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Contrasting root associated fungi of three common oak-woodland plant species based on molecular identification: host specificity or non-specific amplification?

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Abstract An increasingly popular approach used to identify arbuscular mycorrhizal (AM) fungi in planta is to amplify a portion of AM fungal small subunit ribosomal DNA (SSU-rDNA) from whole root DNA extractions using the primer pair AM1-NS31, followed by cloning and sequencing. We used this approach to study the AM fungal community composition of three common oak-woodland plant species: a grass *(Cynosurus echinatus*), blue oak (*Quercus douglasii*), and a forb (*Torilis arvensis*). Significant diversity of AM fungi were found in the roots of *C. echinatus*, which is consistent with previous studies demonstrating a high degree of AM fungal diversity from the roots of various hosts. In contrast, clones from *Q. douglasii* and *T. arvensis* were primarily from non-AM fungi of diverse origins within the Ascomycota and Basidiomycota. This work demonstrates that caution must be taken when using this molecular approach to determine in planta AM fungal diversity if non-sequence based methods such as terminal restriction fragment length polymorphisms, denaturing gradient gel electrophoresis, or temperature gradient gel electrophoresis are used.

Keywords Arbuscular mycorrhizae · Diversity · Molecular identification · Polymerase chain reaction · Primer specificity

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Introduction

Despite the ecological importance of arbuscular mycorrhizal (AM) fungi (Klironomos 2000, 2003; Smith and Read 1993; van der Heijden et al. 1998), little is known about the population biology and diversity of these fungi because they are obligate symbionts that cannot easily be cultured under standard laboratory conditions. Identifying the AM fungi involved in the symbiosis is also difficult because few morphological differences exist among spores, and it is not possible to identify species from the morphology of mycelium either inside or outside of roots (Redecker et al. 2003). For example, the distantly related genera *Glomus* and *Paraglomus* cannot be distinguished based on spore morphology (Morton and Redecker 2001) and there are also phylogenetically divergent groups within the genus *Glomus* (Schwarzott et al. 2001). Moreover, field collected spores are often parasitized by other organisms, which can hinder morphological identification due to degradation and can also lead to erroneous interpretation of phylogenetic analyses due to mixed DNA samples of AM fungi and contaminants (Schüßler et al. 2001a; Clapp et al. 2002; Redecker 2003).

Despite potential contamination problems associated with field- or greenhouse-collected spores, molecular marker techniques such as randomly amplified polymorphic DNA (RAPD) (Wyss and Bonfante 1993), amplified fragment length polymorphisms (AFLP) (Rosendahl and Taylor 1997), and microsatellite primed PCR (Longato and Bonfante 1997; Zézé et al. 1997) have been used to study the diversity and population biology of AM fungi. More recently, a molecular marker technique called amplified fragment length microsatellites (AFLM) has been developed to eliminate problems associated with contaminated spores (Douhan and Rizzo 2003). Nevertheless, spore-based genetic analyses can be problematic because some AM fungi sporulate sparsely, infrequently, or not at all (Schüßler et al. 2001a). Therefore, these techniques may not easily be applied to the many taxa that are directly involved in the symbiosis under natural field conditions, and may not necessarily be applicable to in planta analyses of AM fungal diversity.

To overcome these problems, AM-specific primers have been developed that can be used in planta to preferentially amplify fungal DNA from host roots (Helgason et al. 1998; Kjøller and Rosendahl 2000; Redecker 2000). Most of these primers target ribosomal genes and have been used to identify individual AM fungal species (Millner et al. 2001) to AM fungal community composition (van Tuinen et al. 1998; Helgason et al. 1999; Redecker 2000). Several recent studies have used the primer combination of AM1 (Helgason et al. 1998) and NS31 (Simon et al. 1992) to amplify a portion of the small subunit of AM fungal small subunit ribosomal DNA (SSU-rDNA) from whole root DNA extractions. The amplification products are cloned, screened by restriction fragment length polymorphism (RFLP) analysis, and representative clones are sequenced and subjected to phylogenetic analyses. This approach has been used to contradict the notion that there is low species diversity and lack of host specificity among AM fungi (Daniell et al. 2001; Helgason et al. 1999; Husband et al. 2002; Vandenkoornhuyse et al. 2002).

We are studying mycorrhizal fungal diversity in a Mediterranean oak-grassland ecosystem in the Sierra Nevada Mountains of California in the United States. The diverse plant community makes this a good system to study host specificity and how mycorrhizal fungi may influence nutrient exchange between related and unrelated plant species. The ecosystem is dominated by the deciduous oak, *Quercus douglasii* (blue oak), which is a host to primarily ectomycorrhizal (EM) fungi but may also be colonized by AM fungi. There is also a species-rich herbaceous flora of forbs and grasses whose symbionts are primarily AM fungi. Using the molecular approach of Helgason et al. (1998), we examined the AM fungal community composition of three common plants: *Q. douglasii* (oak), *Cynosorus echinatus* (grass), and *Torilis arvensis* (forb).We found a significant difference in our ability to detect target AM fungi from nontarget fungi based on host. Our results are significant because this primer pair has recently been used to study in planta AM species diversity using terminal restriction length polymorphisms (T-RFLP) (Vandenkoornhuyse et al. 2003; Johnson et al. 2004), a method dependent upon absolute primer specificity.

Materials and methods

Sampling and DNA extraction from roots

Plants were sampled from the Koch natural area within the University of California's Sierra Foothill Research and Extension Center located in Browns Valley, Calif., approximately 100 km northeast of Sacramento (39°15′N, 121°17′W). The site consists of an annual grassland oak-woodland within the Sierra Nevada foothills that has been maintained as an undisturbed natural reserve since 1960. The climate is Mediterranean, with hot, dry summers and mild, rainy winters with approximately 700 mm annual mean precipitation and a mean annual temperature of 15°C (Dahlgren et al. 1997).

Specimens of *C. echinatus* and *T. arvensis* at the flowering stage and *Q. douglasii* seedlings (approximately 1–3 years old) were collected on 29 May 2003 from a 32 m × 32 m plot consisting of 16 (8 m ×8 m) subplots. Three to five plants of each species were collected in each subplot within a 2–3 m radius of each other. Roots of each species were pooled to represent one sample per subplot, washed under running tap water, freeze-dried, and frozen at −80°C until further processing. Dried root samples of each host species were ground using an electric plant tissue grinder, or manually in liquid nitrogen. DNA was extracted from approximately 25–50 mg dried ground roots using an UltraClean Soil DNA Kit (MoBio, Solana Beach, Calif.) following the manufacturer's instructions. Five microliters of each extraction was separated on a 1.5% agarose gel and stained with SYBR Green I nucleic acid stain (Molecular Probes, Eugene, Ore.) to estimate quantity and quality of DNA prior to PCR.

PCR amplification, cloning, and sequencing

The universal eukaryotic primer NS31 (Simon et al. 1992) and the primer AM1 (Helgason et al. 1998) were used to amplify a small portion (~550 bp) of AM fungal SSU-rDNA. PCR was first optimized using several samples of each host by varying the annealing temperature, $MgCl_2$ concentration, cycle lengths, and template concentration. The final 20 μl reaction mixture contained 2 μl of a 1:10 dilution of template DNA, 1× PCR buffer (Invitrogen, Carlsbad, Calif.), 2.5 mM $MgCl_2$, 0.2 mM each dNTP (Invitrogen), 7.5 pmol of each primer, and 0.5 U Platinum *Taq* polymerase (Invitrogen). The final thermocycling conditions used were identical to those in the method of Helgason et al. (1999), using a PE 9700 thermocycler (Perkin-Elmer, Norwalk, Conn.). Five microliters of each reaction was separated on a 1.5% agarose gel and stained with SYBR Green I nucleic acid stain prior to cloning.

PCR products were cloned using the TOPO TA sequencing cloning system (Invitrogen) following the manufacturer's instructions. Positive clones were transferred to 100 μl Luria-Bertani (LB) broth amended with 100 μg/ml ampicillin (Sigma, St. Louis, Mo.) using sterile toothpicks, grown overnight at 37°C in sterile 96-well microtiter plates, and up to 24 clones from each sample were reamplified using M13 forward and reverse primers. Five microliters of each reaction was separated on a 1.5% agarose gel, stained with SYBR Green I nucleic acid stain, and up to eight positive cloned amplification products per sample were cleaned using a Millipore MontagePCR$_{96}$ vacuum filtration system (Millipore, Bedford, Mass.) following the manufacturer's instructions. AM1 was used as a sequencing primer using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.) and the second strand sequenced with NS31 on representative clones after preliminary analyses. The PCR products were cleaned using Millipore's Montage SEQ$_{96}$ vacuum filtration system (Millipore) following the manufacturer's instructions, and the sequences were run through an ABI 3730 XL capillary

sequencer at the Genomic Facility at University of California at Davis.

Data analysis

Sequences were edited and contigs constructed using Sequencher software (version 4.1.2, Gene Codes, Ann Arbor, Mich.). All sequences were subjected to a batch BLAST search (Altschul et al. 1997) using the program blastCl3 downloaded from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) and were also checked for putative chimeric sequences using the online chimera checker program at the Ribosomal Database Project II website (http://rdp.cme.msu.edu). BLAST search results with the closest hits were downloaded and added to the data set. We also included sequences from representative AM taxa from Schüßler et al. (2001b), in which the AM fungi were placed in a monophyletic phylum, the Glomeromycota. The sequences were aligned using ClustalX (version 1.81) (Thompson et al. 1997). The alignment was manually edited in MacClade 4.03 (Maddison and Maddison 2001) with ambiguous sites removed and subjected to neighbor joining (NJ) analyses under the Kimura 2 parameter option, and maximum parsimony (MP) analysis using the heuristic search procedure with 1,000 random-addition-sequence replicates and tree-bisection-reconnection branch swapping using PAUP* (version 4.0 beta 10) (Swofford 2002). Gaps were treated as missing data. Confidence in tree topology was examined using bootstrap with 1,000 replicates for NJ and 500 replicates under the heuristic option for MP. Sequences have been deposited in GenBank (see figure legends). A BLAST search was also conducted using the AM1 primer sequence using the BLAST option of "short to nearly matching" with the number of descriptions and alignments set to 1,000.

Results

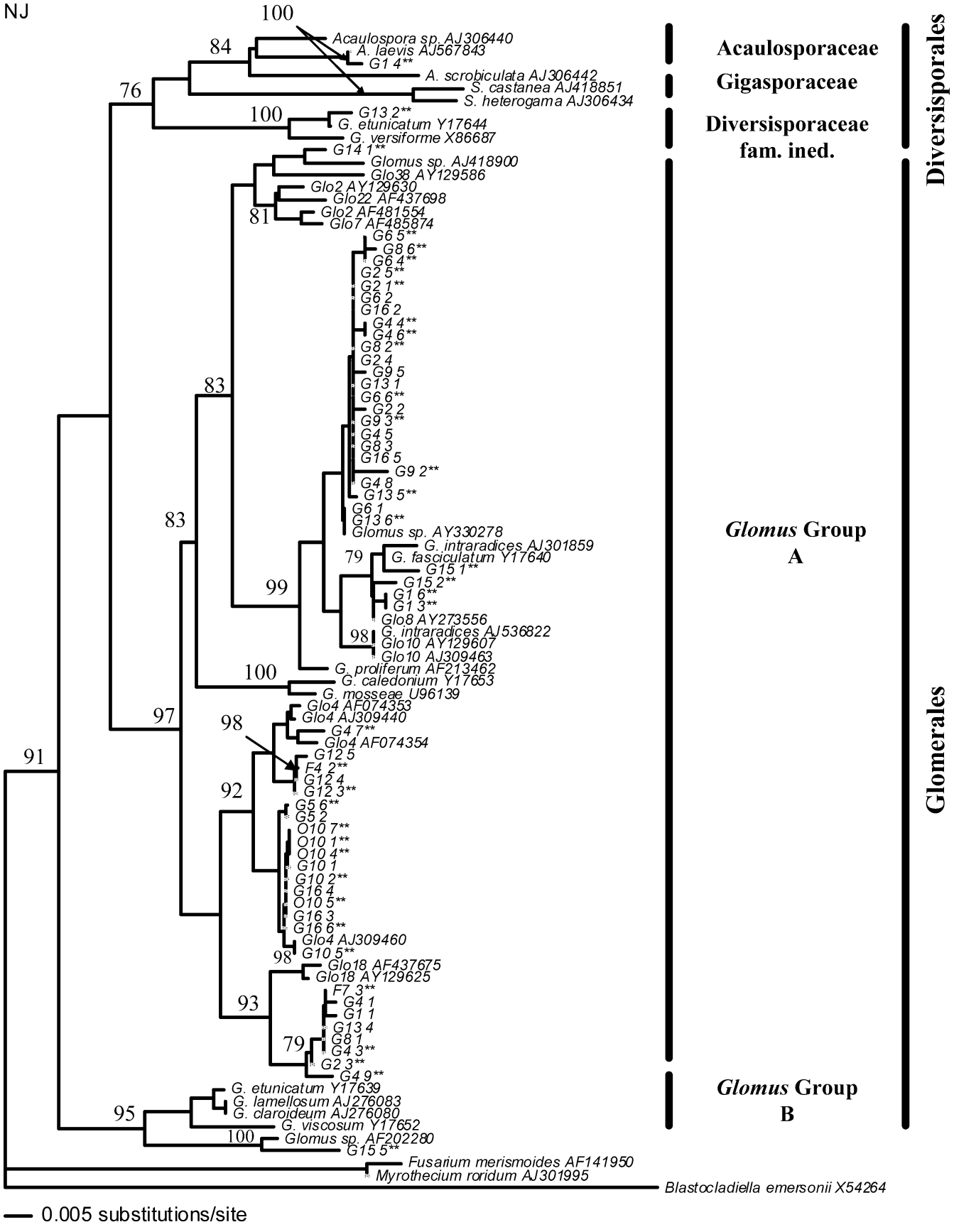
Primer homology and chimeras

A BLAST search of the AM1 primer sequence returned 322 hits of deposited AM sequences, with 241 showing 100% sequence homology. Table 1 shows the AM1 priming site homology to one randomly chosen sequence with 100% similarity as well as randomly chosen representative sequences from the 81 returned hits that were not 100% homologous. Table 1 also shows the closest representative sequences in GenBank to the recovered clones from Fig. 2 and three additional fungal sequences for comparative purposes. AM sequences deposited with GenBank have

Table 1 AM1 primer and priming site comparison to representative members of the Glomeromycota (*G*), the closest GenBank matches to members of the Ascomycota (*A*) and Basidiomycota (*B*) from the recovered clones, and three additional sequences for illustrative purposes. *Glomus etunicatum* was chosen at random to represent a species with 100% homology to AM1 as represented by a dash. The other AM taxa were chosen at random for comparative purposes from the 81 BLAST hits that were not 100% homologous to the AM1 primer. Base changes are shown with respect to the priming site sequence

Accession number			$^{5\prime}$G	T	T	T	C	C	C	G	T	A	A	G	G	C	G	C	C	G	A	A$^{3\prime a}$	
			$^{3\prime}$C	A	A	A	G	G	G	C	A	T	T	C	C	G	C	G	G	C	T	T$^{5\prime b}$	
G	*Glomus etunicatum*	Y17639	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
G	*Glomus spurcum*	Y17650	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	
G	*Glomus* sp	AB076294	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	
G	*Glomus* sp	AF480157	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	
G	*Glomus* sp	AB076299	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	C	
G	*Acaulospora longula*	AJ306439	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	
G	*Archaeospora leptoticha*	AJ006797	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	C	C		
G	*Archaeospora trappei*	AJ006800	–	–	–	–	–	–	–	T	–	–	–	–	–	A	–	–	–	–	–	–	C	
G	*Archaeospora* sp	AF452633	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	C	
G	*Paraglomus occultum*	AJ006799	–	–	–	–	–	–	–	T	–	C	–	–	–	A	–	–	–	–	–	–	C	
B	*Bullera crocea*	D31648	T	–	–	–	–	A	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	
A	*Cephaliophora muscicola*	AB001108	T	–	–	–	–	A	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	
A	*Cladosporium cladosporioides*	AF548071	T	–	–	–	–	A	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	
A	*Gyalecta ulmi*	AF465464	T	–	–	–	–	A	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	
B	*Inocybe geophylla*	AF287835	T	–	–	–	–	A	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	
B	*Marchandiomyces corallinus*	AF289660	T	–	–	–	–	A	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	
A	*Nectria lugdunensis*	AY231639	T	–	–	–	–	A	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	
B	*Russula exalbicans*	AY293156	T	–	–	–	–	A	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	
A	*Aspergillus niger* var. *awamori*	AB030917	T	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
A	Uncultured soil fungus	AF515329	T	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
B	*Grifola frondosa*	AF334914	T	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	

[a]AM1 primer sequence
[b]AM1 binding site sequence


NJ
Acaulosporaceae
Gigasporaceae
Diversisporaceae
fam. ined.
Diversisporales
Glomus Group A
Glomus Group B
Glomerales
Acaulospora sp. AJ306440
A. laevis AJ567843
G1 4**
A. scrobiculata AJ306442
S. castanea AJ418851
S. heterogama AJ306434
G13 2**
G. etunicatum Y17644
G. versiforme X86687
G14 1**
Glomus sp. AJ418900
Glo38 AY129586
Glo2 AY129630
Glo22 AF437698
Glo2 AF481554
Glo7 AF485874
G6 5**
G8 6**
G6 4**
G2 5**
G2 1**
G6 2
G16 2
G4 4**
G4 6**
G8 2**
G2 4
G9 5
G13 1
G6 6**
G2 2
G9 3**
G4 5
G8 3
G16 5
G9 2**
G4 8
G13 5**
G6 1
G13 6**
Glomus sp. AY330278
G. intraradices AJ301859
G. fasciculatum Y17640
G15 1**
G15 2**
G1 6**
G1 3**
Glo8 AY273556
G. intraradices AJ536822
Glo10 AY129607
Glo10 AJ309463
G. proliferum AF213462
G. caledonium Y17653
G. mosseae U96139
Glo4 AF074353
Glo4 AJ309440
G4 7**
Glo4 AF074354
G12 5
F4 2**
G12 4
G12 3**
G5 6**
G5 2
O10 7**
O10 1**
O10 4**
G10 1
G10 2**
G16 4
O10 5**
G16 3
G16 6**
Glo4 AJ309460
G10 5**
Glo18 AF437675
Glo18 AY129625
F7 3**
G4 1
G1 1
G13 4
G8 1
G4 3**
G2 3**
G4 9**
G. etunicatum Y17639
G. lamellosum AJ276083
G. claroideum AJ276080
G. viscosum Y17652
Glomus sp. AF202280
G15 5**
Fusarium merismoides AF141950
Myrothecium roridum AJ301995
Blastocladiella emersonii X54264
0.005 substitutions/site

◀ **Fig. 1** Phylogenetic relationships of arbuscular mycorrhizal (AM) fungi inferred from partial small subunit (SSU) rDNA sequences from clones obtained from the roots of a grass (*G*), *Cynosurus echinatus*, an oak (*O*), *Quercus douglasii*, and a forb (*F*), *Torilis arvensis* as well as sequences from GenBank, based on neighbor-joining (*NJ*) analysis. Letters after the host designation indicate the sampled subplot followed by the individual clone. For example, G2 4 equals grass host, subplot 2, and clone number 4. The tree was rooted with *Blastocladiella emersonii* (X54264) as the outgroup and bootstrap values are based on 1,000 replicates with values over 75% shown. Taxonomic affinities for the Glomeromycota are based on the treatment of Schüßler et al. (2001b). Individual taxon names are as they were originally annotated in GenBank. Of the 51 clones, 36 (indicated by **), were sequenced in both directions. These 36 sequences have been deposited with GenBank under accession numbers AY916391–AY91426

priming site differences ranging from zero to four base pairs within the AM1 priming site region. Three base pair differences were found in the priming sites of the deposited sequences in GenBank that had the closest BLAST hits to the non-AM clones. Only two base pair differences were found in the priming sites of the three additional fungal sequences (two ascomycetes and one basidiomycete) that were compared. No obvious chimeric sequences were detected based on chimera checker.

PCR amplification and cloning

PCR amplification products dramatically decreased once the annealing temperature was raised above 58°C, with no apparent products amplified at 60°C, at all DNA template and $MgCl_2$ concentrations tested (data not shown). Following PCR, the expected amplification product of ~550 bp was detected from all *C. echinatus* samples. PCR amplification of *Q. douglasii* and *T. arvensis* samples also yielded the expected band at ~550 bp, but less than half of the samples for each host produced a visible product in the jority around 500–700 bp. Therefore, more cloning and sequencing efforts were attempted for *C. echinatus* compared to *Q. douglasii* and *T. arvensis.* For cloning and sequencing, only PCR products that were similar in size to the amplicons from *C. echinatus* were chosen from *Q. douglasii* and *T. arvensis*.

Fungal diversity

For all phylogenetic analyses, NJ and MP trees produced similar topologies. Therefore, only NJ trees will be presented. BLAST searches and subsequent phylogenetic analyses revealed that the sequenced clones with affinities to the

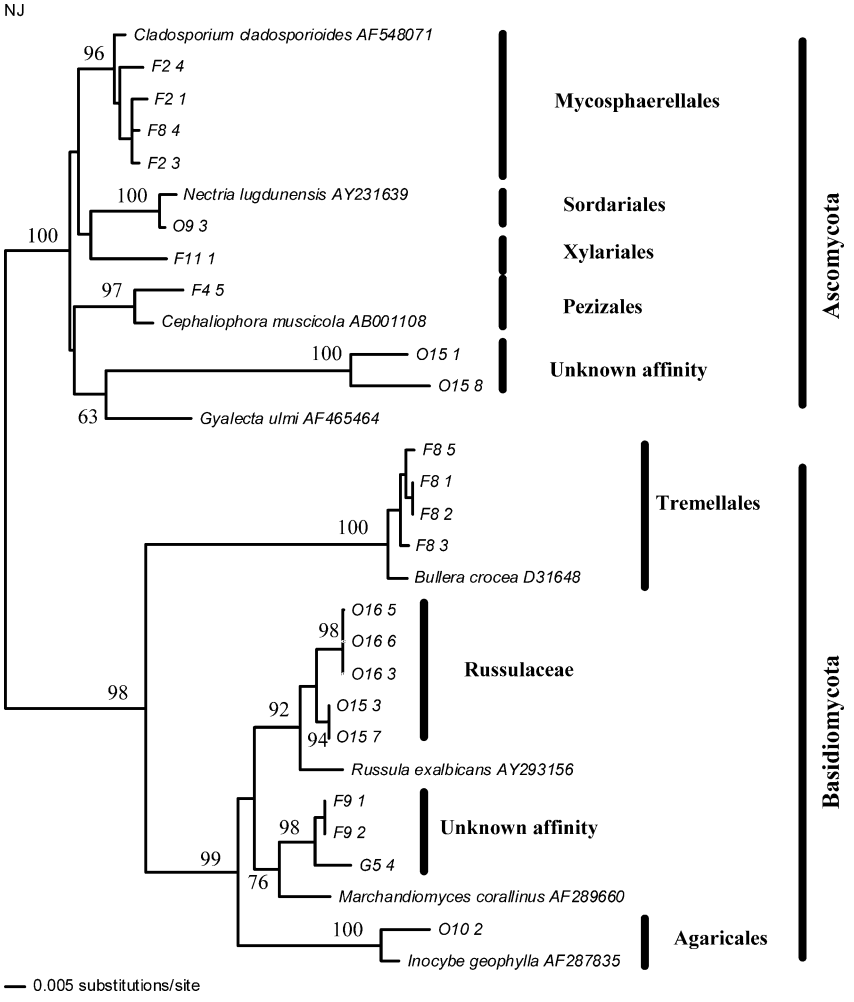


Fig. 2 Phylogenetic relationships of representative members of the Ascomycota and Basdiomycota inferred from partial SSU rDNA sequences from clones obtained from the roots of a grass (*G*), *C. echinatus*, an oak (*O*), *Q. douglasii*, and a forb (*F*), *T. arvensis* as well as sequences from GenBank based on NJ analysis. Letter designations are as in Fig. 1. The tree was midway rooted and bootstrap values are based on 1,000 replicates with values over 50% shown. Family or order affinities are based on the taxonomic treatment of Kirk et al. (2001). Individual taxon names are as they were originally annotated in GenBank. All clones were sequenced in both directions. Sequences have been deposited with GenBank under accession numbers AY916427–AY916448

Glomeromycota fell into two orders: the Glomerales and Diversisporales (Fig. 1). The majority of clones fell into the *Glomus* Group A clade (Schüßler et al. 2001b) with strong bootstrap support (97%), and were distributed across most of the subplots. Within this major clade, up to six minor clades were found with varying support. Members of the Acaulosporaceae, Diversisporaceae, and *Glomus* Group B each clustered with one clone with strong bootstrap support. A total of 51 of 52 clones derived from *C. echinatus* were of AM origin (Fig. 2) whereas only 4 of 13 clones from *Q. douglasii* and 2 of 14 clones from *T. arvensis* were of AM origin (Fig. 2). The single clone from *C. echinatus* that was not of AM origin had sequence similarity to a mitosporic basidiomycete (*Marchandiomyces carollinus*) but with only moderate bootstrap support (Fig. 2). Therefore, the identity of this clone is not certain. In contrast, the majority of clones from *Q. douglasii* and *T. arvensis* were most similar to diverse members of the Ascomycota and Basidiomycota (Fig. 2). Tentative identifications for 19 of 21 clones could be made to genus, family and/or order based on sequence similarities of 97% or to sequences deposited in GenBank and high bootstrap support for the representative clades (Fig. 2). The two clones that could not be identified clustered weakly with the genus *Gyalecta* in the Lecanoromycetidae. *Gyalecta* is a lichenized genus and the family is found primarily in the tropics or in humid environments (Fig. 2). However, saprobic species in the Lecanoromycetidae are commonly found on wood in xeric habitats (Kirk et al. 2001). Therefore, the two unidentified clones are more likely related to saprobic species in the Lecanoromycetidae than to the genus *Gylalecta*.

Discussion

The dominance of non-AM fungal sequenced clones in our *Q. douglasii* and *T. arvensis* samples clearly demonstrates that caution must be used when using this molecular approach to identify AM fungi if DNA sequencing is not applied. For example, Vandenkoornhuyse et al. (2003) and Johnson et al. (2004) have recently applied the T-RFLP technique to study in planta AM diversity. The T-RFLP technique assumes that all amplification products are specific to the organism(s) of interest since only banding patterns are analyzed. Vandenkoornhuyse et al. (2003) rationalized their approach by stating: "the choice of this set of primers was driven by the fact that nothing other than AM fungi is amplified in our stringent PCR conditions." However, one of the earliest studies to use the AM1-NS31 combination following the same PCR regime reported that 7 out of 62 (~10%) sequenced clones were non-AM fungal (ascomycete) or chimeric sequences (Helgason et al. 1999), and more recently, a basidiomycete sequence has been reported using this approach (Helgason et al. 2002). We also know of three independent laboratories that are finding results similar to ours with respect to non-specificity. For example, 78% of clones sequenced from field-grown cacao (*Theobroma cacao*) roots were non-target fungi (Sordariomycetes) (L.C. Mejia and E.A. Herre, personal communication). Moreover, Vandenkoornhuyse et al. (2002) reported that 33 out of 121 (~27%) sequenced clones were chimeric, or suspected of being chimeric. Error rates of this magnitude in fungal species identification could significantly affect microbial community analyses based on T-RFLP or other techniques such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE).

Johnson et al. (2004) have recently used T-RFLP to study the AM fungal diversity from microcosm soils that had been developed for 3 years using various treatments of plant assemblages and a bare soil control. Using *Plantago lanceolata* as a bioassay trap plant, they could not detect AM hyphae in the roots of *P. lanceolata* grown for 3 weeks in the bare soil treatment based on root staining. However, based on T-RFLP, they detected substantial AM fungal diversity in the roots of *P. lanceolata* grown for 12 weeks in the same soil, which was contrary to their expectations (no staining was done on this treatment). Johnson et al. (2004) suggested that the discrepancy between the 3- and 12-week treatments was that more time was needed for AM fungi to colonize the roots due to inoculum primarily in the form of spores compared to mycelial networks. Based on our results, perhaps non-specificity could explain the unexpected outcome of Johnson et al. (2004) based on their T-RFLP data. Therefore, future studies should consider including some cloning and sequencing as a control when estimating AM fungal diversity based on T-RFLP data. This would be especially important if unexpected results are found in initial experiments.

The AM1 primer was developed based on only 12 AM fungal sequences, and Helgason et al. (1999) stated that the primer may not be specific for all AM taxa, which was later demonstrated for the divergent genera *Archaeospora* and *Paraglomus* (Redecker et al. 2000). Since it is not possible to recover the original AM1 priming sites from the amplified fragments, we cannot determine if the amplification products were non-specifically amplified. However, a BLAST search of the AM1 primer sequence demonstrated only slight primer site differences among many related as well as divergent taxa (Table 1). Three deposited sequences, one basidiomycete and two ascomycetes, illustrate this point well. Only two base pair differences were found, and these were at the distal 5′ end of the primer, which is more likely to lead to non-specific amplification. Moreover, the variation among the AM1 priming site region within the Glomeromycota is as divergent as between members of the Ascomycota and Basidiomycota (Table 1). It is also conceivable that additional taxa that have 100% sequence similarity within this region exist but have yet to be sequenced or are not culturable. For example, Allen et al. (2003) found that *Sebacinia* spp. dominated their cloned library but they could never recover these species based on standard culturing procedures.

Regardless of specific or non-specific amplification, our results clearly demonstrate a potential problem with this primer pair for in planta AM fungal identification, and also show that very different results can be found depending on the host. This was true for all PCR conditions tested and

suggests that amplification of AM fungi using the AM1-NS31 primer combination may be related to the amount of mycorrhizal colonization of the host. We did not stain for hyphae within roots of our samples; however, independently sampled *C. echinatus*, *T. arvensis*, and *Q. douglasii* plants from the same field plot and greenhouse grown plants inoculated with field soil were all found to be colonized by AM fungi following root staining (Xinhua He, unpublished data). As expected, *C. echinatus* was colonized more extensively by AM fungi than were *T. arvensis* and *Q. douglasii*. Based on greenhouse studies, *C. echinatus* also produces substantially more extraradical AM fungal hyphae compared with *Q. douglasii* and *T. arvensis* (G.W. Douhan, personal observation). These observations in general support the idea that preferential amplification is dependent upon the amount of colonization. Nevertheless, a primer pair is only as good as its ability to discriminate target from non-target DNA, therefore it should not matter if AM fungi are present or not within the sampled substrate.

PCR-based cloning and sequencing techniques have been powerful tools to study the diversity of organisms from environmental samples, especially for organisms that cannot be, or are not easily, cultured under laboratory conditions (von Wintzingerode 1997). However, these molecular techniques may potentially be compromised by experimental error other than non-specific amplification such as PCR-generated chimeras, heteroduplexes, and mutations (Qiu et al. 2001). The long extension times used during PCR in this study and that of Helgason et al. (1999) should theoretically have reduced the probability of chimeric sequences as has been demonstrated by others (Wang and Wang 1996; Qiu et al. 2001). Chimeric sequences and the formation of heteroduplex molecules between different copies of amplified products are also more likely to occur between closely related taxa (Qiu et al. 2001). Thus, if artifacts were to occur, they should theoretically take place within members of the Glomeromycota, Ascomycota, and Basidiomycota rather than between the different phyla. For example, the unrooted NJ tree (Fig. 2) of sequences from ascomycetes and basidiomycetes clearly separated these distinct phyla, and all but one recovered clone had significant hits to deposited sequences in GenBank. We also did not detect any obvious chimeric sequences from our recovered AM clones and recovered multiple clones for most clades. The clones that were rare also clustered with other deposited sequences with high bootstrap support. Therefore, PCR-related artifacts were likely not a significant problem in this study, but the possibility of chimeric sequences cannot be completely ruled out.

We also found that each of the three plant hosts produced the expected band of ~550 bp, but the grass samples produced more uniform products than the other two hosts. The uniformity of the cloned PCR products of the grass samples compared with the oak and forb samples was also more apparent once clones were reamplified and separated on agarose gels. This was our first indication that we were potentially amplifying many non-AM fungi from *Q. douglasii* and *T. arvensis*. While we sequenced only those clones that were the same relative size as clones produced from the grass samples, we still found that the majority of products from the oaks and forb were not of AM origin. Therefore, many more non-AM taxa would have likely been detected if the clones had not been selected by PCR product size.

The AM1-NS31 primer pair has been used in several recent studies to test for host specificity and t- determine AM fungal diversity. These studies have been important in shedding new light regarding the community ecology and population biology of AM fungi. We found significant diversity of AM fungi in the roots of *C. echinatus*, which is consistent with previous studies demonstrating the high degree of AM fungal diversity from the roots of various hosts (Helgason et al. 1998, 1999; Husband et al. 2002; Vandenkoornhuyse et al. 2002). However, we also report here that this approach may not be suitable for every host since primarily non-AM fungi were amplified from *Q. douglasii* and *T. arvensis*. Therefore, researchers interested in using this molecular approach to identify AM fungi in planta should be aware of the potential drawbacks.

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