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# A taxon-specific oligonucleotide probe for temperate zone soil isolates of *Glomus mosseae*

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**Abstract** The 5.8 S subunit and flanking internal transcribed spacer (ITS) regions in nuclear ribosomal DNA (rDNA) from spores of *Glomus mosseae* FL156 and UK118 were amplified by polymerase chain reaction (PCR) using ITS1 and ITS4 as primers. The amplification product from template DNA of UK118 was cloned and sequenced (569 bp); the amplified DNA from FL156 was sequenced directly (582 bp). There was a 95% sequence similarity between DNAs amplified from the two isolates; in contrast, major dissimilarities with partial sequences of seven other glomalean taxa were observed. Four oligonucleotide sequences unique to *Glomus mosseae* were identified as potential primers. Their specificity to *Glomus mosseae* was assessed by PCR amplification of genomic DNA from spores from 36 glomalean fungi: 13 isolates of *Glomus mosseae*, two *Glomus monosporum*, 10 other *Glomus* isolates, and 11 other glomalean taxa from each of four other genera. The *Glomus mosseae* isolates were from a broad range of temperate zone agricultural soils. Oligonucleotide pair GMOS1:GMOS2 primed specific amplification of an oligonucleotide sequence (approximately 400 bp) present in all *Glomus mosseae* isolates and two isolates of the closely related *Glomus monosporum*. This primer pair did not prime PCR when the template consisted of DNA from any of the other glomalean fungi or any of the nonmycorrhizal controls. In addition, a *24*-mer oligonucleotide, designated GMOS5, hybridized with *Glomus mosseae* and *Glomus monosporum* DNA amplified by PCR using primer pairs ITS1:ITS4 and GMOS1:GMOS2. Colony-blot assays showed that GMOS5 hybridized to 100% and 97% of *E. coli* pUC19 clones of amplification products from *Glomus mosseae* FL156 and UK118 DNA templates, respectively, indicating that nearly all clones contained an homologous sequence. GMOS5 was used successfully to detect specifically *Glomus mosseae* in DNA extracted from colonized sudan grass (*Sorghum sudanense* L.) roots and amplified by PCR using the primer pair GMOS1:GMOS2. The results confirm several previous indications that *Glomus mosseae* and *Glomus monosporum* are indistinguishable taxonomic entities.

**Key words** Arbuscular mycorrhizal fungi · Internal transcribed spacer · Ribosomal DNA

**Supplementary material** Additional documentary material has been deposed in electronic form and can be obtained from: http://link.springer.de/journals/myco/

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## Introduction

Recent studies have shown the benefit of arbuscular mycorrhizal fungi (AMF) in sustainable crop and land management systems, especially through their contribution of hyphal networks and exudates to the formation of water-stable aggregates (Miller and Jastrow 1992; Tisdall 1991; Wright and Millner 1994; Wright and Upadhyaya 1996; Wright et al. 1996). However, biodiversity and other ecological studies of AMF have been limited by difficulties in the identification of the taxa and isolates. Although hyphae are the most physiologically active states of the fungal part of the symbiosis, hyphal morphology is inadequate for identification at the taxonomic levels needed in most studies. Traditionally, spore characteristics, abundance, and root length colonized during host growth have been the major means for identification and measurement of AMF response to environmental factors. Relatively large spore counts associated with some taxa very likely indicate simply a propensity to sporulate abundantly, rather

than any inherently greater capacity to colonize roots and exert their activity in the rhizosphere.

Research reported here was directed toward the construction of taxon-specific oligonucleotides (TSOs) for detection of *Glomus mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe in roots collected from temperate agricultural soils. We envision that such TSOs, along with those constructed for other taxa, will facilitate studies of AMF biodiversity, colonization sequence, and ecology in response to agroecosystem management practices.

Recent development and use of molecular tools for detection, identification, and quantitation of AMF as a group of root symbionts (Simon et al. 1992a, b, 1993a, b; Wyss and Bonfante 1993; Bonito et al. 1995) has been followed by evidence that DNA polymorphisms occur among and within spores of individual isolates of glomalean fungi (Sanders et al. 1995; Zézé et al. 1997). The latter potentially complicates the work of constructing taxon-specific probes. Also, evidence from other molecular studies now shows that AMF can exist as nonsporulating, multispecies communities on single plants (Clapp et al. 1995). Presently, a large probe (655 bp, designated P0-M3) derived from a sequence-characterized, randomly amplified PCR product is the only one available which detects most of nine *Glomus mosseae* isolates tested and no other *Glomus* species or single isolates of *Acaulospora*, *Scutellospora*, or *Gigaspora* (Lanfranco et al. 1995). As such, P0 and M3 (Lanfranco et al. 1995) are the only TSOs available for priming PCR and/or detection of glomalean fungi more specifically than at the family level (Simon et al. 1993a). The TSOs reported here are smaller, well-characterized ITS region alternatives to P0 and M3.

Given the high amount of sequence homology reported for the 18s nuclear ribosomal DNA (rDNA) region (Simon et al. 1992a) and our goal of developing TSOs for several AMF taxa, we chose the more variable, but defined ITS regions flanking the 5.8s rDNA subunit. This choice was based on several reports describing successful use of the internal transcribed spacer (ITS) regions flanking the 5.8s subunit of the nuclear rRNA gene to detect and identify fungi (White et al. 1990; Kim 1992; Lee and Taylor 1992; Carbone and Kohn 1993; Levesque et al. 1994), and our successful preliminary PCR assays with several glomalean fungi.

## Materials and methods

### Fungal isolates and spore collection

A total of 36 isolates (Table 1) of endomycorrhizal fungi were used in this study, including 13 isolates of *Glomus mosseae* and at least two taxa from *Entrophospora*, *Gigaspora*, *Glomus*, and *Scutellospora*, and one from *Acaulospora*. *Pythium ultimum* Trow and *Endogone pisiformis* Link & Fries were included as nonmycorrhizal fungus controls; *Zea mays* L. (corn) was used as the plant DNA control (Table 1). Single mycorrhizal fungus isolates were grown with *Z. mays* L. in soil-less pot cultures in a greenhouse for 6–12 weeks (Millner and Kitt 1992). Spores were harvested by standard wet-sieving, cleaned with repeated, forcible sprays of water on a sieve, rinsed in a very dilute (0.01%) solution of Ivory detergent (Proctor & Gamble, Cincinnati, Ohio), washed twice with water, and by a final rinse in distilled water. Spores in a water-filled watchglass were examined stereoscopically using a Wild microscope (×10–40 magnification) to assess surface cleanliness and overall spore condition. Intact, clean spores were hand-picked with sterile, ultrafine-tip forceps into 0.5-ml Eppendorf tubes containing 50–100 μl of PCR-grade water (Gibco BRL, cell culture, endotoxin-free, membrane-filtered, distilled water, Bethesda, Md.) . Spore suspensions were vortexed twice for 10 s, before all excess water was removed from the tube. Spores were moved into the lid of the tube by tapping the closed inverted tube on the lab bench. Fresh PCR-grade water was added to this lid, and the spores were individually moved to a clean 0.5-ml tube with forceps that were flame-sterilized between picking each spore; spores were suspended in 5–10 μl of PCR grade water. Roots from inoculated and uninoculated corn and *Sorghum sudanense* (sudan grass) pot cultures grown in soil:sand (1:1, v:v) were washed free of adhering soil and processed for DNA extraction. The soil was a silt loam, fine loamy, mixed mesic Aeric Ochraqauaif and the sand was 4-Q quartz (Pennsylvania Glass Sand Corp., Pittsburgh, Pa.). Plants had been inoculated with 30 field-picked spores of *Glomus mosseae* unless they were uninoculated controls.

### DNA extraction

Spores were crushed in the Eppendorf tubes using polycarbonate micropestles that had been sequentially soaked in 1 N NaOH and 1 N HCl then rinsed in distilled water prior to use. Pestles treated in this manner were found to be DNA-free in PCR assays in our lab. Each spore crush was resuspended in 10 volumes of water and a one-third volume of Chelex 100 resin (BioRad, Hercules, Calif.) solution (20% w/v). Spore crushes were sonicated 15 s in a jewelry cleaning unit (Model 77, Electromation Components Corp., N.Y.), then freeze-thawed at –20 °C and room temperature three times. Preparations were stored at –20 °C and diluted as needed with PCR-grade water before use as templates.

Roots (150 mg) of corn and sudan grass were frozen at –20 °C and ground to a fine powder with liquid nitrogen in a prechilled (–80 °C) mortar and pestle. DNA was extracted from the powdered root tissue using the rapid DNA extraction protocol of the IsoQuick Nucleic Acid Extraction kit (MicroProbe Corp., Bothell, Wash.).

### Oligonucleotides

Oligonucleotides used in this study (Table 2) were synthesized commercially and used unpurified. Their locations in the 18s, 5.8s, and 26s rDNA sequences are shown in Fig. 1.

### PCR conditions and PCR product analysis

PCR was performed with an automated temperature cycling instrument (MJ Research, Inc., Watertown, Mass.) with mixtures containing the following (final concentrations): 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 200 μM each of the four deoxynucleotide triphosphates (dNTPs), 0.25 μM (each) primer, and 2.5 Units of Taq polymerase/100 μl total reaction mix (Boehringer Mannheim, Indianapolis, Ind.), and template DNA. Reaction mixtures contained a 1/10 volume of diluted template DNA (dilutions ranged from 1/10 to 1/100 for glomalean templates and 1/100 to 1/1000 for control templates); 100-μl reaction volumes were used after suitable template dilutions (frequently 1/50 for glomalean templates) and other PCR conditions ($Mg^{++}$, template, and dNTP concentrations, annealing temperatures,

**Table 1** Isolates,designations, number of spores extracted, geographic place of origin and contributor of each isolate used in this study

| Isolate name (taxonomic authority) | INVAM No. | No. of spores extracted | Geographic origin | Contributor |
|---|---|---|---|---|
| *Glomus mosseae* (Nicolson & Gerdemann) Gerd. & Trappe | FL 156 | 50 | Florida, USA | Schenck |
| *G. mosseae* | FR 113 | 150 | France | Gianinanzzi |
| *G. mosseae* | UK 144 | 175 | United Kingdom | Hepper |
| *G. mosseae* | UK 143 | 235 | United Kingdom | Hepper |
| *G. mosseae* | UK 118 | 435 | United Kingdom | Hepper |
| *G. mosseae* | UK 142 | 530 | United Kingdom | Hepper |
| *G. mosseae* | UK 125 | 270 | United Kingdom | Jeffries |
| *G. mosseae* | UK 126 | 170 | United Kingdom | Jeffries |
| *G. mosseae* | UK 127 | 575 | United Kingdom | Jeffries |
| *G. mosseae* | NV 102 | 105 | Nevada, USA | Bethlenfalvay |
| *G. mosseae* | CA 196 | 200 | California, USA | Mihara |
| *G. mosseae* | SP 108 | 200 | Spain | Roldan-Fajardo |
| *G. mosseae* | KS 888 | 100 | Kansas, USA | Hetrick |
| *Glomus monosporum* Gerdemann & Trappe | FR 115 | 60 | France | Gianinazzi |
| *G. monosporum* | IT 12 | 85 | Italy | Bonfante-Fasolo |
| *Glomus claroideum* Schenck & Smith | SC 186 | 250 | South Carolina, USA | Skipper |
| *G. claroideum* | MD 125 | 70 | Maryland, USA | Watson |
| *Glomus clarum* Nicolson & Schenck | WV 751 | 235 | West Virginia, USA | Morton |
| *Glomus deserticola* Trappe, Bloss, & Menge | CA 113 | 250 | California, USA | Menge |
| *Glomus etunicatum* Becker & Gerdemann | FL 906 | 100 | Florida, USA | Skipper |
| *G. etunicatum* | MD 107 | 300 | Maryland, USA | Millner |
| *G. etunicatum* | UT 316 | 15 | Utah, USA | Native Plants, Inc. |
| *Glomus fistulosum* Skou & Jakobsen | DN 987 | 190 | Denmark | Morton |
| *Glomus gerdemannii* | AU 215 | 20 | Australia | Morton |
| *Glomus intraradices* Schenck & Smith | FL 208 | 100 | Florida, USA | Nemec |
| *Glomus occultum* Walker | IA 702 | 1000 | Iowa, USA | Morton |
| *Gigispora albida* | BR 203 | 40 | Brazil | Morton |
| *Gigaspora gigantea* (Nicolson & Gerdemann) Gerd. & Trappe | PA 149 | 8 | Pennsylvania, USA | Millner |
| *Gigaspora rosea* Nicolson & Schenck | FL 105 | 1 | Florida, USA | Schenck |
| *G. rosea* | FL 185 | 50 | Florida, USA | Perez |
| *G. rosea* | UT 102 | 20 | UTAH, USA | Morton |
| *Acaulspora gerdemannii* Schenck & Nicolson | FL 130 | 20 | Florida, USA | Morton |
| *Entrophosphora contigua* | WV 201 | 20 | West Virginia, USA | Morton |
| *E. infrequens* (Hall) Ames & Schneider | NY 101 | 20 | New York, USA | Watson |
| *Scutellospora heterogama* (Nicolson & Gerdemann) Walker & Sanders | WV 858 | 50 | West Virginia, USA | Morton |
| *S. coralloidea* (Trappe & Gerdemann) Walker & Sanders | CA 260 | 1 | California, USA | Ames |
| *Endogone pisiformis* Link ex Fries | EP1[a] | hypal mat[b] | Canada | Berch |
| *Zea mays* L. cv. IO Chief | Corn[a] | root extract[b] | USA | Millner |
| *Pythium ultimum* Trow | PuZ1[a] | hypal mat[b] | Maryland, USA | Lumsden |

[a] Not INVAM numbers; designations used to refer to non-AMF controls
[b] No spores extracted from non-AMF controls, see Materials and methods for details

number of cycles, type of *Taq* polymerase enzyme, hot start and two-step cycling) had been determined for each template in 10 μl-reactions. Template dilutions which produced adequate product yields with the ITS1:ITS4 primer pair also produced good yields when amplified with GMOS1 and GMOS2; all amplifications were done at least two or three times.

PCR components were assembled on ice and then transferred to a thermal cycler block which had been pre-heated to 95 °C (to reduce non-specific priming). For reactions using ITS primers, including those modified for cloning, the preheated components were initially heated for 2.5 min at 95 °C, and were then subjected to 40 cycles of 30 s at 95 °C, 45 s at 58 °C, and 60 s at 72 °C, with a final extension step of 72 °C for 5 min. For reactions using the primer pair GMOS1:GMOS2, the components were initially heated for 2.5 min at 95 °C, then subjected to 35 cycles of 60 s at 95 °C, 60 s at 53 °C, and 60 s at 72 °C, with a final extension step of 72 °C for 5 min.

Asymmetric PCR (Nichols and Raben 1994) was used to generate single-stranded product for sequencing of *Glomus mosseae* FL156, which was initially amplified as a double-strand using primers ITS1 and ITS4 in the above-described protocol. Asymmetric PCR was performed using a 1:2 dilution of the ITS1-ITS4 double-stranded product without purification as template, and ITS1 or ITS4 as primer, to generate the forward and reverse DNA strands in two separate reactions. Components for asymmetric PCRs were assembled on ice and then transferred to a thermal cycler block preheated to 95 °C. The components were initially heated at 95 °C for 30 s, and then subjected to 20 cycles of 30 s at 95 °C, 30 s at 60 °C, and 180 s at 72 °C, with a final 7 min extension at 72 °C.

**Table 2** Designations and sequences of synthetic oligonucleotides used in this study

| Designation | Nucleotide sequence (5′→3′) |
|---|---|
| ITS1 | TCC GTA GGT GAA CCT GCG G |
| ITS1-26 | TCC GTA GGT GAA CCT GCG GAA GGA TC |
| ITS4 | TCC TCC GCT TAT TGA TAT GC |
| 5.8s | CGC TGC GTT CTT CAT CG |
| 5.8Sr | TCG ATG AAG AAC GCA GC |
| ITS4Pst | CCC TGC AGT CCT CCG CTT ATT GAT ATG C |
| ITS1Eco | TAG GTA CCG TAG GTG AAC CTG CGG AAG GAT C |
| GMOS1 | CTG ANG ACG CCA GGT CAA AC |
| GMOS2 | AAA TAT TTA AAA CCC CAC TC |
| GMOS3 | CGA CGC GAT CAC CCT NAA AAA |
| GMOS4 | GCG AGG CTT GCG AAA ATA |
| GMOS5 | GGC TCA ATT CCG ACG CGA TCA CCC |
| GMOS6 | AAA AAA AGA GCG ACG CCT CG |

## DNA cloning

DNA products from 100-μl PCRs using ITS1Eco and ITS4Pst primers were diluted and washed twice with 10-fold volumes of water using Centricon-30 units (Amicon, Inc., Boston, Mass.). After digestion with EcoR1 and Pst1, the products were purified from an 0.8% agarose gel using a GeneClean kit (Bio101, La Jolla, Calif.), and ligated with 0.25 μg of pUC19 DNA which had been digested with EcoR1 and Pst1. The ligated products were used to transform electrocompetent cells of *E. coli* DH5α (Gibco BRL, Gaithersburg, Md.). A boiling miniprep procedure (Maniatis et al. 1982) was used to identify transformants containing plasmid inserts of the correct size (590 bp). Plasmid DNA preparations for double-stranded sequencing were purified by alkaline lysis with at least two PEG precipitation steps to remove contaminating RNA (Ausubel et al. 1987).

## Sequencing

Direct sequencing of single-stranded PCR product generated by asymmetric PCR was used to determine the rDNA sequence from *Glomus mosseae* FLA156; products from two or three 100 μl-reactions were pooled to compensate for the low yields. The pooled product was washed four times with 1.0 ml PCR-grade water, and concentrated to approximately 35 μl using a Centricon-30 unit. For sequencing reactions, an annealing mixture containing 3.5 μl of the concentrated single-stranded (ss) DNA product, 1 μl of a 5-μM stock solution of sequencing primer and Sequenase reaction buffer (U.S. Biochemical Corp., Cleveland, Ohio) was incubated at 65 °C for 2 min followed by 37 °C for 5 min. Primers ITS1, ITS4, 5.8s, or 5.8Sr were used in these sequencing reactions, which were performed using the standard Sequenase protocol and $^{35}$S dATP, except that 2 μl of a 50-fold dilution of labelling mix was used in the labelling reaction. Reaction products were resolved on 8% denaturing acrylamide gels.

Double-stranded sequencing of cloned DNA was used to determine the nucleotide sequence of the rDNA from *Glomus mosseae* UK118, *Glomus etunicatum* UT316, *Glomus intraradices* FL208, *Glomus occultum* IA702, *Gigaspora albida* BR203, *Gigaspora rosea* FL185, *Gigaspora gigantea* PA149, *Scutellospora coralloidea* CA260, and *S. heterogama* WV858. Sequencing reactions were performed using standard Sequenase protocols, purified plasmid DNAs, primers ITS1, ITS1Eco and ITS4, ITS4Pst, 5.8s, 5.8Sr, and $^{35}$S dATP. Reaction products were resolved on 8% denaturing acrylamide gels.

Sequences were aligned using DNAstar software (Madison, Wisc.). Selected oligonucleotide regions were analyzed individually using OLIGO version 5.0 (National Biosciences, Plymouth, Minn.) to evaluate their suitability as primers for PCR (Sommer and Tautz 1989) or as hybridization probes.

## Electrophoresis and DNA hybridization

PCR products were separated by electrophoresis using 1.0–3.0% agarose gels and TAE buffer (Maniatis et al. 1982) containing 0.5 μg/ml ethidium bromide. Gels were stained with ethidium bromide, and photographed with a Polaroid camera (type 57 and 665 film) while illuminated with UV light. DNA transfers and hybridizations were performed using GeneScreen Plus membranes (Dupont NEN Research Products, Boston, Mass.) according to the manufacturer's instructions. Blotted membranes were subjected to UV cross-linking (Stratagene, La Jolla, Calif.), followed by prehybridization and hybridization steps at 60 °C in a solution of 10% dextran sulfate, 1% SDS and 1 M NaCl for 1 and 16 h, respectively. Following hybridization with $^{32}$P-end-labelled GMOS probes (Ausubel et al. 1987), membranes were washed twice for 5 min each at room temperature in 2× SSC, twice for 30 min each at 60 °C in 2× SCC and 1% SDS (1× SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), and twice in 0.1× SSC for 30 min each at room temperature. Colony hybridizations were performed using Colony/Plaque Screen membranes (Dupont NEN Research Products). DNAs were fixed to these membranes by autoclaving for 1 min at 100 °C, according to the manufacturer's suggestions. Prehybridization, hybridization, and washing steps were identical to those used for Southern blots with GeneScreen Plus membranes described above.

## Nucleotide sequence accession numbers

Nucleotide sequences from *Glomus mosseae* UK118 and FL156 rDNAs were submitted to GenBank and given accession numbers U49264 and U49265, respectively.

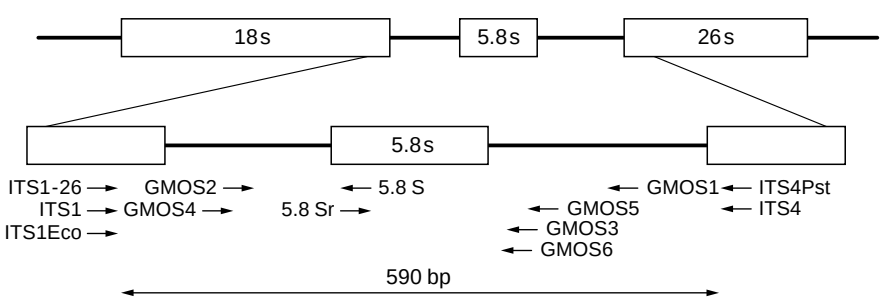



**Fig. 1** Schematic representation of the rDNA of *Glomus mosseae*. *Open boxes* represent the ribosomal gene subunits. *Arrows* show approximate positions and reading direction of the original PCR primers (ITS1, ITS1–26, and ITS4), the modified PCR primers used for cloning (ITS1Eco and ITS4Pst), taxon-specific PCR primers (GMOS1–GMOS4), hybridization probes (GMOS5 and GMOS6), and internal sequencing primers (5.8s and 5.8Sr)

## Results and discussion

### DNA amplification and sequencing

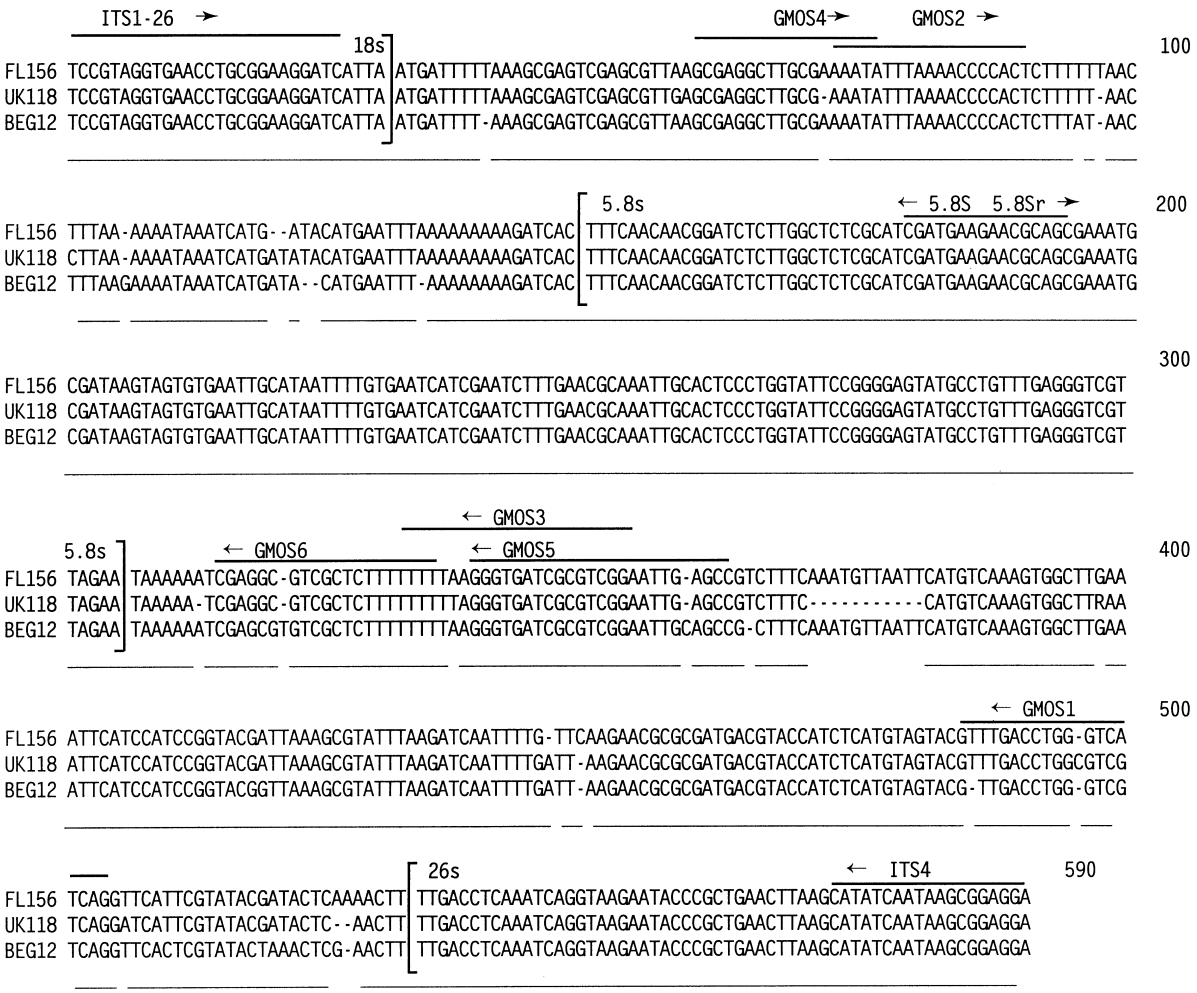
The ITS region from *Glomus mosseae* FL156 and UK118, as well as other glomalean taxa and non-mycorrhizal, control templates, was reproducibly amplified by PCR using ITS1 and ITS4, or ITS1-26 and ITS4. A major double-stranded product of approximately 550–600 bp, was generated from all templates. The products were sequenced as described and sequences were aligned. Figure 2 shows a sequence alignment for the two *Glomus mosseae* isolates, FL156 and UK118. The alignment shows that the 5.8s gene subunit regions are identical, whereas the flanking ITS regions are 95.9% similar. When aligned with partial sequences of the corresponding ITS and 5.8s gene regions from *Glomus etunicatum* UT316, *Glomus intraradices* FL208, *Glomus occultum* IA702, *Gigaspora rosea* FL105, *Glomus albida* BR203, and *S. coralloidea* CA260, (see http://link.springer.de/journals/myco/) several regions appeared unique to the *Glomus mosseae* isolates.

**Fig. 2** Nucleotide sequences (5′–3′) and alignment of ITS regions from three isolates of *Glomus mosseae*, FL156, UK118, and BEG12 (as reported for clone 1 by Sanders et al. 1995). Locations of the 18s, 5.8s, and 26s gene subunits are shown as *bracketed regions*. Positions of the non-specific PCR primers (ITS 1–26, 5.8S, ITS4), the modified PCR primers used for cloning (ITS1EcoR and ITS4Pst), *Glomus mosseae*-specific PCR primers (GMOS1 and GMOS2) and *Glomus mosseae*-specific hybridization probe (GMOS5) are indicated (*underlined designations*)

### Selection and testing of GMOS oligonucleotides

Analyses of sequences identified as potentially specific for *Glomus mosseae* indicated that six of the numerous possible sequences had physico-chemical properties suitable for primers and/or hybridization probes. Four of these oligonucleotides, GMOS1 through GMOS4 (shown in Table 2), were tested in pairwise comparisons to determine their capacity to generate a sufficient quantity of the expected size PCR product, exclusively from *Glomus mosseae* templates.

Results of these PCR tests with various combinations of GMOS1 through GMOS4 as primers suggested that the GMOS1 and GMOS2 pair gave the most reproducible and the fewest nonspecific PCR products, and they were used for additional tests. Reactions con-



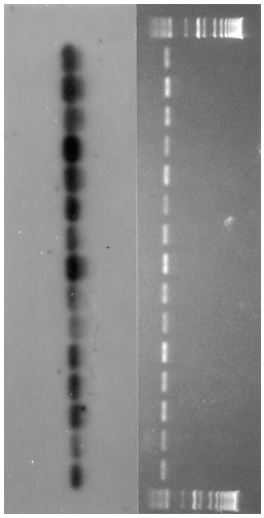



**Fig. 3** Amplified products from PCR using primers GMOS1 and GMOS2 and genomic DNA preparations from spores of *Glomus mosseae* and *Glomus monosporum* isolates as templates. *a* Southern blot hybridization of *b* with GMOS 5 with a 2-day film exposure; *b* electrophoresis in 0.8% agarose stained with ethidium bromide (*MW* molecular weight)

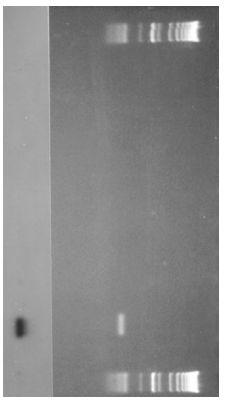



**Fig. 4** Comparison of PCR products generated with primers GMOS 1 and GMOS2, and Southern blot hybridizations with GMOS5 for *Glomus mosseae* UK143 with seven other *Glomus* species; *a* blot and hybridization of *b* with a 2-day film exposure; *b* electrophoresis in 0.8% agarose stained with ethidium bromide. Approximate size of product is 0.40 kb

taining GMOS1 and GMOS2 primed amplification of all *Glomus mosseae* and *Glomus monosporum* isolates (Fig. 3b), without amplification of other *Glomus* species (Fig. 4b) or other glomalean taxa (Fig. 5b). In contrast, the GMOS1 and GMOS4 pair did not prime amplification of template DNA from two *Glomus mosseae* isolates, yet DNA from *Gigaspora rosea* FL105 and FL185, *Glomus etunicatum* UT316, *Glomus occultum* IA702, and *Glomus claroideum* SC186 were amplified in some of the replicate PCRs (data not shown).

The PCR competency of all genomic DNA templates not amplified in reactions with GMOS1 and

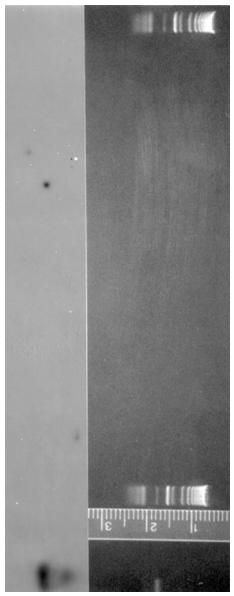



**Fig. 5** Comparison of PCR products generated with primers GMOS 1 and GMOS2, and Southern blot hybridizations with GMOS5 for *Glomus mosseae* UK118 with 11 isolates from four other genera of glomalean fungi, two soil-plant fungi (*Endogene pisiformis* and *Pythium ultimum*), and *Zea mays; a* blot and hybridization of *b* with a 2-day film exposure; *b* electrophoresis in 0.8% agarose stained with ethidium bromide

GMOS2 (Figs. 4b, 5b) were verified by PCR assays primed by the ITS1 and ITS4 primer pair (Fig. 6); PCR products of the expected size were obtained from all those templates. Thus, we conclude that the primer pair GMOS1:GMOS2 was highly specific for *Glomus mosseae* and *Glomus monosporum*.

## Hybridizations

For reactions in which GMOS1 and GMOS2 were primers, GMOS5 was successfully used to detect the presence of *Glomus mosseae* and *Glomus monosporum* on gel-blotted membranes (Figs. 3a, 4a, 5a); no hybridizing products resulted from the PCRs containing other templates (Figs. 4a, b, 5a, b).

Detection of *Glomus mosseae* in colonized sudan grass roots by PCR depended on which primer pair was used; the GMOS1:GMOS2 primer pair worked better than the ITS1:ITS4 pair. With the GMOS1:GMOS2 primer pair and DNA from extracts of nonmycorrhizal roots, no PCR product was observed in gels and no signal was obtained on gel blots hybridized with GMOS5, as expected for a nonmycorrhizal control (Fig. 7a, b). When GMOS1 and GMOS2 were used with DNA extracts of mycorrhizal roots, the PCR product was of the expected size and the corresponding gel blots produced a signal when hybridized with GMOS5. When nonmycorrhizal root DNA was the template for PCRs primed by the ITS1:ITS4 pair, an appropriately sized PCR

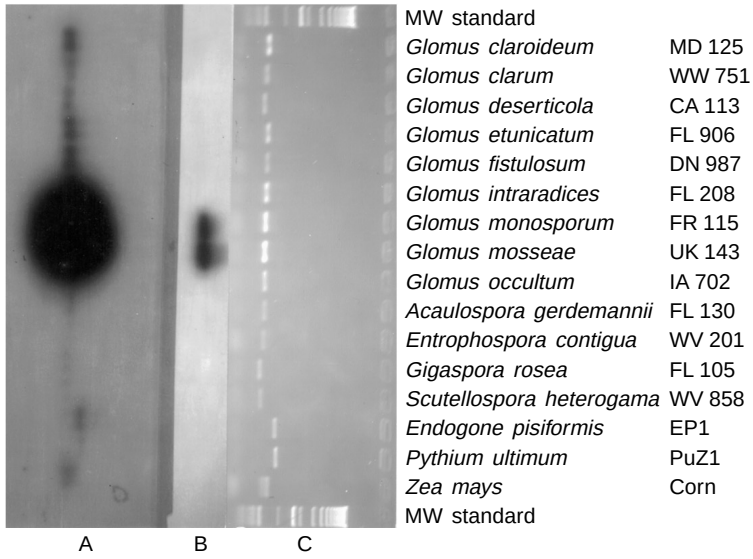



**Fig. 6** Comparison of PCR products of rDNA amplified with primer pair ITS1:ITS4 from AMF, nonmycorrhizal fungi, and corn; *a* Southern blot of *c* hybridized with GMOS 5, with a 2-day film exposure; *b* same as *a* but 2-h film exposure; *c* electrophoresis in 0.8% agarose stained with ethidium bromide. Products range in size from approximately 0.42 to 0.59 kb

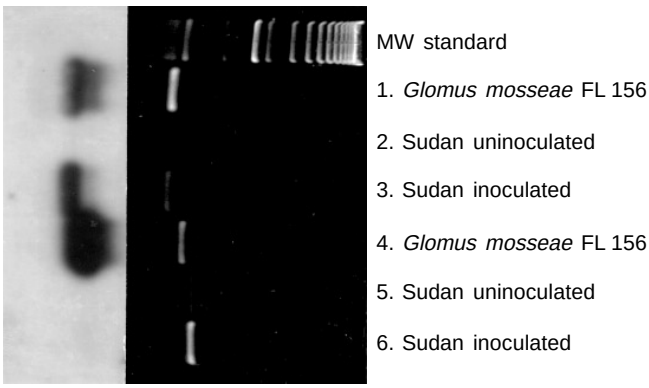



**Fig. 7** Comparison of PCR products generated with primer pairs GMOS1:GMOS2 and ITS1:ITS4 and hybridization tests with GMOS5; *a* Southern blot hybridization of *b* with GMOS5, and 2-day film exposure; *b* electrophoresis in 0.8% agarose stained with ethidium bromide. Products range in size from approximately 0.40 to 0.59 kb. PCRs were performed using the templates shown and primer pairs GMOS1:GMOS2 (lanes 1–3) or ITS1:ITS4 (lanes 4–6)

product was visible on the electrophoretic gel, but no hybridization occurred with GMOS5 (Fig. 7a, b). This indicated that root DNA was amplifiable by the ITS1:ITS4 primer pair, but no sequence homologous to the *Glomus mosseae* probe, GMOSS5, was detectable, as was expected for a nonmycorrhizal control. However, when DNA from colonized sudan grass roots was amplified in PCR with primer pair ITS1:ITS4, no product corresponding to *Glomus mosseae* was detectable (Fig. 7a). This indicated that the GMOS1:GMOS2 primer pair was essentially specific for detection of *Glomus mosseae* in DNA extracts of sudan roots, and that the ITS1:ITS4 pair is not a good choice for amplifications of root extracts to be hybridized with GMOS5.

The extent of possible intragenomic sequence heterogeneities to GMOS5 was determined by testing hybridization to 100 clones each of FL156 and UK118 with GMOS5. Results showed that nearly all rDNA copies from the genomic preparation of these two *Glomus mosseae* isolates contained sequences recognizable by GMOS5:100% of the FL156 PCR product clones and 97% of the UK118 PCR product clones hybridized. Nonhybridizing clones were not characterized further. These results suggest that GMOS5 would reliably detect *Glomus mosseae*, even though sequence heterogeneities are known to naturally occur in the ITS regions (Sanders et al. 1995).

## Discussion

In undertaking the development of a TSO for *Glomus mosseae*, we initially chose what we thought would be a direct approach for producing adequate ssDNA for sequencing, i.e., asymmetric PCR. However, product yields from asymmetric PCR of *Glomus mosseae* FL156 were low and only partial sequences were readable; thus several sequencing reactions were necessary. For these reasons, PCR products from all the other glomalean templates were subsequently cloned using modified ITS primers. Sequencing cloned PCR products was less problematic than generating and sequencing asymmetric PCR products, as was also reported by Lloyd-MacGilp et al. (1996).

Design of GMOS-specific oligonucleotides for PCR primers and hybridization probes was based on the sequence of a 590-bp segment of rDNA from *Glomus mosseae* isolates FL156 and UK118. The ITS1 and ITS2 regions in the segment were much more variable than the 5.8s subunit gene region. Major sequence differences identified in the ITS1 and ITS2 regions of *Glomus mosseae* FL156 and UK118 relative to those found in partial sequences of *Glomus etunicatum, Glomus intraradices, Glomus occultum, Gigaspora* spp., and *Scutellospora* spp. were used to design unique primers, GMOS1, GMOS2, and GMOS5, for *Glomus mosseae*.

Reactions primed by primer pair GMOS1:GMOS2 yielded single PCR products of the appropriate size (400 bp) only when *Glomus mosseae* or *Glomus monosporum* DNA were templates. Reactions using DNA templates from other glomalean taxa, i.e., *Acaulospora, Entrophospora, Endogone, Gigaspora, Scutellospora*, and from nonglomalean control *Zea*, did not yield products visible on agarose gels.

Results from hybridization experiments using the GMOS5 and the ITS1:ITS4 primed PCR products from a variety of AMF templates showed that hybridization was specific to *Glomus mosseae* templates (approximately 20 times more intense signal produced against *Glomus mosseae* templates than toward other templates). Not surprisingly, hybridization experiments using GMOS5 and PCR products generated using the GMOS1:GMOS2 primer pair also hybridized only to *Glomus mosseae* DNA. For reasons not presently understood, hybridization of GMOS5 to PCR products generated with primer pair GMOS1:GMOS2 is considerably weaker than is hybridization of this probe to the ITS1:ITS4 primer-generated PCR products from *Glomus mosseae*.

Results from PCR and hybridization tests with *Glomus monosporum* DNA templates and the *Glomus mosseae* TSOs provide valuable additional data needed to resolve the identification and taxonomic confusion which occurs with these two closely allied taxa. The morphological similarities between these taxa are so strong that many investigators cannot satisfactorily distinguish them (Dodd et al. 1996). The PCR and hybridization results indicate that these morphological similarities coincide with a high degree of sequence similarity. Ultimately, however, satisfactory resolution of the relatedness of these two taxa will require a comprehensive comparison and review of their morphological, biochemical, and molecular characteristics as modeled in the recent comparative study of *Glomus mosseae* and *Glomus coronatum* (Dodd et al. 1996).

The *Glomus mosseae* – *Glomus monosporum* identification issue is merely one of perhaps several instances in which the specificity of a TSO designed for glomalean fungi must be evaluated at several hierarchical levels. The intended uses of the TSOs, i.e., at single or several proximally located field sites instead of at globally distant sites or with ecologically diverse collections of AMF, will influence the rigor of cross-hybridization testing which may be needed to satisfy criteria for the robustness of the TSO. For example, in recent studies of genetic diversity and variability within (Sanders et al. 1995) and among (Lloyd-MacGilp et al. 1996) individual isolates of *Glomus mosseae*, and *Gigaspora margarita* BEG34 (Zézé et al. 1997), the nature and basis of concerns about sequence variability were described and illustrated. Sanders et al. (1995) showed that ITS region sequence diversity is high among individual spores for several glomalean taxa, including *Glomus mosseae* BEG12. Likewise, Lloyd-MacGilp et al. (1996) showed up to 6% sequence divergence among clones from the same spore for several *Glomus mosseae* isolates. Despite these differences, enough similarity exists within parts of the ITS region of *Glomus mosseae* to support the use of TSOs. For example, alignment of the ITS region sequences of *Glomus mosseae*, BEG 12 clone 1 (Sanders et al. 1995), with those from FL156 and UK118 (see Fig. 2) shows that BEG12 is 100% identical to the 5.8s gene regions of FL156 and UK118, overall approximately 95% (562 of 590 nucleotide bases) similar to *Glomus mosseae* FL156, and about 92% (545 of 590 nucleotide bases) similar to UK118. Furthermore, the GMOS2 sequence is 100% identical to one found in clones 1 and 2 of BEG 12 (Sanders et al. 1995); the 19-mer GMOS1 is identical to 18 nucleotide bases in clone 1 of BEG 12 and all 19 nucleotide bases in clone 2 of BEG12, as reported by Sanders et al. (1995); the 24-mer GMOS5 is identical to 23 of 24 and 17 of 24 nucleotide bases of BEG12 clones 1 and 2, respectively (Sanders et al. 1995). Such similarities are considerably greater than those occurring simply at random and with the other *Glomus* isolates for which we had partial sequences.

The BEG 12 isolate exemplifies the situation with any unidentified AMF isolate and its response to the TSOs. Based on the results presented here with GMOS5 hybridization tests for GMOS1 and GMOS2 (Figs. 3a, 4a, 5a) and with the cloned DNAs, we conclude that starting from extracts of colonized roots or directly from extracts of spores, PCR products would contain at least enough of the target TSO sequences for *Glomus mosseae* to be reliably detected by GMOS5. Clearly, at this juncture in the development of TSOs for glomalean fungi, accurate predictions of successful, valid identifications made using the TSO for each representative of its respective taxon would be premature, if not presumptuous. As PCR and hybridization assays become more readily used in taxonomic studies with these fungi, such predictions will be more easily made. In addition, TSOs will facilitate the study of AMF biodiversity in agricultural and soil systems and provide a means to advance biodiversity analysis beyond the limited interpretations and understanding that morphologically based detection and identification approaches provide (Morton and Bentivenga 1994).

The utility of the TSOs for detecting *Glomus mosseae* DNA from colonized roots was demonstrated using samples of sudan grass. Presently, we have no information on the detection limits using these oligonucleotides, but this is the focus of further studies using this and other probes with taxa of other glomalean genera and species.

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## References


Ausubel FM, Brent R, Kingston RE, Kingston DD, Moore DD, Seidman JG, Smith JA, Struhl K (eds) (1987) Current protocols in molecular biology. Wiley, New York

Bonito R Di, Elliott ML, Des Jardin EA (1995) Detection of an arbuscular-mycorrhizal fungus in roots of different plant species with the PCR. Appl Environ Microbiol 61:2809–2810

Carbone L, Kohn LM (1993) Ribosomal DNA sequence divergence within internal transcribed spacer I of the Sclerotiniaceae. Mycologia 85:415–427

Clapp JP, Young JPW, Merryweather JW, Fitter AH (1995) Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol 130:259–265

Dodd JC, Rosendahl S, Giovannetti M, Broome A, Lanfranco L, Walker C (1996) Inter- and intraspecific variation within the morphologically-similar arbuscular mycorrhizal fungi *Glomus mosseae* and *Glomus coronatum.* New Phytol 133:113–122

Kim WK (1992) A region of heterogeneity adjacent to the 5S ribosomal RNA gene of cereal rusts. Curr Genet 22:101–105

Lanfranco L, Wyss P, Marzachi C, Bonfante P (1995) Generation of RAPD-PCR primers for the identification of isolates of *Glomus mosseae*, an arbuscular mycorrhizal fungus. Molec Ecol 4:61–68

Lee SB, Taylor JW (1992) Phylogeny of five fungus-like protoctistan *Phytophthora species*, inferred from the internal transcribed spacers of ribosomal DNA. Molec Biol Evol 9:636–653

Levesque BA, Vrain TC, De Boer SH (1994) Development of a species-specific probe for *Pythium ultimum* using amplified ribosomal DNA. Phytopathology 84:474–478

Lloyd-MacGilp SA, Chambers SM, Dodd JC, Fitter AH, Walker C, Young JPW (1996) Diversity of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi. New Phytol 133:103–111

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor

Miller RM, Jastrow JD (1992) The role of mycorrhizal fungi in soil conservation. In: Bethlenfalvay GJ, Linderman RG (eds) Mycorrhizae in sustainable agriculture. Am Soc Agron Special Publication, No 54, Madison, Wisc, pp 29–44

Millner PD, Kitt DG (1992) The Beltsville method for soilless production of V-A mycorrhizal fungi. Mycorrhiza 2:9–15

Morton JB, Bentivenga SP (1994) Levels of diversity in endomycorrhizal fungi (Glomales, Zygomycetes) and their role in defining taxonomic and non-taxonomic groups. Plant Soil 159:47–59

Nichols RC, Raben N (1994) Hints for direct sequencing of PCR-generated single-stranded DNA. BioTechniques 17:413–414

Sanders IR, Alt M, Groppe K, Boller T, Wiemken A (1995) Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol 130:419–427

Simon L, Lalonde M, Bruns TD (1992a) Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Appl Environ Microbiol 58:291–295

Simon L, Levesque RC, Lalonde M (1992b) Rapid quantification of endomycorrhizal fungi colonizing roots. PCR Methods Appl 2:76–80

Simon L, Levesque RC, Lalonde M (1993a) Identification of endomycorrhizal fungi colonizing roots by fluorescent single-strand conformation polymorphism-polymerase chain reaction. Appl Environ Microbiol 59:4211–4215

Simon L, Bousquet J, Levesque RC, Lalonde M (1993b) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:67–69

Sommer R, Tautz D (1989) Minimal homology requirements for PCR primers. Nucleic Acids Res 17:6749

Tisdall JM (1991) Fungal hyphae and structural stability of soil. Aust J Soil Res 29:729–743

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TF (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, Calif, pp 315-322

Wright S, Upadhyaya A (1996) Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. Soil Sci 161:575–586

Wright S, Franke-Snyder M, Morton JB, Upadhyaya A (1996) Time-course study and partial characterization of a protein on hyphae of arbuscular mycorrhizal fungi during active colonization of roots. Plant Soil 181:193–203

Wright SF, Millner PD (1994) Dynamic processes of vesicular-arbuscular mycorrhizae: a mycorrhizosystem within the agroecosystem. In: Hatfield JL, Stewart BA (eds) Soil biology: effects on soil quality. Advances in Soil Science. Lewis, Ann Arbor, Mich, pp 29–59

Wyss P, Bonfante P (1993) Amplification of genomic DNA of arbuscular-mycorrhizal (AM) fungi by PCR using short arbitrary primers. Mycol Res 97:1351–1357

Zézé A, Sulistyowati E, Ophel-Keller K, Barker S, Smith S (1997) Intersporal genetic variation of *Gigaspora margarita*, a vesicular arbuscular mycorrhizal fungi, revealed by M13 minisatellite-primed PCR. Appl Environ Microbiol 63:676–678