Mycorrhiza (2001) 10:203–207
© Springer-Verlag 2001

ORIGINAL PAPER

Daniel Schwarzott · Arthur Schüßler

A simple and reliable method for SSU rRNA gene DNA extraction, amplification, and cloning from single AM fungal spores

Accepted: 25 October 2000

Abstract We describe a method that allows quick and easy PCR amplification and cloning of nearly complete SSU rRNA genes from arbuscular mycorrhizal fungi. The procedure tested on spores from 37 different glomalean isolates was based on magnetic separation with Dynabeads, followed by nested PCR with two primer pairs. All trials led to visible amplification products of the expected size. Thereafter, the PCR fragments could be quickly and efficiently cloned by means of a topoisomerase-activated vector (pCR2.1-TOPO). The technique is rapid, uncomplicated and comparatively inexpensive. The use of single spores for DNA extraction has some advantages over multispore-preparations, e.g. it is less susceptible to contamination with other organisms present in the cultures. The method can be used for the quick and reliable preparation of a large number of samples and is highly reproducible. It could also be used for genes other than the SSU rRNA gene.

Keywords DNA extraction · Glomales · Single spores · SSU rRNA · TOPO cloning

Introduction

Arbuscular mycorrhizal fungi (AMF), which are placed in the order Glomales (Morton and Benny 1990; Smith and Read 1997), represent a monophyletic group (Gehrig et al. 1996; Redecker et al. 2000; Schüßler 1999). On the basis of spore morphology, about 150 species in six genera have been described (Walker and Trappe 1993). However, phylogeny within the Glomales is still unclear, as is the evolution of these obligate symbionts. Fossils 400–460 million years old show that glomalean fungi formed symbioses with ancient land plants (Redecker et al. 2000; Remy et al. 1994), possibly influencing or even being crucial to their colonisation of land. This hypothesis is supported by sequence analyses (Redecker et al. 1999). At least one AMF species forms symbioses with both higher plants and bryophytes (Schüßler 2000). *Geosiphon pyriforme* represents a symbiosis of a glomalean fungus with cyanobacteria (Schüßler and Kluge 2000).

To investigate the biodiversity of AMF, molecular techniques were developed, e.g. isozyme analyses (Rosendahl 1989), RAPD-PCR (Lanfranco et al. 1995), PCR and DNA fingerprinting of microsatellite regions (Gadkar et al. 1997; Longato and Bonfante 1997; Zézé et al. 1997) and specific antibodies (Hahn et al. 1993). The most recent approaches focus on the use of PCR combined with restriction analysis (Abbas et al. 1996; Redecker et al. 1997; Sanders et al. 1995) or with isolate- or group-specific primers (e.g. Bago et al. 1998; Chelius and Triplett 1999; Edwards et al. 1997; Helgason et al. 1998; Lanfranco et al. 1999; Millner et al. 1998; Redecker 2000; Redecker et al. 1997; Schüßler et al. 2000; van Tuinen et al. 1998). However, many of the published primers do not show the predicted specificity (Redecker 2000; Schüßler et al. 2000) because the underlying dataset is too small. Therefore, in order to develop DNA-based probes for biodiversity studies, and also to understand the phylogeny and evolution of AMF, it is necessary to deliver larger sets of sequence data.

The characterisation of ribosomal genes, including the ITS1 and ITS2 regions, is a suitable tool for revealing phylogenetic relationships and developing molecular probes to identify glomalean fungi (Redecker 2000; Schüßler et al. 2000; van Tuinen et al. 1998). The requirements for processing a large number of samples are: reproducibility, use of DNA from single spores, rapid, uncomplicated and reliable DNA-extraction procedures and low cost (Sanders et al. 1995). Several protocols for DNA extraction from multiple or single AMF spores have been described. All of them require a me-

D. Schwarzott · A. Schüßler (✉)
Technische Universität Darmstadt, Institut für Botanik,
Schnittspahnstrasse 10,
64287 Darmstadt, Germany
e-mail: schuessler@bio.tu-darmstadt.de
Fax: +49–6151–166049

chanical step to break up the spore wall. The suspension containing the crushed spores can be directly used for PCR (Vandenkoornhuyse and Leyval 1998) but DNA is usually extracted by more complex procedures, e.g. using proteinase K and heat treatment (Biancotto et al. 1996), freezing and heating cycles (Gehrig et al. 1996), Chelex-100 (Sanders et al. 1995; Simon 1996; Simon et al. 1992), NaOH, heat, and subsequent neutralisation (Redecker et al. 1997).

During our attempts to clone full-length SSU rRNA genes, all these methods were tested for DNA extraction from single AMF spores. Many were found to be time consuming and none of them was comparable in PCR reliability and cloning efficiency with the strategy described in this paper.

Materials and methods

Origin and cleaning of spores

Spores of AMF were obtained from the culture collections of BEG (La Banque Européenne des Glomales, INRA, Dijon, France; http://wwwbio.ukc.ac.uk/beg/), INVAM (International Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi, Morgantown, USA; http://invam.caf.wvu.edu/), Chris Walker (Biological Research and Imaging Laboratory, New Milton, UK) and Kartini Kramadibrata (Research and Development Centre for Biology, The Indonesian Institute of Sciences, Bogor, Indonesia). They were harvested under a dissection microscope and cleaned from soil by several washing steps in double-distilled water. Single spores were transferred into 0.5-ml Eppendorf tubes containing 200 μl sterile 1% SDS in double distilled water and sonicated at 35 kHz for 10–30 s in a Sonorex Super 10P (Bandelin Electronic, Berlin) with the power set to 100% and the degas function on. Sonication time depended on the mechanical stability of the spores, which can be damaged by this step. After washing with sterile double-distilled water, spores were examined under a dissection microscope. If material was still adhering, the sonication step was repeated. Thereafter, the spores were washed again, transferred to a 1.5-ml Eppendorf tube on ice containing 1.5 μl sterile double-distilled water and used for DNA extraction within 1 h or stored at –80 °C for later use.

DNA isolation

The cleaned spores were crushed within the Eppendorf tube with a sterile pipette tip under a dissection microscope and 200 μl Dynabeads solution (Dynabeads DNA Direct System 1, Dynal A.S., Oslo, Norway) was added immediately. The DNA extraction procedure by means of magnetic particles was performed following the instructions of the manufacturer, except that the first incubation step ("lysis" for 5 min) was extended to 10 min and only one instead of two washing steps of the Dynabeads/DNA complex was carried out. The resulting Dynabeads/DNA complex was resuspended in 30 μl TE buffer, put on ice and then directly used for PCR.

First PCR amplification

SSU rRNA genes were amplified using primers which overlap with the universal primers NS1 and NS8 (White et al. 1990). These are GeoA1 (5′GGTTGATCCTGCCAGTAGTC3′; Tm 62 °C) and ART4 (5′TCCGCAGGTTCACCTACGG3′; Tm 62 °C). PCR was performed in a final volume of 50 μl, containing 10 μl of the Dynabeads/DNA complex, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCL, 0.1% Triton X-100, 0.2 mM of each dNTP, 10 pmol of each primer and 2 units DyNAzyme II polymerase (Finnzymes OY, Espoo, Finland). The amplification reaction was performed as follows: 1×2 min at 94 °C; 35×30 s at 94 °C, 60 s at 59 °C, 180 s at 72 °C; 1×10 min at 72 °C. Control reactions contained no template DNA. For visualisation of the PCR product, 10 μl of the amplification products were separated electrophoretically on 0.8% agarose gels and stained with ethidium bromide.

Second (nested) PCR amplification

The PCR solution was diluted 1:10 if a fragment was visible in the agarose gel after first PCR, or was left undiluted if no fragment could be visualised. An aliquot (1 μl) of this solution was then used as template for nested PCR reamplification. We tested a set of different primers, e.g. NS1 (5′GTAGTCATATGCTTGTCTC3′; Tm 54 °C) or GeoNS1 (5′ATGGCTCATTAAATCAGTTAT3′; Tm 54 °C) combined with Geo10 (5′ACCTTGTTACGACTTTTACTTC3′; Tm 60 °C). We now use routinely GeoA2 (5′CCAGTAGTCATATGCTTGTCTC3′; Tm 64 °C), which represents NS1 elongated by 3 bp at the 5′ end, combined with Geo11 (5′ACCTTGTTACGACTTTTACTTCC3′; Tm 64 °C) representing Geo10 elongated by 1 bp at the 3′ end. Nested PCR was performed in a total volume of 50 μl, containing 1 μl template solution, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCL, 0.2 mM of each dNTP, 10 pmol of each primer, 2 units SAWDY Taq-DNA-Polymerase (Peqlab, Erlangen, Germany). We were also successful with other more expensive polymerases (e.g. AmpliTaq-DNA-polymerase, Perkin Elmer; AGS Gold, Hybaid). The amplification reaction was performed as follows: 1×2 min at 94 °C; 35×30 s at 94 °C, 60 s at 63 °C, 180 s at 72 °C; 1×10 min at 72 C. Aliquots (5 μl) of the PCR products where electrophoretically analysed on 0.8% agarose gels and stained with ethidium bromide.

Cloning and sequence analysis

PCR fragments were cloned directly, without further cleaning steps, into pCR2.1-TOPO vector (Invitrogen, Netherlands) according to the instructions of the manufacturer. This highly efficient "5-min ligation" by means of a topoisomerase-activated vector saves a lot of time-consuming steps. An aliquot (1.5 μl) of the fresh (not frozen) PCR product, 2.5 μl sterile water and 1 μl vector solution were mixed and incubated for exactly 5 min at 25 °C in a waterbath. This ligation solution was then placed on ice to be used immediately for cloning. During the set up of the ligation procedure the –80 °C stored competent cells were thawed on ice. *β*-mercaptoethanol (2 μl of 0.5 M) was added and mixed by gentle stirring with the pipette tip. Thereafter, 2 μl of the ligation solution was added and gently mixed. After another 30 min on ice, followed by a 30-s heat shock at 42 °C in a waterbath, the tube was immediately cooled on ice for 2 min. Subsequently, 250 μl SOC medium was added and the cells shaken at 37 °C and 225 rpm in an incubator for 1 h. After this procedure, the cells were ready to be spread on LB plates containing kanamycin and x-gal for blue/white screening. Plates were incubated overnight at 37 °C and six white clones were picked and analysed by restriction analysis for the insertion of the expected PCR fragment.

To prove the glomalean origin of the cloned DNA fragments, they were partly sequenced (ca. 600 bp) with the universal sequencing primer M13 forward and/or M13 reversed by Sequence Laboratories (Seqlab) Göttingen GmbH, Germany and a sequence analysis was performed as described by Schüßler (1999).

Results

With the method described, we amplified and cloned PCR fragments from single spores of 37 different iso-

lates (Table 1). Spores from all glomalean families, including the recently identified ancestral branches (Redecker et al. 2000), were used, varying in size from about 50 to 300 μm. All trials led to PCR fragments of SSU rRNA genes. For the results shown here, we used one third (10 μl Dynabeads-solution) of the single spore extract for the first PCR but one sixth (5 μl) of the extracted DNA is also sufficient. Stronger PCR amplification was obtained if the second washing step during Dynabeads isolation was omitted, indicating that template was lost during this step. With the small amounts of biological material used, the second washing step appears unnecessary.

Only six of the 37 isolates investigated led to a weak amplification product of about 1.800 bp visible after the first PCR, as illustrated representatively in Fig. 1A. In the other cases, a second, nested PCR was necessary to obtain a visible product. All these trials were successful with respect to the amplification of the expected fragment. Fig. 1B shows such a nested PCR with the primers GeoA2/Geo11, leading to an amplification product of about 1.760 bp. A single PCR carried out with GeoA2/Geo11 never produced a visible amplification product.

All fragments could be successfully cloned into the pCR2.1-TOPO vector. This “5-min cloning” is extremely efficient compared with other methods, saving many time-consuming steps. Normally, we confirmed six clones by restriction analysis and about 95% of the clones were positive for the expected fragment length.

Table 1 Nearly full-length SSU rRNA gene clones obtained with the method reported. Cloned fragments were validated by restriction analysis, partial sequencing (about 600 bp) and phylogenetic analysis. Clones validated by sequence analyses to be of glomalean origin are shown in the upper part of the table (no. 1–28), those of contaminant origin in the lower part (no. 29–37)

No.	Species	Accession number	Culture identity	Culture number	Voucher	Primers used for nested PCR	Provider	DNA clone number
1	*Acaulospora gerdemannii*	AJ301862	NC176			GeoA2/Geo11	Redecker	pWD147–1-1
2	*A. laevis*	Y17633	WUM46	Att896–8	W3107	NS1/Geo10	Walker	pWD95–1-4
3	*A. trappei*	Y17634		Att186–1	W3179	NS1/Geo10	Walker	pWD103–3-8
4	*Geosiphon pyriforme*	Y15904	GEO1			GeoNS1/Geo10	Schüßler	pHG-Geo1Aa
	Ge. pyriforme	Y15905	GEO1			GeoNS1/Geo10	Schüßler	pHG-Geo1Ba
	Ge. pyriforme	AJ276074	GEO1			GeoNS1/Geo10	Schüßler	pHG-Geo1Ca
5	*Gigaspora candida*	AJ276091	BEG17	Att26–19	W3292	GeoA2/Geo11	BEG/INRA	pWD131–7
6	*Gi. aff. margarita*	AJ276090			W2992	GeoA2/Geo11	Walker	pWD143–12
7	*Glomus caledonium*	Y17653	BEG15			NS1/Geo10	BEG/INRA	pKL10–2
8	*G. caledonium*	Y17635	BEG20	Att263–15	W3294	GeoA2/Geo11	Walker	pWD135–1
9	*G. claroideum*	AJ276075	BEG14			GeoNS1/Geo10	BEG/INRA	pKL2–9a
10	*G. claroideum*	AJ276080	BEG23			GeoNS1/Geo10	BEG/INRA	pKL14–4a
11	*G. claroideum*	AJ276079	BEG31	Att79–3	W1843	GeoNS1/Geo10	Walker	pKL4–2
12	*G. clarum*	AJ276084	BR147B-8	Att72–1	W3163	NS1/Geo11	INVAM	pWD125–1
13	*G. clarum*	AJ276083		Att672–13	W3161	GeoA2/Geo11	Walker	pWD116–1-2
14	*G. coronatum*	AJ276086	COG1	Att143–5	W3153	NS1/Geo10	Walker	pWD93–2-1
15	*G. etunicatum*	Y17639	UT316–8	Att678–4	W3093	NS1/Geo10	INVAM	pWD106–3-2
16	*G. etunicatum*	Y17644		Att382–16	W2423	GeoNS1/Geo10	Walker	pASGE-10
17	*G. fasciculatum*	Y17640	BEG53			GeoNS1/Geo10	BEG/INRA	pKL5–3
18	*G. fragilistratum*	AJ276085		Att112–6	W3238	NS1/Geo10	Walker	pWD114–3-3
19	*G. geosporum*	AJ245637	BEG11			GeoNS1/Geo10	BEG/INRA	pKL11–1a
20	*G. lamellosum*	AJ276087		Att244–13	W3160	NS1/Geo10	Walker	pWD100–2-6
21	*G. luteum*	AJ276089	SA101–1	Att676–0	W3184	GeoA2/Geo11	INVAM	pWD141–1
22	*G. manihotis*	Y17648		Att575–9	W3224	GeoA2/Geo11	Walker	pWD113–4-1
23	*G. occultum*	AJ276082	IA702–3	Att677–3	W3091	NS1/Geo10	INVAM	pWD117–1-1
	G. occultum	AJ276081	IA702–3	Att677–4	W3166	GeoA2/Geo11	INVAM	pWD108–2-1
24	*G. spurcum*	Y17650	Ex-Type	Att246–4	W2396	GeoNS1/Geo10	Walker	pHG-17
	G. spurcum	AJ276077	Ex-Type	Att246–4	W3239	NS1/Geo10	Walker	pWD115–1-9
25	*G. versiforme*	AJ276088		Att475–21	W3221	GeoA2/Geo11	Walker	pWD111–2-1
26	*G. viscosum*	Y17652	BEG27	Att179–8	W3207	NS1/Geo10	Walker	pWD107–1-2
27	*Scutellospora gilmorei*	AJ276094		Att590–1	W3085	GeoA2/Geo11	Walker	pWD140–3
28	*S. aurigloba*	AJ276092	WUM53	Att475–21	W3121	GeoNS1/NS8	Walker	pWD66–5
	S. aurigloba	AJ276093	WUM53	Att475–21	W3121	GeoNS1/NS8	Walker	pWD66–26
29	*A. lacunosa*	Basidiomycete	BEG78	Att626–8	W3289	GeoA2/Geo11	BEG/INRA	pWD158–2-3
	A. lacunosa	Ascomycete	BEG78	Att626–8	W3289	GeoA2/Geo11	BEG/INRA	pWD148–1-4
30	*A. laevis*	Ascomycete	BEG13	Att192–4	W3291	GeoA2/Geo11	BEG/INRA	pWD133–1
31	*A. scrobiculata*	Ascomycete	BEG33	Att209–37	W2393	GeoA2/Geo11	BEG/INRA	pWD149–1-4
32	*Entrophospora infrequens*	Ascomycete		Ȃtt672–5	W3067	GeoA2/Geo11	Walker	pWD137–23
33	*G. etunicatum*	Basidiomycete		Att367–1	W3248	NS1/Geo10	Walker	pWD122–1
34	*G. occultum*	Basidiomycete	CL700C-2	Att679–6	W3099	NS1/Geo10	INVAM	pWD118–1-4
35	*G. versiforme*	Basidiomycete	BEG47	Att475–22	W3180	GeoA2/Geo11	Bianciotto	pWD136–6
36	*S. calospora*	Basidiomycete	BEG32	Att333–17	W3290	GeoA2/Geo11	BEG/INRA	pWD146–2-1
37	*S. aurigloba*	Plant	WUM 47	Att871–3	W3105	GeoNS1/NS8	Walker	pWD49–2-4

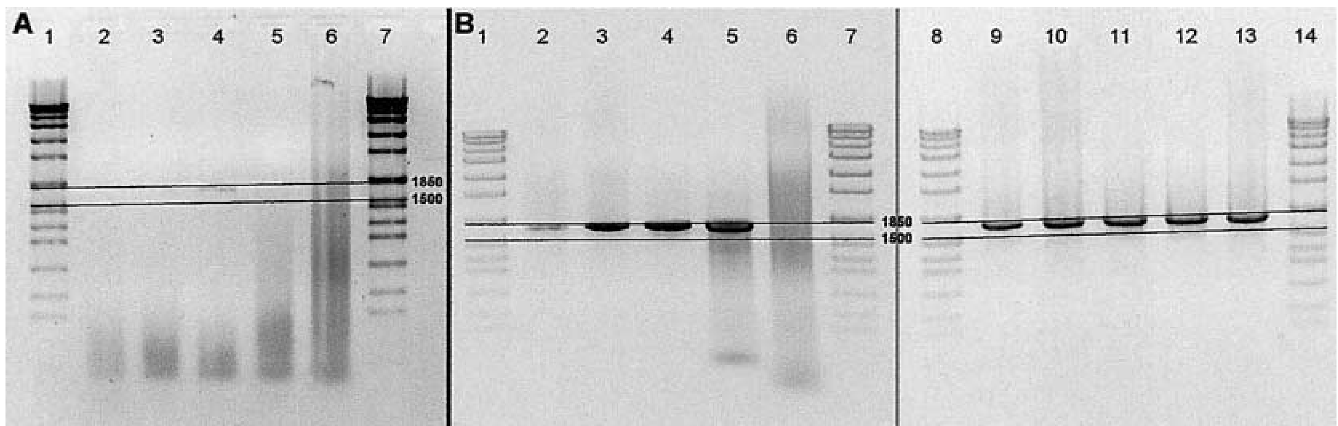

Fig. 1A,B Nested PCR with single spore extracts. The lines show the sizes of the 1500 bp and 1850 bp fragments of the SPP1 marker (bacteriophage SPP1 *Eco*R I digest; fragment lengths: 370, 490, 680, 1,000, 1,150, 1,400, 1,500, 1,850 1,900, 2,700, 3,500, 4,800, 6,000, 7,100, 8,100 bp). **A** First PCR from single-spore extracts. *Lanes 1 and 7* SPP1 marker, *6* control, *2–5* PCR products of single-spore extracts of: *2 Glomus luteum* SA101, *3 Scutellospora gilmorei* W3085, *4 Gigaspora aff. margarita* W2992, *5 Glomus caledonium* BEG20; 10 µl of the PCR reaction was loaded in each lane. Only one of four spores (*lane 4*) gave a visible amplification product after first PCR. **B** Second (nested) PCR. *Lanes 1, 7, 8 and 14* SPP1 marker, *2–6* nested PCR of products shown in A, *9–13* nested PCR from another representative set of different AMF (*Acaulospora gerdemannii* NC176, *Glomus versiforme* W3221, *Glomus manihotis* W3224, *Glomus clarum* W3161, *Glomus occultum* IA702) which showed no visible products after first PCR. All reactions led to products of the expected size; 5 µl of the PCR reaction was loaded in each lane

To verify the glomalean origin of the PCR fragments, one clone of each isolate was partly sequenced and analysed phylogenetically (not shown; see Schüßler 1999). The glomalean origin of SSU rRNA sequences was confirmed for 28 of 37 sequenced clones (76%), while in all other clones SSU rRNA sequences of contaminants from other fungal orders or green plants (1 clone) were identified. The work described here is still in progress and we are presently trying to amplify and clone fragments from newly obtained spores for those cases which resulted in sequences of contaminants.

Discussion

The extraction of DNA from single spores with Dynabeads in combination with nested PCR and the highly efficient TOPO-cloning system is a suitable and highly reliable method to clone nearly full-length SSU rRNA genes of glomalean fungi.

There are several reasons for using DNA extracted from single spores for phylogenetic analysis. First, the number of healthy spores available is sometimes low, in which case methods needing large numbers of spores (e.g. Hosny et al. 1999) are not useful. Another disadvantage is the extensive time required to collect and clean such a large number of spores. Probably the most important reason for using single spores for DNA extraction is the reduction of contamination by other organisms. This includes the possibility off cross-contamination in open pot cultures by other glomalean species. The removal of contamination attached to the spore surface is easier for one spore. In some cases, with the help of a taxonomist, single spores with clear characters can be sorted from mixed cultures and the sequence analysed, for example as was done for the newly described species *Scutellospora projecturata* (Kramadibrata et al. 2000).

Amplifying SSU rRNA genes from contaminants has caused severe problems in molecular biological investigations of AMF (Redecker 2000; Schüßler 1999). Since nested PCR is also very sensitive to contaminating DNA and because spores can be contaminated or parasitised, the origin of the sequences must be verified. Our strategy to avoid false SSU rDNA sequences in our phylogenetic studies is to first sequence about 600 bp of the cloned fragment with a universal primer. Sequences are then aligned using a wide-based dataset (see Schüßler 1999) and phylogenetic analyses performed to validate the glomalean origin before the complete fragment is sequenced. Details of the alignment dataset can be downloaded from http://www.geosiphon.de, where links and information about the software are also given.

The Dynabeads kit was developed to extract DNA from a larger amount of material (1–3 mg) but also works well for single AMF spores. It is possible to extract DNA from many samples simultaneously within less than 30 min with the method described. The DNA concentration is low since, in most cases, a PCR product can be visualised only after reamplification, but an amount equivalent to one sixth of a single-spore extract is sufficient for nested PCR, and inhibiting substances do not seem to be present. Although we have used the method for SSU rRNA genes, it should also be applicable to cloning of other chromosomal gene fragments obtained with specific primer sets.

Acknowledgement We thank the Deutsche Forschungsgemeinschaft for supporting this work (SCHU 1203; SFB199).

References

Abbas JD, Hetrick BAD, Jurgenson JE (1996) Isolate specific detection of mycorrhizal fungi using genome specific primer pairs. Mycologia 88:939–946

Bago B, Bentivenga S, Brenac V, Dodd JC, Piché Y, Simon L (1998) Molecular analysis of *Gigaspora* (Glomales, Gigasporaceae). New Phytol 139:581–588

Biancotto V, Bandi C, Minerdi D, Sironi M, Tichy HV, Bonfante P (1996) An obligately endosymbiotic mycorrhizal fungus itself harbours obligately intracellular bacteria. Appl Environ Microbiol 62:3005–3010

Chelius MK, Triplett EW (1999) Rapid detection of arbuscular mycorrhizae in roots and soil of an intensively managed turfgrass system by PCR amplification of small subunit rDNA. Mycorrhiza 9:61–64

Edwards SG, Fitter AH, Young JPW (1997) Quantification of an arbuscular mycorrhizal fungus, *Glomus mosseae*, within plant roots by competitive polymerase chain reaction. Mycol Res 101:1440–1444

Gadkar V, Adholeya A, Satyanarayana T (1997) Randomly amplified polymorphic DNA using the M13 core sequence of the vesicular-arbuscular mycorrhizal fungi *Gigaspora margarita* and *Gigaspora gigantea*. Can J Microbiol 43:795–798

Gehrig H, Schüßler A, Kluge M (1996) *Geosiphon pyriforme*, a fungus forming endocytobiosis with *Nostoc* (Canyobacteria), is an ancestral member of the Glomales: evidence by SSU rRNA analysis. J Mol Evol 43:71–81

Hahn A, Bonfante P, Horn K, Pausch F, Hock B (1993) Production of monoclonal antibodies against surface antigens of spores from arbuscular mycorrhizal fungi by an improved immunization and screening procedure. Mycorrhiza 4:69–78

Helgason T, Daniell TJ, Husban R, Fitter AH, Young JJW (1998) Ploughing up the wood-wide web? Nature 394:431

Hosny M, Hijiri M, Passerieux E, Dulieu H (1999) rDNA units are highly polymorphic in *Scutellospora castanea* (Glomales, Zygomycetes). Gene 226:61–71

Kramadibrata K, Walker C, Schwarzott D, Schüßler A (2000) A new species of *Scutellospora* with a coiled germination shield. Ann Bot 86:21–27

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. Mol Ecol 4:61–68

Lanfranco L, Delpero M, Bonfante P (1999) Intrasporal variability of ribosomal sequences in the endomycorrhizal fungus *Gigaspora margarita*. Mol Ecol 8:37–46

Longato S, Bonfante P (1997) Molecular identification of mycorrhizal fungi by direct amplification of microsatellite regions. Mycol Res 101:425–432

Millner PD, Mulbry WW, Reynolds SL, Patterson CA (1998) A taxon specific oligonucleotide probe for temperate zone soil isolates of *Glomus mosseae*. Mycorrhiza 8:19–27

Morton JB, Benny GL (1990) Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): A new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. Mycotaxon 37:471–491

Redecker D (2000) Specific PCR primers to identify arbuscular mycorrhizal fungi (Glomales) within colonised roots. Mycorrhiza 10:73–80

Redecker D, Thierfelder H, Walker C, Werner D (1997) Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. Appl Environ Microbiol 63:1–6

Redecker D, Hijri M, Dulieu H, Sanders IR (1999) Phylogenetic analysis of a dataset of fungal 5.8 S rDNA sequences shows that highly divergent copies of internal transcribed spacers reported from *Scutellospora castanea* are of ascomycete origin. Fungal Genet Biol 28:238–244

Redecker D, Kodner R, Graham LE (2000a) Glomalean fungi from the Ordovician. Science 289:1920–1921

Redecker D, Morton JB, Bruns TD (2000b) Ancestral lineages of arbuscular mycorrhizal fungi. Mol Phylogenet Evol 14:276–284

Remy W, Taylor TN, Hass H, Kerp H (1994) Four hundred million year old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci USA 91:11841–11643

Rosendahl S (1989) Comparisons of spore-cluster forming *Glomus* species (Endogonaceae) based on morphological characteristics and isoenzyme banding pattern. Op Bot 100:215–223

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

Schüßler A (1999) Glomales SSU rRNA gene diversity. New Phytol 144:205–207

Schüßler A (2000) *Glomus claroideum* forms an arbuscular mycorrhiza-like symbiosis with the hornwort *Anthoceros punctatus*. Mycorrhiza 10:15–21

Schüßler A, Kluge M (2000) *Geosiphon pyriforme*, an endocytosymbiosis between fungus and cyanobacteria, and its meaning as a model system for AM research. In: Hock B (ed) The Mycota IX. Springer, Berlin Heidelberg New York, pp 151–161

Schüßler A, Gehrig H, Schwarzott D, Walker C (2000) Analysis of partial Glomales SSU rRNA gene sequences: implications for phylogeny and primer design. Mycol Res (in press)

Simon L (1996) Phylogeny of the Glomales: deciphering the past to understand the present. New Phytol 133:95–101

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

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic, San Diego

Vandenkoornhuyse P, Leyval C (1998) SSU rDNA sequencing and PCR-fingerprinting reveal genetic variation within *Glomus mosseae*. Mycologia 90:791–797

Van Tuinen D, Jacquot E, Zhao B, Gollotte A, Gianinazzi-Pearson V (1998) Characterisation of root colonisation profiles by a microcosm community of arbuscular mycorrhizal fungi using 25 S rDNA-targeted nested PCR. Mol Ecol 7:879–887

Walker C, Trappe JM (1993) Names and epithets in the Glomales and Endogonales. Mycol Res 97:339–344

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 (eds) PCR protocols, a guide to methods and applications. Academic, San Diego, pp 315–322

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

Note added in proof Because of changes in the taxonomic concept, names of some species used in this study will change (former names in parenthesis): *Archaeospora trappei* (*Acaulospora trappei*), *Archaeospora leptoticha* (*Acaulospora gerdemannii*), *Archaeospora gerdemannii* (*Glomus gerdemannii*), *Paraglomus brasilianum* (*Glomus brasilianum*), *Paraglomus occultum* (*Glomus occultum*) (Morton, J.B., Redecker, D. (2001) Two new families of Glomales, Archaeosporaceae and Paraglomaceae, with two new genera *Archaeospora* and *Paraglomus*, based on concordant molecular and morphological characters. *Mycologia*, in press).