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# A DGGE-cloning method to characterize arbuscular mycorrhizal community structure in soil

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## Abstract

Although arbuscular mycorrhizal fungi (AMF) are crucial for ecosystem functioning, characterizing AMF community structure in soil is challenging. In this study, nested polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) were combined with cloning of fungal 18S ribosomal gene fragments for the rapid comparison of AMF community structure in soil. Reference AMF isolates, representing four major genera of AMF, were used to develop the method. Sequential amplification of 18S rDNA fragments by nested PCR using primer pairs AM1-NS31 and Glo1-NS31GC followed by DGGE analysis yielded a high-resolution band profile. In parallel, 18S rDNA fragment clone libraries were constructed and clones screened by DGGE. Sequence identity was inferred by matching the electrophoretic mobility of the sample fingerprint bands to that of bands from individual clones. The effectiveness of this approach was tested on soil samples from different ecosystems, yielding reproducible, complex DGGE band patterns specific to each site. The coupling of PCR–DGGE with clone library analysis provides a robust, reliable, and precise means to characterize AMF community structure in soils.

**Keywords:** 18S rDNA, nested PCR, DGGE, clone, AMF community structure

## 1. Introduction

Arbuscular mycorrhizal fungi (AMF) belonging to the phylum Glomeromycota are abundant in most soil ecosystems, where they form mutualistic associations with the majority of higher plants. AMF can efficiently absorb and transport mineral nutrients, such as phosphorous and zinc from the soil to the host plant, through an extended, intricate hyphal network, in exchange for carbohydrates. Thus, AMF influence the growth, reproduction, and health of the plant (Subramanian and Charest, 1997). AMF also improve aggregate stability, thereby building up a macroporous soil structure, that allows the penetration of water and air and prevents erosion (Jastrow *et al.*, 1998). Therefore, AMF play a key role in the functioning of terrestrial ecosystems, being critical for

both plant community structure and ecosystem productivity (Klironomos *et al.*, 2000).

Despite their ecological importance, little is known about the population biology and diversity of AMF in natural ecosystems, mainly because of methodological limitations. Traditional studies on AMF diversity are based mainly on spore morphology (Walker, 1992), but taxonomic identification of AMF spores collected directly from the field is quite difficult because they are often unidentifiable due to degradation or parasitization by other organisms (Rousseau *et al.*, 1996). Furthermore, as they are obligate symbionts, they cannot be cultivated in the absence of their host (De Souza and Barbara, 1999). Catch plants are often used to produce identifiable spores (Bever *et al.*, 2001). Although useful for the isolation and propagation of some AMF species, this indirect

culture strategy is time consuming and biases are often introduced by plant preference for AMF species, different growth conditions, and other environmental factors, which hinder its suitability for characterization of AMF communities (Jansa *et al.*, 2002; Oehl *et al.*, 2003). Additionally, recovery of spores from soil is often problematic and the abundance of spores in the soil may not accurately reflect AMF community composition and dynamics (Clapp *et al.*, 1995).

Molecular techniques circumvent problems associated with morphological identification of AMF. The polymerase chain reaction (PCR) can target specific AMF DNA sequences, the majority being ribosomal RNA (rDNA) genes. Sequence variation can then be visualized with electrophoresis (see Clapp *et al.*, 2002 for a review). Molecular techniques provide a vigorous means of characterizing AMF to enhance our understanding of the phylogeny (Schussler *et al.*, 2001), ecology (Helgason *et al.*, 1998), and evolution (Sanders, 2002) of this group of fungi. Simon *et al.* (1992) initiated molecular characterization of AMF. Since then, especially after the appearance of primers with improved specificity to the glomalean SSU rRNA gene (Helgason *et al.*, 1998; Redecker, 2000), a number of PCR-based methods have been applied to AMF, including restriction fragment length polymorphism (RFLP) (Helgason *et al.*, 1999; Daniell *et al.*, 2001), single-stranded conformation polymorphism (SCCP) (Kjoller and Rosendahl, 2000), terminal RFLP (T-RFLP; Vandenkoornhuyse *et al.*, 2003), denaturing gradient gel electrophoresis (DGGE) (Kowalchuk *et al.*, 2002; Opik *et al.*, 2003; Ma *et al.*, 2005), and temperature gradient gel electrophoresis (TGGE) (Cornejo *et al.*, 2004).

In DGGE or TGGE, DNA fragments of the same length but of different sequence can be separated (Muyzer and Smalla, 1998). Separation is based on the melting behavior of fragments with different sequence composition under increasing gradients of denaturants or temperature. Since its introduction for the analysis of bacterial community structure (Muyzer *et al.*, 1993), this method has been widely used in the characterization of soil bacterial (Kozdroj and Van Elsas, 2000) and fungal (Pennanen *et al.*, 2001; Kowalchuk *et al.*, 2006) as well as micro-fauna communities (Waite *et al.*, 2003; Foucher *et al.*, 2004) from various environments. Kowalchuk *et al.* (2002) was the first to apply DGGE to assess AMF diversity in sand dune soil and root samples. Other studies used DGGE to detect AMF in forest and grassland soil (Opik *et al.*, 2003), agricultural ecosystems (Ma *et al.*, 2005), and for the discrimination of AMF species (De Souza *et al.*, 2004).

To obtain phylogenetic information from DGGE analysis, bands are often excised and the recovered DNA reamplified and separated by repeated cycles of PCR–DGGE until single bands are obtained for sequencing. This approach has numerous limitations (Gonzalez *et al.*, 2003; Handschur *et al.*, 2005), including (1) the likelihood of obtaining multiple DNA sequences from a single band of interest (Gonzalez *et al.*, 2003) due to the co-migration of different sequences in DGGE (Rolleke *et al.*, 1999), (2) the excision of

bands that are very close to each other may be difficult, (3) excessive exposure to UV light during excision and repeated amplifications could introduce sequence variation artifacts (Schabereiter-Gurtner *et al.*, 2001), which may result in unusable sequences (Ma *et al.*, 2005), and (4) the target length of DNA fragments (200–500 bp, in this study 230 bp) may limit detailed phylogenetic analysis. Alternatively, the recovered DNA fragments from the excised bands could be cloned and sequencing performed on each single clone. This is accurate but more costly. Without exception, the above-mentioned studies used one of the band-excision methods for phylogenetic analysis of AMF.

One way to overcome the drawbacks of recovering sequence data from excised bands is to combine DGGE analysis with the construction of clone libraries. A global view of the microbial diversity can be obtained by DGGE profiling of a sample from a specific environment. Later, sequence information for bands of interest can be acquired from a single clone that produces a DGGE band at the same position as the DGGE band from the environmental sample. Our objective was to develop a rapid and reliable DGGE-cloning-based approach for the characterization of AMF, based on sequence variation in a region of the 18S rDNA gene. The method was tested using DNA from cultured species within the phylum Glomeromycota. Its feasibility was evaluated using DNA extracted from soil samples with various physical, chemical, and biological properties. To our knowledge this is the first comprehensive application of a DGGE-cloning-based approach to assess AMF community structure in environmental samples.

## 2. Materials and methods

### 2.1. Reference AMF species

The AMF strains, representing three families of Glomeromycota, used for the development of this DGGE-cloning protocol (Figure 1) are listed in Table 1. Cultures (200 g) of standard whole inoculum consisting of soil, infected roots, and spores were obtained from INVAM (International Culture Collection of Arbuscular and Vesicular–Arbuscular Mycorrhizal Fungi, Morgantown, USA; <http://invam.caf.wvu.edu>), after which cultures were stored at 4 °C for less than 15 days before spore extraction.

### 2.2. Field samples

Soil samples were collected from the following four ecosystems: grassland (NG) and eastern red cedar (*Juniperus virginiana* L., NR) forest ecosystems at the Nebraska National Forest in the Nebraska Sandhills, Halsey, NE (41° 51' 45" N, 100° 22' 06" W); grassland (PG) from Nine Mile Prairie, Lincoln, NE (40° 52' N, 96° 49' W); and a corn–soybean cropping system (CS) located on the University of Nebraska–Lincoln east campus, Lincoln, NE (40° 49' 12" N, 95° 39' W).

The dominant vegetation types at the two grasslands are prairie sandreed, kentucky bluegrass, little bluestem, and switchgrass for the NG site and big bluestem, indiangrass, switchgrass, and smooth bromegrass for the PG site. Soil samples from NG and NR were collected in July 2006 and from PG and CS in August 2006. Soil cores (2.54 × 10 cm<sup>2</sup>) were randomly collected from 30 locations within each site, pooled, and thoroughly mixed by sieving to 2 mm. A 100 g sub-sample was stored at -20 °C prior to DNA extraction.

2.3. Spore extraction and preparation for DNA extraction

Standard wet sieving and centrifugation procedures modified from INVAM (<http://invam.caf.wvu.edu/methods/spores/extraction.htm>) were used to extract spores from reference cultures. Briefly, 50 g of inoculum was blended in ddH<sub>2</sub>O at maximum speed for 5 s, after which the suspension was immediately poured through a pair of sieves (250 and 53 μm mesh). Material on the top sieve (250 μm, spores and plant root residues together with some sand) was washed and transferred to a large Petri dish for collecting spores from *Gigaspora gigantea*. For the remaining species, the material on the bottom sieve (53 μm) was suspended in water and centrifuged at 960g for 4 min. The resulting pellet was resuspended in a

20–60% gradient of sucrose solution and centrifuged (960g) for another 2 min. The suspension was then poured through a sieve of 38 μm, washed thoroughly under tap water, and transferred to Petri dishes. Spores were collected manually under a binocular stereomicroscope with an extruded 23 cm glass pipette. They were then stored at 4 °C for 3 weeks before further cleaning. Parasitized and degraded spores were removed during weekly checks. Intact spores were further cleaned by ultrasonication and rinsing (De Souza *et al.*, 2004). Individual clean spores were collected and transferred to a 1.5 ml microcentrifuge tube (30–100 spores each) and stored at -80 °C until DNA extraction.

2.4. DNA extraction from spore and bulk soil

DNA extraction from multiple extracted spores was performed according to Kowalchuk *et al.* (2002) with the following modifications. After the spores were crushed in 1.5 ml eppendorf microcentrifuge tube on ice with a glass micropestle, 40 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), buffer and 20 μl 20% Clelex 100 (Bio-Rad Laboratories, Hercules, CA, USA) were added. After the spores were crushed again, four freeze-thaw cycles were carried out using liquid N<sub>2</sub> and boiling water. Lysed spores were incubated at 95 °C for 5 min and then centrifuged at 10,000g for 5 min. The supernatant was transferred to a new microcentrifuge tube and stored at -20 °C until use.

Initially, PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Solana beach, CA, USA) was used to extract DNA from fresh soil according to the manufacture's instruction; however, when using AMF-specific primers to amplify AMF 18S rDNA fragments from the extracted DNA, amplification was inconsistent. The following modifications were made to obtain more uniform DNA extraction: (1) prior to DNA extraction, 10 g of freeze-dried field-collected soil was homogenized and ground in liquid N<sub>2</sub> with a mortar and pestle, (2) soil processed for each kit was increased from 0.25 to 0.5 g, (3) 0.2 g of glass beads (0.1 mm diameter, Cat. no. 11079101, Bio-spec Products Inc., Bartlesville, OK, USA) was added to each lysis tube before lysing, (4) bead beating for 3 min at 4600 rev min<sup>-1</sup> in a mini-bead beater cell disrupter (type BX-4, Catalog No. 311OBX; Bio-Spec Products Inc., Bartlesville, OK, USA) was substituted for vortex mixing, (5) the spin filter was air dried for 10 min at room temperature

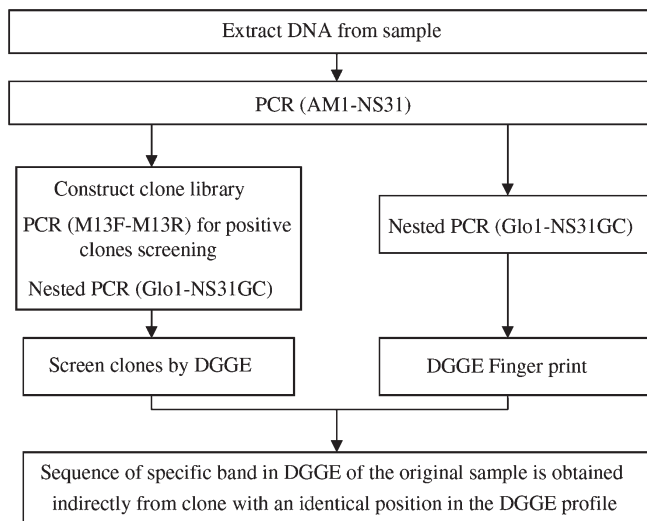


Figure 1. Description and flow chart illustrating the experimental procedures used in this study to analyze AMF community structure.

Table 1. Reference AMF species used to develop the DGGE-cloning protocol

Accession no.	Species (authority)	Source	No. of bands in Fig. 2 dominant/faint
BR601B	<i>Acaulospora scrobiculata</i> Trappe	INVAM	3/1
CA201	<i>Glomus mosseae</i> (Nicol. & Gerd.) Gerd. & Trappe	INVAM	1/1
IA506	<i>Glomus intraradices</i> Schenck & Smith	INVAM	2/8
IL203A	<i>Scutellospora heterogama</i> (Nicol. & Gerdemann) Walker & Sanders	INVAM	2/1
MN922A	<i>Gigaspora gigantea</i> (Nicol. & Gerd.) Gerd. & Trappe	INVAM	5/0

(25 °C), after it was placed in a clean 2 ml collection tube to evaporate the residual volatile solvent, which may inhibit subsequent amplification reaction, (6) the volume of the final elution solution was reduced to 50 µl. The yield and fragmentation of the DNA were checked by agarose gel electrophoresis (0.8%), followed by ethidium bromide staining and visualization under UV light. The DNA extract was stored at -20 °C for future use.

### 2.5. Nested PCR strategy for amplification of 18S rDNA fragments

Initially the amplification product of primer pair AM1/NS31GC was used for DGGE analysis; however, amplified spore DNA yielded a single fuzzy band for all five AMF species. Moreover because of the low concentration of AMF 18S rDNA fragments in the soil DNA extraction, none of the soils tested yielded detectable signals on the DGGE gel profiles, indicating minimal amplification. The lower resolving power of AM1/NS31GC amplification product on DGGE or TGGE was also observed by Opik *et al.* (2003) and Cornejo *et al.* (2004). To increase the resolution and yield, nested PCR was used as described below.

All PCR reactions were performed in 0.5 ml thin wall PCR tubes, using 1 unit Taq DNA polymerase (Invitrogen) in a final volume of 25 µl, containing 1×PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4) overlaid with 50 µl mineral oil. Negative controls consisted of 1 µl of molecular grade water in all sets of PCR reaction to check for contamination. In nested PCR, a second control consisted of a reamplified negative control from the first round of PCR. PCR was carried out under sterile conditions and all disposable plasticware was autoclaved prior to use.

DNA isolated from spores and soil samples were first amplified with the AMF specific primer AM1 (5'-GTTTCCC-GTAAGGCGCCGAA-3'; Helgason *et al.*, 1998) in combination with the universal eukaryotic primer NS31 (5'-TTGGAGGGCAAGTCTGGTGCC-3'; Simon *et al.*, 1992) to get an approximate 580 bp fragment of the 18S rRNA gene. Five microliters of spore DNA extract or 1 µl of 1/10 dilution of soil DNA extract was used as the template. The PCR mixture was composed of 200 µM each of the four deoxynucleoside triphosphates, 1.5 mM MgCl<sub>2</sub>, and a 0.2 µM concentration of each primer. An aliquot of 0.3 µg µl<sup>-1</sup> acetylated bovine serum albumin (BSA, Promega, Cat# R3961) was added to each reaction to enhance the activity of DNA polymerase. PCR amplifications were carried out with a PerkinElmer Cetus DNA thermal cycler (PerkinElmer, Boston, MA, USA) using one cycle of 1 min at 94 °C, 1 min at 66 °C, and 1 min 30 s at 72 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 66 °C, and 1 min 30 s at 72 °C, and a final extension at 72 °C for 10 min. Five microliters of the PCR product was analyzed by agarose gel electrophoresis (0.8% (w/v) agarose; 100 V, 60 min) and ethidium bromide staining to confirm the presence of product.

Amplification product from the first PCR reaction was diluted 1/10 and 1 µl of this dilution was used as the template in a second round of PCR using the NS31-GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCAC-GGGGGTTGGAGGGCAAGTCTGGTGCC-3'; Kowalchuk *et al.*, 2002) and the Glo1 (5'-GCCTGCTTTAAACTCTA-3'; Cornejo *et al.*, 2004) primers with the same reaction mixture as the first round of PCR, except for primers and no BSA addition. The following thermocycling conditions were used: initial denaturation for 5 min at 95 °C, followed by 35 cycles with denaturation for 45 s at 94 °C, annealing for 45 s at 52 °C, and extension for 1 min at 72 °C. A final extension step at 72 °C for 30 min was conducted to allow complete extension for all fragments (Janse *et al.*, 2004). The amplicons were analyzed by agarose gel electrophoresis (0.8% (w/v) agarose, 80 V, 60 min) and ethidium bromide staining to check integrity and yield, and stored at -20 °C for subsequent DGGE analysis.

### 2.6. Analysis of PCR products by DGGE

Twenty microliters of nested PCR product generated by Glo1 and NS31-GC primer pair was subsequently analyzed by DGGE on a 20 slot Hoefer™ SE 600 Standard dual cooled gel electrophoresis unit (Amersham Biosciences, Piscataway, NJ, USA). Denaturing gels were generated using an SG series gradient mixer (Hoefer Scientific Instruments, San Francisco, CA, USA) and a P-1 peristaltic pump (Pharmacia Fine Chemicals), by standard procedures. The gradient was made at an approximate rate of 4 ml min<sup>-1</sup>. Gels (18 × 16 cm<sup>2</sup>) containing 8% (w/v) polyacrylamide (37.5:1 acrylamide/bis-acrylamide; Sigma Cat# A7168) and 1×TAE (Tris/acetic acid/EDTA, pH 8.0) buffer were 0.75 mm thick. A linear gradient from 35% to 55% denaturant was used for all analysis, where 100% denaturant contains 7 M urea (Sigma, Cat# U5378) and 40% (v/v) formamide (Sigma Cat# F9037, Muzer *et al.*, 1993). A 3 ml stacking polyacrylamide gel containing no denaturant was added after the denaturing gel polymerized for 10 min. The electrophoresis tank was placed on a stirrer for uniform distribution of heat during electrophoresis. All DGGE analysis was performed in 1×TAE buffer at a constant temperature of 60 °C at 80 V for 10 min, followed by 50 V for 990 min. For comparison of the DGGE pattern, a reference marker, prepared as described below, was added to both sides of the gel. Gels were stained for 20 min in 0.5 mg l<sup>-1</sup> ethidium bromide and destained twice for 15 min in MilliQ water. Pictures were captured with Gel Doc 2000 Gel documentation system (Bio-rad, Hercules, CA, USA) and digitized by the Quality One Quantitation Software version 4 (Bio-rad, Hercules, CA, USA).

### 2.7. Cloning of 18S rDNA fragments

Clone libraries were constructed based on the 18S rDNA fragments generated with primer pair AM1 and NS31 us-

ing the same PCR conditions as described above. To obtain enough DNA for cloning, products of 10 PCR from the same sample were pooled and concentrated to a final volume of 30  $\mu$ l using Wizard DNA clean-up system (Promega, Madison, WI, USA). The entire concentrated product was subjected to agarose gel electrophoresis (0.8% agarose, 100 V, 60 min). The gel portion containing the desired DNA fragment was excised with a blade and the DNA purified with QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. The purified product was cloned into plasmid vector pCR 2.1 and the ligation product transformed into *Escherichia coli* (strain TOP10) by electroporation using the TOPO TA Cloning Kit (Invitrogen, <http://www.invitrogen.com>, Cat# K4560-01) following manufacturer instructions. The transformed cells were plated onto LB (Luria-Bertani) medium (1.0% Bacto-Tryptone, 0.5% Bacto-yeast extract, 1.0% NaCl, 1.5% Bacto-agar, pH 7.0) containing ampicillin (50  $\mu$ g ml<sup>-1</sup>) and X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -d-galactopyranoside: 0.1 mM) to identify white-colored recombinant colonies (Sambrook *et al.*, 1989).

### 2.8. Screen of clone libraries by PCR–DGGE

Screening of the clone libraries by PCR and DGGE was carried out as described by Schabereiter-Gurtner *et al.* (2001). To confirm the presence of inserts, white colonies of each sample were selected, resuspended in 40  $\mu$ l TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), followed by cell lysis by three freeze–thawing cycles using boiling water and ice. Three microliters of the lysate was used as template DNA for PCR, in a volume of 25  $\mu$ l, with 0.4  $\mu$ M each of the vector-specific primers M13 forward (–20) (M13F) (5'-GTAAAACGACGGCCAG-3') and M13 reverse (M13R) (5'-CAGGAAACAGCTATGAC-3') using Taq DNA polymerase. PCR amplifications were carried out using initial denaturation at 94 °C for 4 min, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 48 °C and 1 min 30 s extension at 72 °C, and a final extension at 72 °C for 10 min. Five microliters of product was analyzed by agarose gel electrophoresis (0.8%, 100 V for 60 min). Positive clones were confirmed based on their length of about 780 bp, including the 580 bp insert, and the two flanking regions of the plasmid. Clones without insert contained a single 200 bp PCR product.

One microliter of the amplification product (diluted 1/10), from the positive clones with M13F and M13R primers, was used as template in nested PCR with primer pair Glo1 and NS31-GC as described above in a volume of 25  $\mu$ l. Ten microliters of the PCR product from each positive clone was run on DGGE gel to determine the electrophoretic mobility. Inserts with different DGGE band types were compared with the band pattern of the original sample. Representative inserts for each band type matching the band patterns for the original samples were sequenced as described below.

### 2.9. Creation of a reference marker for DGGE

To compare the DGGE patterns from different gels, 10 different clones of isolates *G. gigantea*, *Acaulospora scrobiculata*, and *Scutellospora heterogama*, exhibiting different band positions, were used to produce a DGGE marker. One hundred microliters of PCR product obtained from each clone with primer pair Glo1 and NS31GC was pooled and precipitated overnight in chilled 96% ethanol, and then resuspended in 100  $\mu$ l ddH<sub>2</sub>O. Ten microliters was used as marker for DGGE analysis.

### 2.10. Sequencing of cloned inserts and DGGE bands and affiliation analysis

For sequencing of the clone inserts, 100  $\mu$ l PCR product generated with primers M13F and M13R were concentrated to a final volume of 30  $\mu$ l using Wizard DNA clean-up system (Promega, Madison, WI, USA), after which they were subjected to agarose gel electrophoresis (0.8% (w/v), 100 V, 80 min). The gel portion containing the expected DNA fragment was excised and DNA purified with QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA) with a final elution volume of 30  $\mu$ l. To obtain sequences from DGGE bands, gel-containing fragments of interest were excised and DNA eluted, reamplified with primer pair Glo1 and NS31GC, separated, and confirmed as described by Ma *et al.* (2005). PCR products were purified as described above. Purified DNA was sent to the Genomics Core Research Facility (GCRF) at University of Nebraska Lincoln (<http://greengene.unl.edu>) for sequencing. Sequencing was performed for both strands of each DNA sample using M13F and M13R as sequencing primers. Sequences were deposited in the European Molecular Biology Laboratory (EMBL) database under accession numbers AM746132, AM746133, AM746134, AM746135, AM746136, AM746137, AM746138, AM746139, AM746140, AM746141, AM746142, AM746143, AM746144, AM746145, AM746146, AM746147, AM746148, AM746149, AM746150, AM746151, AM746152, AM746153, AM746154, AM746155, AM746156 and AM746157–AM746157. Possible chimeric sequences were screened using Ribosomal Database Project (RDP release 8.1) online Chimera Check program (<http://rdp8.cme.msu.edu/html/analyses.html>) (Maidak *et al.*, 2001). Similarity comparisons, to known 18S rDNA sequences in the database, were performed using the online (Basic Local Alignment Search Tool) program (BLAST; <http://www.ncbi.nlm.nih.gov/BLAST>).

## 3. Results

Total genomic DNA isolated either from reference species or from environmental samples was of high molecular weight and of sufficient purity for subsequent PCR amplification. Nested PCR successfully produced AMF 18S rDNA fragments of the expected size (about 230 bp), whose fingerprints were achieved after separation of PCR products by DGGE.

This PCR–DGGE approach produced a fairly high number of distinct, sharp, and intense DGGE bands for AMF. No discernable difference in the DGGE patterns between replicates was detected. All intense DGGE bands in the environmental samples could be matched in the clone library. Sequence information of the dominant bands and some less dominant bands could be inferred from the clones that share the identical position in DGGE, thus circumventing problems associated with excised DGGE bands for phylogenetic analysis.

### 3.1. PCR–DGGE analysis of reference species

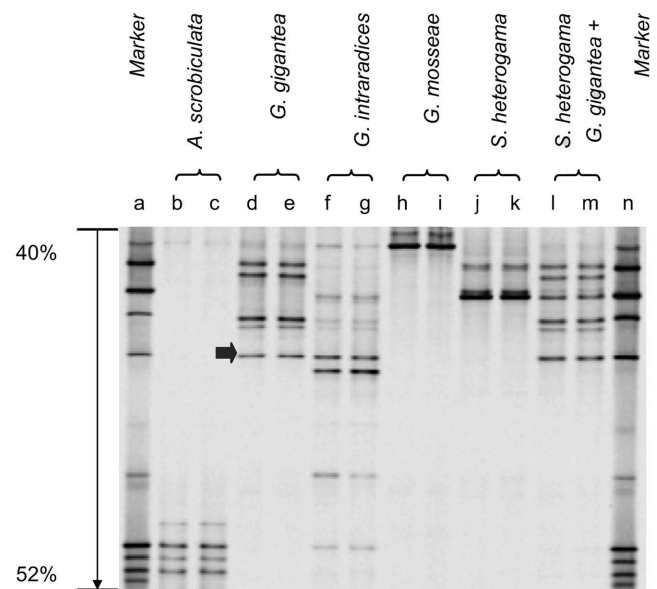
Amplification of DNA from reference species with primer pair AM1–NS31 yielded products of the correct size (approximately 560 bp) when visualized on an agarose gel, although slight size differences could be detected in some cases from the sequence analysis (results not shown). DGGE profiles of nested PCR products generated fragments from the five species that migrated to different positions in the range of 40–52% denaturant concentration in the DGGE gel (Figure 2). In contrast with the other species, the DGGE profile of *A. scrobiculata* had bands in the lower regions (GC-rich region). All five species yielded multiple bands. A single dominant band and a faint band were obtained for *Glomus mosseae* and *S. heterogama*, respectively. The other three reference isolates yielded multiple bands with three dominant bands and one faint band for *A. scrobiculata*, two dominant bands and eight faint bands for *Glomus intraradices*, and five dominant bands for *G. gigantea*. BLAST search indicated that the sequences of the five bands produced by *G. gigantea* and the three dominant bands by *A. scrobiculata* had high similarity (98.5–99.6% identity) to the 18S rDNA sequences of *G. gigantea* and *A. scrobiculata*, respectively, in the database (Table 2), confirming their origins. One dominant band of *G. gigantea* had the same migratory behavior as that of one dominant band of *G. intraradices* (marked with arrow in Figure 2). Each species had a unique DGGE banding profile visually distinguishable from other species.

To check if this PCR–DGGE approach could discriminate mixed dissimilar AMF species, five spores from *G. gigantea* and ten spores from *S. heterogama* were combined, to form an artificial simple AMF community, and subjected to the spore DNA extraction and PCR–DGGE approach described above. In Figure 2, lanes l and m contained bands with same mobility to the dominant bands of isolate *G. gigantea* (lanes d and e) and *S. heterogama* (lanes j and k). Thus, bands of the different species can be clearly separated in one lane. The faint band for *S. heterogama*; however, became less visible in lanes l and m of the mixed community compared with the corresponding band for the single species in lanes j and k.

### 3.2. Characterization of AMF community structure in soil samples by PCR–DGGE

The nested PCR–DGGE procedure described above was validated by studying AMF community structure in soil sam-

ples from four different ecosystems with different physical, chemical, and biological properties. Analysis was performed in duplicate on the same DGGE gel. The resulting DGGE profiles contained well-separated intense and faint bands (Figure 3) and revealed high and consistent AMF complexity. Profiles for the same sample were always similar, while different samples yielded dissimilar patterns. Overall, between five (NG) and fourteen (NR) bands were visualized in each profile. Soil samples from forest (NR) yielded two dominant bands and up to 12 faint bands. There were four dominant bands and four less intense bands for samples from cropped soils (CS). The grassland soils contained two dominant bands and nine less intense bands for PG and three dominant and two faint bands for NG. One dominant band, marked with arrow C in Figure 3, was visible in the DGGE pattern derived from most samples except NR. In addition, one band from CS marked with arrow D was of very high intensity, compared to bands from other soil samples. These distinct characteristics distinguished samples of different origins from each other. The two dominant bands in the DGGE profile of NR samples (marked as A and B in Figure 3) were excised, reamplified, rechecked by DGGE, and sequenced for comparison purposes.

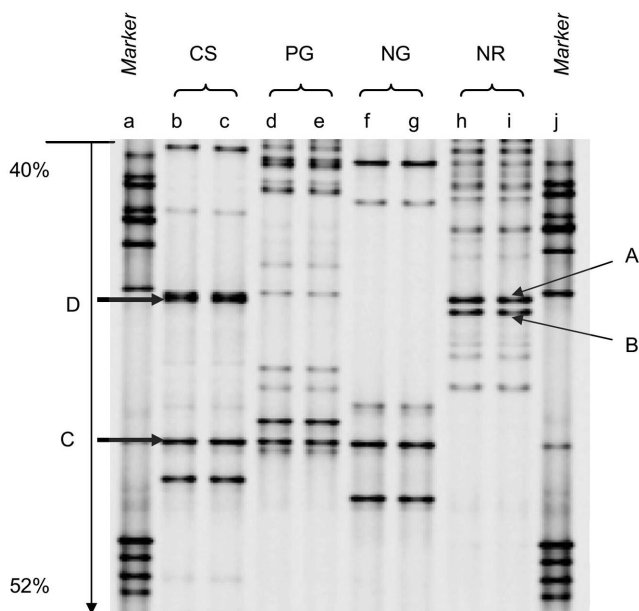


**Figure 2.** DGGE profile of 18S rDNA fragments from reference AMF species. Lanes a and n: marker; lanes b and c: *A. scrobiculata* BR601B; lanes d and e: *G. gigantea* MN922A; lanes f and g: *G. intraradices* IA509; lanes h and i: *G. mosseae* CA201; lanes j and k: *S. heterogama* IL203A; lanes l and m: mixture of *G. gigantea* MN922A and *S. heterogama* IL203A. Horizontal arrow indicates the band common to both *G. gigantea* MN922A and *G. intraradices* IA509. Vertical arrow indicates that the denaturant gradient increases from 40% on the top to 52% on the bottom of the gel (100% denaturant concentration is defined as containing 7 M urea and 40% (v/v) formamide).



**Table 2.** Identity of selected DGGE bands from clones of reference isolates *Gigaspora gigantea* and *Acaulospora scrobiculata*

Sequence designation	INVAM classification	Closest match from GenBank Sequence	similarity by BLAST (%)
G1	<i>G. gigantea</i> MN922A	<i>G. gigantea</i>	99.4
G2	<i>G. gigantea</i> MN922A	<i>G. gigantea</i>	98.5
G3	<i>G. gigantea</i> MN922A	<i>G. gigantea</i>	98.5
G4	<i>G. gigantea</i> MN922A	<i>G. gigantea</i>	98.7
G5	<i>G. gigantea</i> MN922A	<i>G. gigantea</i>	98.9
A2	<i>A. scrobiculata</i> BR601B	<i>A. scrobiculata</i>	99.6
A3	<i>A. scrobiculata</i> BR601B	<i>A. scrobiculata</i>	99.4
A4	<i>A. scrobiculata</i> BR601B	<i>A. scrobiculata</i>	99.3



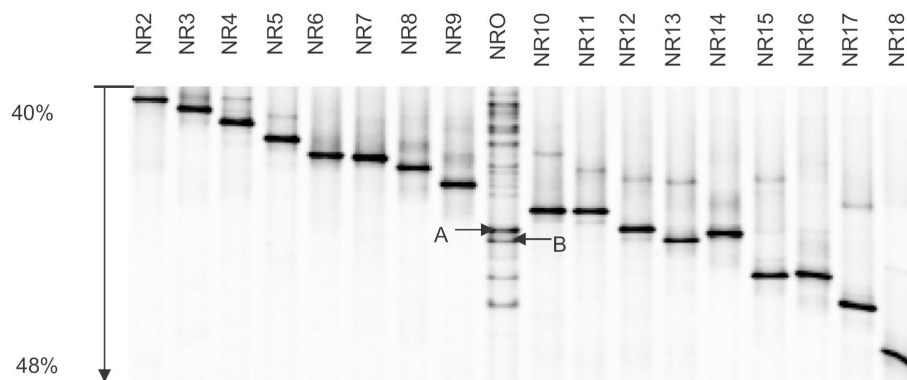
**Figure 3.** Band pattern of DGGE analysis of AMF 18S rDNA fragments from soils of different origins. Lanes a and j: marker; lanes b and c: CS; lanes d and e: PG; lanes f and g: NG; lanes h and i: NR. Bands marked with A and B were excised, amplified, purified, and sequenced. Arrow marked with C indicates a dominant band common in all the samples except NR. Arrow marked with D indicates a band in CS with exceptional intensity. Vertical arrow indicates that the denaturant gradient increases from 40% on the top to 52% at the bottom of the gel with 100% denaturant defined as containing 7 M urea and 40% (v/v) formamide. (CS: soil sample from corn–soybean cropping system, Lincoln, Nebraska; PG: grassland at Nine Mile Prairie, Lincoln, Nebraska; NG: grassland at Nebraska National Forest in Sandhill area, Halsey, Nebraska; NR: red cedar forest at Nebraska National Forest in Sandhill area, Halsey, Nebraska.)

### 3.3. Clone library analysis for select reference species and soil samples

PCR amplification products of the 18S rDNA fragment with primer pair AM1 and NS31 from representative species *G. gigantea* and *A. scrobiculata* and from soil sample NR, which had the most complex DGGE profile, were cloned for further analysis. The clone libraries obtained were designated G, A, and NR, respectively (G for clone library of *G. gigantea*, A for clone library from *A. scrobiculata*, and

NR for clone library from red cedar forest soil sample). In all 40 clones were picked from library G, 38 clones were picked from library A, and 144 clones were picked from library NR; 37, 35, and 136 positive clones from each clone library, respectively, were produced. Subsequent screening by DGGE produced six different bands types for clones G, five of which could be matched to bands from the *G. gigantea* DGGE profile. The entire four band types present in clone library A matched to DGGE profile of *A. scrobiculata*. All the dominant band types and most of the less dominant band types in the original DGGE profiles from the NR sample could be matched to the corresponding clone library (Figure 4). Clones showing different mobility on DGGE were coded to indicate their origins and relative positions (the topmost band on the gel was no. 1, with numbers increasing to the lower part of the gel), e.g., NR5 indicates the clone was from NR, band no. 5. One representative of each clone type was further amplified, purified, and sequenced. Sequence output contained very few ambiguous positions, especially in the range of the inserts, indicating high quality. Sequence identities for the bands in the original DGGE profile were inferred from the sequences of the clones that migrated to the same vertical position.

A BLAST similarity search in the GenBank database (Table 2) indicated that all five *G. gigantea* and three *A. scrobiculata* derived sequences belonged to members of *G. gigantea* and *A. scrobiculata*, respectively, with high similarity (98.5–99.6% identity). All of the sequences from NR had high similarity (97–99.8% identity) to AMF sequences in the database (Table 3) and belonged to members of the genus *Glomus* in the phylum Glomeromycota, with the exception of NR1 and NR11. The former was proven through Chimera Check to be chimeric and the latter had a maximum sequence similarity of only 94.1% identity to one uncultured *Glomus* species. Four sequences (NR9, NR10, NR12, and NR13) had high similarities (99.3–99.8% identity) to the same *Glomus* sequence (uncultured *Glomus* isolate Glo60 clone F9AGMyc37). Three pairs of sequences (NR4 and NR6, NR8 and NR14, NR15 and NR17) were affiliated closely to three uncultured *Glomus* isolates. In ad-



**Figure 4.** DGGE profile of AMF 18S rDNA clone library from NR (red cedar forest ecosystem at the Nebraska National Forest, Halsey, Nebraska) soil sample. NR2–NR18: coded single clone (clone NR1, which is chimeric is not shown on the gel). Lane NRO: original fingerprint of NR sample. Arrows marked with A and B represent the two dominant bands shown in Figure 3 that were excised and sequenced. Vertical arrow indicates that the denaturant gradient increases from 40% on the top to 48% at the bottom of the gel with 100% denaturant defined as containing 7 M urea and 40% (v/v) formamide.

**Table 3.** Identity of selected DGGE bands from clones of soil from eastern red cedar forest (NR)

Sequence designation <sup>a</sup>	Closest match from GenBank (% sequence similarity by BLAST)	Genbank accession no.	
NR1 <sup>b</sup>	?	?	
NR2	Uncultured <i>Glomus</i> clone ETC6 (99.1%)	DQ388618.1	GI:86990635
NR3	Uncultured <i>Glomus</i> clone ET2 (99.3%)	AY459194.1	GI:38639330
NR4	Uncultured <i>Glomus</i> clone CCons2 (99.1%)	DQ357080.1	GI:113715636
NR5	<i>Glomus</i> sp. MUCL 43203 (99.6%)	AJ852527.1	GI:121488184
NR6	Uncultured <i>Glomus</i> clone CCons2 (98.9%)	DQ357080.1	GI:113715636
NR7	Uncultured <i>Glomus</i> clone G10_2L2SP (98.9%)	EF177547.1	GI:122893774
NR8	Uncultured <i>Glomus</i> clone JPC053 JP1 (97%)	DQ085224.1	GI:72537348
NR9	Uncultured <i>Glomus</i> isolate Glo60 clone F9AGMyc37 (99.3%)	EF041100.1	GI:117380994
NR10	Uncultured <i>Glomus</i> isolate Glo60 clone F9AGMyc37 (99.5%)	EF041100.1	GI:117380994
NR11 <sup>c</sup>	Uncultured <i>Glomus</i> clone OC6_09C2Z (94.1%)	EF177624.1	GI:122893851
NR12	Uncultured <i>Glomus</i> isolate Glo60 clone F9AGMyc37 (99.8%)	EF041100.1	GI:117380994
NR13	Uncultured <i>Glomus</i> isolate Glo60 clone F9AGMyc37 (99.3%)	EF041100.1	GI:117380994
NR14	Uncultured <i>Glomus</i> clone JPC053 JP1 (97%)	DQ085224.1	GI:72537348
NR15	Uncultured <i>Glomus</i> clone p3819 (98.7%)	AJ563884.1	GI:40644687
NR16	Uncultured <i>Glomus</i> isolate Glo8 clone F9AGMyc61 (99.8%)	EF041068.1	GI:117380962
NR17	Uncultured <i>Glomus</i> clone p3819 (99.1%)	AJ563884.1	GI:40644687
NR18 <sup>d</sup>	<i>Glomus clarum</i> isolate UFPE08 (98.5%)	AJ852597.1	GI:84617234

<sup>a</sup> Sequence designation was defined in the text.

<sup>b</sup> Sequence was proven to be chimeric by Chimera Check Program.

<sup>c</sup> Sequence similarity values below 97% are not considered to be identical (Stackebrandt and Goebel, 1994), thus, NR11 is not identical to *Glomus* clone OC6\_09C2Z. They are associated at no more than the species level.

<sup>d</sup> Only sequence closely related to morphologically defined AMF species.

dition, six sequences (NR2, NR3, NR5, NR7, NR11, and NR16) showed high similarity to six *Glomus* isolates or clone sequences of unknown taxonomic affiliation. Only one sequence (NR18) corresponded to a morphologically defined AMF species (*Glomus clarum* Nicolson & Schenck). The sequence for the excised band A (Figure 3) was 99.1% identical to the sequence of clone NR12 (Table 3), which migrated to the same position as band A. The sequence for excised band B was 99.7% identical to the clone that had the same mobility on DGGE gel, clone NR13 (Figure 4).

## 4. Discussion

### 4.1. Nested PCR and DGGE

Nested PCR, which strongly increases the sensitivity of PCR-based fingerprinting (Hijri *et al.*, 2006; Randazzo *et al.*, 2006), was used to solve the problem of low resolution and yield using the primer pair AM1/NS31GC. An initial PCR amplification with the AMF-specific primer pair AM1 and NS31 was followed by a second round using Glo1 and NS31GC, with the first primer pair being specific to AMF and the second pair enhancing the yields of AMF product and improving the resolution on DGGE. This approach successfully ampli-

fied the AMF 18S rDNA fragment both from AMF reference species and from different soil samples, producing distinguishable DGGE bands profiles for rapid characterization of AMF communities. This two-step approach has been shown to yield well-separated band patterns on TGGE for tested AMF species (Cornejo *et al.*, 2004). The DGGE profile of the nested PCR products contained sharp and intense bands, compared to those that based on amplification products of AM1/NS31GC published elsewhere (Kowalchuk *et al.*, 2002; Ma *et al.*, 2005), greatly improving gel profile-based microbial community characterization.

The primer pair AM1/NS31 is one of the most widely used group-specific primer pairs in studies of AMF communities and large amounts of DNA sequence information derived from this primer pair are available; however, this primer pair does not amplify 18S rDNA fragments from all known AMF (Daniell *et al.*, 2001). Although the three well-established families of the Glomeromycota, *i.e.*, Glomaceae, Acaulosporaceae, and Gigasporaceae, can be amplified, some species in the two deeply branching and less commonly found families of Archaeosporaceae and Paraglomeraceae may not be detected when this primer pair is applied to environmental samples (Redecker, 2000). ARCH1311, which is specific to the two deeply branching families (Redecker, 2000), together with primers targeting other groups of AMF species, has been used to detect the largest portion of taxon diversity in the Glomeromycota (Redecker, 2000; Redecker *et al.*, 2003; Hijri *et al.*, 2006). DGGE analysis using the combination of those primers, however, has not been worked out yet. Recently, the primer pair AM1/NS31 was shown to amplify some non-AMF sequences (Douhan *et al.*, 2005; Ma *et al.*, 2005) making sequence information necessary for accurate community characterization. In the present study, we used this primer pair with the above limitations in mind.

#### 4.2. 18S rDNA clone analysis

To retain the advantages of DGGE analysis while reducing the number of clones, we applied them in parallel to determine AMF community structure of soils. The sequences retrieved from clones were unique and contained more information than those in excised DGGE bands. All the dominant bands of two reference isolates, *A. scrobiculata* and *G. gigantea*, were matched by clones from their clone libraries. Both dominant bands and most of the less dominant bands in the DGGE profile from the tested NR forest soil were also found in the corresponding clone library. Moreover, clones NR10, NR11, and NR18 did not have corresponding bands in the original DGGE profile, indicating more comprehensive inclusion of ribotypes in the clone library. Because DGGE detects dominant species that comprise more than 1–2% of the whole community (Muyzer and Smalla, 1998), it is likely that sequences of less dominant species that escape detection by DGGE could be recovered through cloning. Thus, sequence types detected in both DGGE and the

clone library would be the dominant AMF ribotypes in the DNA extract and the relative abundance of ribotypes may be inferred by this difference.

We propose that the sequence of the bands in the original DGGE profile can be inferred from clones that migrate to the same position on DGGE analysis. This is a reasonable inference as the PCR products arise from the same genomic DNA sample using the same primer pairs. The high similarity between the sequences of the two excised bands (A and B in Figure 3) and those of the matching clones supports this inference. This combined approach of DGGE and cloning has been applied to bacterial communities (Schabereiter-Gurtner *et al.*, 2001; Handschur *et al.*, 2005) from diverse environmental samples.

Interestingly, sequence similarity analysis indicated that clone inserts with highly similar sequences did not necessarily migrate close to each other on the DGGE gel (Opik *et al.*, 2003). For instance, although NR9 and NR10, which shared a sequence similarity of 99%, migrated close to each other on DGGE, NR9 and NR12 with a sequence similarity of 99.5% did not. Neither did NR8 and NR14, which were 99% similar to each other. Clones NR10 and NR11, which migrated to the same position in DGGE, had a sequence similarity of only 92%. Thus, inference of sequence identity of DGGE bands based on matching mobility to clones still needs to be done with caution.

BLAST similarity search demonstrated the overwhelming dominance of *Glomus*-like ribotypes in the NR soil, although only one sequence (NR18) was affiliated to a cultured *Glomus* species, *G. clarum*. NR11, which had a maximum sequence similarity of 94.1% to one *Glomus* clone, may be a new undescribed *Glomus* ribotype. The dominance of the genus *Glomus* in AMF community has been reported from a number of different ecosystems, ranging from forest (Opik *et al.*, 2003; Wubet *et al.*, 2003), wetland (Wirsel, 2004), and grassland (Scheublin *et al.*, 2004), to highly disturbed agricultural fields (Daniell *et al.*, 2001), using either morphological or molecular tools. The aforementioned primer specificity of AM1 may contribute to the detected dominance of *Glomus* studied here. Ribotypes belonging to the deeply branching clades may escape detection. Another reason for the dominance of *Glomus* ribotypes may stem from their versatile propagation and survival strategies. *Glomus* species are capable of establishing a symbiosis via spores or mycelium, and forming anastomoses between mycelia allows them to quickly reestablish a hyphal network after disruption (Giovannetti *et al.*, 1999). These traits would have favored *Glomus* species during afforestation of the Nebraska Sand Hills with eastern red cedar.

#### 4.3. AMF ribosomal DNA polymorphism

In this study, DGGE displayed a high degree of variation in ribosomal RNA gene sequences for all the reference AMF isolates studied, with a maximum of up to 10 bands ob-

served in the gel profile of *G. intraradices*. This polymorphic nature of ribosomal DNA sequences has been repeatedly described across both variable (ITS; Pringle *et al.*, 2000; Kuhn *et al.*, 2001) and conserved regions (SSU and 5.8S; Clapp *et al.*, 1999; Helgason *et al.*, 1999) of the ribosomal RNA genes of AMF belonging to different genera. This high genetic variation may be either intersporal (Pringle *et al.*, 2000) or intrasporal (Clapp *et al.*, 1999). Hypothesis of either heterokaryosis (Kuhn *et al.*, 2001) or homokaryosis (Pawłowska and Taylor, 2004) was proposed to explain the origin of the unusual polymorphism of AMF ribosomal DNA. We were unable to address this issue in our study because DNA was extracted from multiple spores of the reference isolates for PCR–DGGE analysis. Because of the polymorphism of ribosomal RNA genes and the lack of a clear species concept, it is not proper to define AMF species by molecular methods at present (Wubet *et al.*, 2003). Further examination of polymorphism is needed, before suitable sequences can be identified, to infer AMF taxonomic status from ecological studies undertaken in the field (Rodríguez *et al.*, 2004). AMF communities are primarily identified as sequence group by molecular studies. In our study we used the term ribotype, the group of closely affiliated 18S rDNA sequences that showed high similarity by sequence analysis, to describe the sequence group obtained by the method used.

#### 4.4. Conclusion

In this study a combination of DGGE and cloning was used to rapidly and accurately characterize AMF diversity in environmental samples based on 18S rDNA fragments. The nested PCR–DGGE strategy produced distinct banding patterns that provided non-subjective discrimination among reference isolates and soil samples. Construction of clone libraries enabled collection of reliable sequence information for bands of interest. The high throughput of DGGE combined with selected cloning makes this approach suitable for tracking AMF communities in ecological studies.

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