB048683	0 / 1
Scutellospora heterogama	AF004692, AJ239120	0 / 2
Scutellospora pellucida	AJ239121	0 / 1

Glom1 primer. This was done by a restriction digest before the second reaction with the ITS5/ITS4 primer pair. Of six restriction enzymes tested (*Alu*I, *Bsu*RI, *Eco*RI, *Hin*fI, *Msp*I, *Taq*I), *Alu*I appeared to be the only one fulfilling the requirements. A search among ITS sequences available in GenBank (http://www.ncbi.nlm.nih.gov/) indicated that for most members of the Glomeromycota, the ITS displays no restriction site for *Alu*I (Table 3), whereas restriction sites are very common in other fungal groups (data not shown). However, potential *Alu*I restriction sites were detected in some single sequences within AMF species (Table 3). Additionally, the ITS region of the phylogenetically isolated species belonging to *Paraglomus* appeared to systematically contain *Alu*I restriction sites (Table 3).

Assessment of the method in the field

With root material from the five field sites, the nested PCR with restriction by *Alu*I led to proportions of PCR products corresponding to AMF varying between 40 and 90% according to the site and the sampling season. The rate was constantly about 90% for roots from sites 3 and 4 (mountain meadows). On the most difficult site (no. 1, postindustrial grassland), rates of 70–80% were obtained only between April and August, while in early spring, autumn and winter, rates decreased to about 40%.

Of the contaminants, 48% corresponded to yeasts in the Tremellales (Basidiomycota), 8% to members of the Aphyllophorales, Auriculariales or Cystofilobasidiales, 10% to Agaricales and 8, 7 and 3% to the ascomycetous orders Hypocreales, Helotiales and Pleosporales, respectively. The residual 16% of the contaminants belonged to further fungal orders within the Asco- and Zygomycota. DNA from plants was never amplified.

Of all 120 AMF sequences obtained, 32 representatives of the whole diversity were used in a neighbor joining analysis of the 5.8S rDNA region (Fig. 1). This showed that members of almost all families of the Glomeromycota (i.e. Acaulosporaceae, Gigasporaceae, Glomeraceae, Diversisporaceae fam. ined. and Paraglomeraceae) except the Archaeosporaceae were detected with the developed technique (Fig. 1). The highest diversity and also the highest number of PCR products were found within the Glomeraceae (so far exclusively taxa of *Glomus* group A as designated by Schwarzott et al. 2001 and Schüßler et al. 2001). Members of this group

were detected at four of the five investigated sites (no. 1–4). Sequences within the other families were only detected so far at one or at most two field sites (for example, *Glomus versiforme* at site 3 and 5). The mountain meadows (sites 3 and 4) displayed the highest diversity, with AMF from the Glomeraceae, Acaulosporaceae, Diversisporaceae fam. ined. and even Paraglomeraceae (Fig. 1, see Discussion). No Gigasporaceae were detected in the field, but an assessment with culture material of *Gigaspora rosea* (BEG 9) indicated that the technique also works in this group.

Discussion

Nested PCR in the rDNA region is a powerful tool to directly identify AMF in roots (Jacquot et al. 2000; Kjøller and Rosendahl 2000; Turnau et al. 2001; van Tuinen et al. 1998a). In most of the reports cited, amplification of DNA from contaminant fungi was limited by using primer sets with narrow specificity, which only allowed monitoring of one species group of the Glomeromycota at a time. Redecker (2000), therefore, proposed the use of mixed sets of specific primer pairs to enlarge the number of registered AM species within each experiment. However, even in this case, analysis of AM diversity in the field remains limited; in particular, new sequence-types might be not detected. Other authors tried to use single primer sets with broad specificity to amplify AMF DNA from roots and met the following difficulties: the VANS1 primer excludes many Glomeromycota (Clapp et al. 1995; Sanders et al. 1996), and the AM1 primer fails to amplify the deeply-branching Glomeromycota (Helgason et al. 1999; Daniell et al. 2001). More universal fungal primers (e.g. MH2/MH4) increase the number of contaminating fungi recorded (Tonin et al. 2001).

Within the rDNA, the non-coding ITS displays a higher polymorphism than the SSU and LSU (White et al. 1990). It is routinely used to identify ectomycorrhizas (Buscot et al. 2000) and was demonstrated to be also adequate for AMF identification (Lloyd-MacGilp et al. 1996; Redecker 2000; Redecker et al. 1997). ITS was chosen as the target region in the present study.

Based on the preliminary assays comprising sequence data based on the use of either ITS5/ITS4 or ITS1/ITS4 for the PCR, ITS5/ITS4 rather than ITS1/ITS4 were selected as primers for the second reaction of the nested PCR. Most studies of ITS on AM spores have used ITS1/ITS4 (Antoniolli et al. 2000; Hildebrandt et al. 2001; Lloyd-MacGilp et al. 1996), but good results with ITS5/ITS4 have also been reported (Hildebrandt et al. 1999). The new primer pair SSU-Glom1/LSU-Glom1, developed here for the first reaction of the nested PCR, illustrates the difficulty in designing PCR primers amplifying target regions in the rDNA from all Glomeromycota, while at the same time excluding all other fungi. LSU-Glom1 was designed to be compatible with all Glomeromycota so far known. It was expected to largely exclude the Zygo- and Ascomycota but to display compatibility with some orders in the Basidiomycota. As it appeared impossible to amplify exclusively members of the Glomeromycota, we tried to eliminate contaminating fungi by a restriction digest with *Alu*I between the two reactions of the nested PCR. One expected inconvenience of this step was the elimination of the Paraglomeraceae and single strains in other groups of the Glomeromycota, without the total elimination of contaminants.

Despite these limitations, assessment of the combined nested PCR with intermediary *Alu*I restriction digest on root material from different field sites gave very satisfying results. To our knowledge, direct detection of such a wide range of AMF ITS sequences on roots using a single primer set and a contamination rate fully acceptable for work on field material has been reported rarely so far. In most of our analyses, less than 20% of the amplification products corresponded to contaminants. At several times of the year, higher rates were noted at field site 1. This could be related to the ecological disturbance of this post-industrial soil: Langer and Günther (2001) found that low soil biomass coupled with high microbial activities at this site were correlated with the contamination level. As a possible consequence, AM spores were also at low density and had a short turnover at this site (data not shown). Besides the spectrum of the AMF detected, it is noteworthy that the method gave good results with very diverse types of ecosystems, with vegetation ranging from forest to grassland, soil pH varying from 5.5 to 7.7, and with variable levels of nutrient richness or even of pollution.

Due to the presence of an *Alu*I restriction site in their ITS, members of the Paraglomeraceae should not have been detected with this technique. Nevertheless, one sequence obtained matched this group. As this sequence displayed a restriction site for *Alu*I, the intermediary restriction digest obviously failed in this case. However, as expected from the data set in Table 3, most contaminants did belong to basal Basidiomycota lineages without an *Alu*I site in their ITS. Thus, even though it is not completely efficient, the restriction digest eliminated many contaminants. In this context, the fact remains that the restriction digest eliminates most Paraglomeraceae. This real inconvenience is balanced by the fact that this group is limited to two species so far. In studies focusing on this group, the specific primers proposed by Millner et al. (2001) can be used to complete the species spectrum. For the other groups within Glomeromycota, restriction by *Alu*I will surely eliminate some strains. However, as shown in Table 3, *Alu*I sites are rare in the ITS of Glomeromycota.

It is worth noting that the method revealed sequences from most recognized families, in particular Glomeraceae, Acaulosporaceae, Diversisporaceae fam. ined. (former *Glomus* group C), Gigasporaceae and Paraglomeraceae.

The ITS is not adequate to phylogenetically reconstruct the phylum Glomeromycota. Nevertheless, in accordance with Redecker et al. (1999), the topology of the 5.8S neighbor joining tree (Fig. 1) allowed the separation of all major groups as given by Schwarzott et al. (2001) and Schüssler et al. (2001). Besides this, the

whole ITS gives access to fine population analyses, due to the high level of variability in the ITS1 and ITS2 (e.g. Lloyd-MacGilp et al. 1996).

In conclusion, even though not resolving all difficulties, the presented method represents significant progress towards molecular tools to monitor AMF diversity in the field.

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