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Taxon-specific oligonucleotide primers for detection of *Glomus etunicatum*

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Abstract The 5.8S subunit and flanking internal transcribed spacer (ITS) regions in nuclear ribosomal DNA (rDNA) from spores of *Glomus etunicatum* MD107, MD127, TN101, and FL329 were amplified by polymerase chain reaction (PCR) using ITS1*Kpn* and ITS4*Pst* as primers. The amplification products (597, 599, 598, and 613 bp, respectively) were cloned and sequenced. The similarity among ITS region sequences from MD107, MD127, and TN101 was 99%, whereas the sequence similarity between the ITS regions of these three DNAs and that from FL329 was 91%. The 5.8S rDNA sequences of all four *G. etunicatum* isolates were identical. In contrast, major dissimilarities in the corresponding rDNA sequence regions of other glomalean taxa were observed. Oligonucleotide sequences unique to *G. etunicatum* were tested for their specificity in PCR amplification of genomic DNA from spores of 55 isolates comprising 29 glomalean fungi: 18 isolates of *G. etunicatum*, five *G. intraradices*, three *G. claroideum*, 16 other *Glomus* isolates, and 11 other glomalean taxa from each of four other genera. The *G. etunicatum* isolates were from a broad range of geographic regions and soils. The oligonucleotide pair GETU1:GETU2 primed specific amplification of an oligonucleotide sequence (approximately 400 bp) present in all *G. etunicatum*. This primer pair did not prime PCR when template consisted of DNA from any of the other glomalean fungi or any of the non-mycorrhizal controls, including roots of corn (*Zea mays*). In addition, the pair successfully detected *G. etunicatum* in nested PCR using a primary PCR product amplified from highly diluted extracts of colonized corn roots using modified ITS1:ITS4 primers. In the phylogenetic analysis of *Glomus* 5.8S and ITS2 rDNA region sequences, which included 500 bootstrap data sets, confidence in the *G. etunicatum* branch was very strong (90%) and clearly independent of *G. claroideum* and *G. intraradices*, to which it is very closely related.

Keywords Arbuscular mycorrhizal fungi · Internal transcribed spacer · Ribosomal DNA · Polymerase chain reaction primers · Phylogenetic tree

Introduction

Several recent reports describe the use of polymerase chain reaction (PCR) methods to amplify genomic DNA from arbuscular mycorrhizal fungi (AMF) and the subsequent nucleotide sequencing of the amplified products (for reviews see Franken 1998; Lanfranco et al. 1998). Some sequences have been used to analyze phylogenetic relationships among taxa and genera of Glomales (Simon et al. 1993b; Redecker et al. 2000). Others have been used to analyze genetic diversity among isolates of a single taxon and among spores of a single isolate (Clapp et al. 1995; Sanders et al. 1995; Lloyd-MacGilp et al. 1996). In addition, single-strand conformation polymorphism analysis was applied to PCR products of 18S and 28S rDNA regions to distinguish AMF in colonized roots (Simon et al. 1993a; van Tuinen et al. 1998; Kjøller and Rosendahl in press). Also, Glomalean sequence data were used to construct oligonucleotide primers that distinguish taxa or genera in roots colonized by specific taxa or clusters of taxa (Simon et al. 1993a; Bonito et al. 1995; Abbas et al. 1996; Millner et al. 1998). In addition, PCR/restriction analysis (Redecker et al. 1997) and its variant RAPD-PCR (Wyss and Bonfante 1993) have been used to distinguish glomalean taxa and then to develop taxon-specific primers (Lanfranco et al. 1995).

Additional documentary material has been deposited in electronic form and can be obtained from http://link.springer.de/link/service/journals/00572/index.htm

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The development of taxon-specific oligonucleotide (TSO) primers or probes requires a certain degree of sequence heterogeneity. However, too much heterogeneity within and among isolates of single species can make selection of species-specific nucleotide sequences virtually unattainable. Evidence from some reports (Clapp et al. 1995; Sanders et al. 1995; Lloyd-MacGilp et al. 1996) indicated that this might be an impediment to development of a TSO hybridization probe or primers for *G. mosseae,* if based on ITS region sequence. However, Millner et al. (1998) developed PCR primers and a TSO probe to detect 13 different isolates of *G. mosseae* based on ITS rDNA sequence. The known sequence heterogeneity in the primer regions was insignificant relative to the occurrence of homogeneous sequence, as evidenced by the 100% positive PCR amplifications and probe hybridization reactions with the *G. mosseae* isolates.

The purpose of the study reported here was to develop PCR primers for use in detecting *G. etunicatum*, one of the commonly reported (Musoko et al. 1994; Douds and Millner 1999) AMF in roots. Based on previous success with *G. mosseae* and preliminary results with several *G. etunicatum* isolates, we decided to pursue our study of the ITS regions of rDNA. In addition, we decided to expand the homology test group to include isolates encountered in various field studies.

Materials and methods

Fungal isolates and spore collection

A total of 55 isolates comprising 29 taxa of endomycorrhizal fungi were used in this study. These included 18 isolates of *Glomus etunicatum* and at least three taxa from *Acaulospora*, *Entrophospora*, *Gigaspora*, and *Glomus*, and two from *Scutellospora* (a complete list of isolates has been deposited in electronic form and can be obtained from http://link.springer.de/link/service/journals/00572/index.htm). *Pythium ultimum* Trow and *Endogone pisiformis* Link & Fries were included as non-mycorrhizal fungus controls and *Zea mays* L. (corn) was used as the plant DNA control. Spores were obtained from mycorrhizal fungi grown with corn in pot cultures containing soil:sand (1:1 v:v) mixture as previously described (Millner et al. l998), or they were obtained directly from INVAM (J. Morton). Isolates from a sustainable farming research site in Beltsville Md. were obtained directly from field soil. Soil for pot cultures was a silt loam, fine loamy, mixed mesic Aeric Ochraqauaif. Plants were inoculated with pot-culture inoculum or hand-picked spores unless they were non-inoculated controls. Spores were harvested by standard wet-sieving and cleaned, examined, and hand-picked as previously described (Millner et al. 1998). Intact, clean spores were placed into 0.5-ml Eppendorf tubes, excess water was removed from the tube, and the spores were suspended in 5–10 µl of PCR-grade water (Gibco BRL, cell culture, endotoxin-free, membrane filtered, distilled water, Bethesda, Md.). Roots from inoculated and non-inoculated corn pot cultures grown in soil:sand (1:1 v:v) were washed free of adhering soil and processed for DNA extraction as described below.

DNA extraction

Spores were crushed in the Eppendorf tubes using autoclaved disposable (single-use) polycarbonate micropestles (VWR Scientific). The number of spores of each isolate used for DNA extraction ranged from 1–1,200 per preparation (see additional documentary material at http://link.springer.de/link/service/journals/00572/index.htm). Each spore crush was resuspended in 10 volumes of water and a one-third volume of Chelex 100 resin (BioRad, Hercules, Calif.) solution (20% w/v). Spore crushes were sonicated 15 s in a jewelry cleaning unit (Model 77, Electromation Components Corp., N.Y.), then freeze-thawed at –20°C and room temperature three times. Preparations were stored at –20°C and diluted as needed with PCR-grade water before use as templates.

Corn roots (150 mg) were frozen at –20°C and ground to a fine powder with liquid nitrogen in a pre-chilled (–80°C) mortar and pestle. DNA was extracted from the powdered root tissue using a DNAzol ES DNA extraction kit (Molecular Research Center, Cincinnati, Ohio).

Oligonucleotides

Oligonucleotides used in this study were synthesized commercially and used unpurified. The sequence of ITS4*Pst* was described previously (Millner et al. 1998). Other oligonucleotides were ITS1*Kpn* (5′- TAG GTA CCG TAG GTG AAC CTG CGG AAG GAT C), GETU1 (5′-GTA TTC AAA ACC CAC ACT), GETU2 (5′-CTC ATC AAG CAA TTA CGA), GETU3 (5′-T CTC ATC AAG CAA TTA CG), and GETU4 (5′-TT CTC ATC AAG CAA TTA CG).

PCR conditions and PCR product analysis

PCRs were performed with an automated temperature cycling instrument (MJ Research, Inc., Watertown, Mass.) with mixtures containing the following (final concentrations): 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 250 µM each of the four deoxynucleotide triphosphates (dNTPs), 0.25 µM (each) primer, 5 units/100 µl of Amplitaq Gold DNA polymerase (PE Applied Biosystems, Foster City, Calif.) and template DNA. Reaction mixtures for use with spore preparations contained a 1/10 volume of diluted template DNA (dilutions ranged from 1/10 to 1/100 for glomalean templates and 1/100 to 1/1,000 for non-mycorrhizal control templates). Dilutions of *G. etunicatum* template DNA that produced adequate product yields with the ITS1:ITS4 primer pair also produced good yields when amplified with GETU1 and GETU2; all amplifications were carried out at least twice. Reaction mixtures used for detecting *G. etunicatum* in root fragment preparations contained dilutions of root extract DNA ranging from 1/500 to 1/5,000.

PCR components were assembled on ice and then transferred to a thermal cycler block pre-heated to 95°C (to reduce non-specific priming). For reactions using ITS primers, the components were initially heated for 9 min at 95°C to activate the enzyme and were then subjected to 40 cycles of 30 s at 95°C, 45 s at 55 °C, and 60 s at 72°C, with a final extension step of 72°C for 5 min. For reactions using the primer pair GETU1:GETU2, the components were initially heated for 9 min at 95°C, then subjected to 35 cycles of 60 s at 95°C, 60 s at 53°C, and 60 s at 72°C, with a final extension step of 72°C for 5 min.

DNA cloning

DNA products from 100 µl PCRs using ITS1*Kpn* and ITS4*Pst* primers were diluted and washed twice with ten-fold volumes of water using Centricon-30 units (Amicon, Inc., Boston, Mass.). After digestion with *Kpn*I and *Pst*I, the products were purified from an 0.8% agarose gel using a GeneClean kit (Bio101, La Jolla, Calif.), and ligated with 0.25 µg of pUC19 DNA that had been digested with *Kpn*I and *Pst*I. The ligated products were used to transform electrocompetent cells of *E. coli* DH5 α (Gibco BRL, Gaithersburg, Md.). An alkaline lysis miniprep procedure (Sambrook et al. 1989) was used to identify transformants containing plasmid inserts of the correct size (590 bp). Plasmid DNA preparations for sequencing were purified by alkaline lysis with at least one PEG precipitation step to remove contaminating RNA (Ausubel et al. 1987).

Sequencing

Cloned DNA from 24 glomalean isolates was sequenced; sequences of two to four clones from each strain were determined. GenBank accession numbers are available in the additional documentary material at http://link.springer.de/link/service/journals/00572/index.htm. Sequencing reactions were performed at the Center for Agricultural Biotechnology, University of Maryland (College Park, Md.) using purified plasmid DNAs, forward and reverse M13 sequencing primers and an ABI Prism sequencing kit (PE Applied Biosystems). Reaction products were analyzed using an ABI Prism 377 Genetic Analyzer and sequences were aligned using programs from DNAstar and GCG (Madison, Wisc.). Oligonucleotide regions were analyzed individually using OLIGO version 5.0 (National Biosciences, Plymouth, Minn.) to evaluate their suitability as primers for PCR.

Electrophoresis

PCR products were separated by electrophoresis using 0.8% agarose gels and 0.04 M, pH 8.0, TAE buffer (Sambrook et al. 1989) containing 0.5 µg/ml ethidium bromide. Gel images were captured using a CCD camera and image software (Biophotonics Corporation, Ann Arbor, Mich.) while illuminated with ultraviolet light.

Phylogenetic analysis

Multiple sequences were aligned and edited using programs (SeqLab, Pileup, Pretty) in the Wisconsin (GCG) package version 10.0: Unix (Genetics Computer Group Inc., Madison, Wisc.). For the pileup program, a gap creation penalty of 5 and a gap length weight of 2 were used; fine adjustments were made manually in SeqLab after visual inspection. Phylogenetic analysis of aligned sequences was performed using the Farris algorithm for sequence addition with the heuristic search option of PAUP (version 4.0.0d55 for Unix; Swofford 1997) run in SeqLab of GCG with Exceed (Hummingbird Communications Ltd., Toronto, Canada) X-windows. Analyses (PAUP) were performed with all characters unweighted. Phylogenetic trees were constructed using the original data set and 500 and 100 bootstrap data sets for the *Glomus* and the Glomales trees, respectively. Bootstrapping was used to calculate confidence indices for tree branches and thereby indicate the robustness of the tree structure. Trees show only groupings with bootstrap values of more than 50 percent. Others were collapsed to polytomies in order to avoid misinterpretation of the low bootstrap supports.

Nucleotide sequence accession numbers

GenBank accession numbers for the amplified ITS1, ITS2, and 5.8S sequences used to develop the GETU primers are available in the additional documentary material at http://link.springer.de/link/service/journals/00572/index.htm.

Results

DNA amplification and sequencing

The ITS region from the *G. etunicatum* strains MD107, MD127, TN101, and FL329, as well as other glomalean taxa and non-mycorrhizal control templates, was reproducibly amplified by PCR using ITS1*Kpn* and ITS4*Pst*. A major double-stranded product of approximately 550–600 bp was generated from all templates. Amplification products were sequenced and aligned as described. Alignments of the entire sequences of these and other isolates used in the study are available in the additional documentary material at http://link.springer.de/link/service/journals/00572/index.htm. Sequence alignment of the DNAs from *G. etunicatum* isolates MD107, MD127, and TN101 shows that the 5.8S gene subunit regions (155 bp) and ITS2 regions (230 bp) are identical, whereas the ITS1 regions (135 bp) are 98% similar. Alignment of these three sequences with that from the *G. etunicatum* isolate FL329 shows 100% similarity in the 5.8S gene subunit region, but significantly less similarity in the ITS1 and ITS2 regions (81 and 89% similarity, respectively). When aligned with sequences of the corresponding ITS and 5.8S gene regions from *G. claroideum* SC186, *G. mosseae* UK118, *G. monosporum* IT102, *G. intraradices* FL208, *G. occultum* IA702, *Gigaspora rosea* FL105, *Gigaspora albida* BR201, and *S. coralloidea* CA260 (data not shown), several regions appeared unique to the *G. etunicatum* isolates.

Selection and testing of GETU oligonucleotides

Comparison of sequence alignments for regions unique to *G. etunicatum* (see the additional documentary material at http://link.springer.de/link/service/journals/00572/index.htm) resulted in few sequences that would have the physico-chemical properties suitable for design of PCR primers. Initial tests with primer pairs GETU1:GETU3 and GETU1:GETU4 detected amplified product from isolates of *G. etunicatum*, but also detected *G. intraradices* WY ith-4. Reactions containing GETU1 and GETU2 primed amplification of all 18 isolates of *G. etunicatum* (data not shown), without amplification of other *Glomus* species or other glomalean taxa (Fig. 1). Subsequent assay results with this latter primer pair showed a robust capacity to generate a sufficient quantity of the expected size PCR product (400 bp) exclusively from *G. etunicatum* templates.

The PCR competency of all genomic DNA templates not amplified in reactions with GETU1 and GETU2 (Fig. 1A) was verified by PCR assays primed by the ITS1*Kpn*:ITS4*Pst* primer pair (Fig. 1B); PCR products of the expected size (550–600 bp) were obtained from all those templates. Thus, we conclude that the primer pair GETU1:GETU2 is highly specific for *G. etunicatum*.

The detection of *G. etunicatum* in root samples is an important intended use of these primers and, therefore, detection was tested by both direct and nested PCR assays. Detection of *G. etunicatum* in colonized corn roots by PCR was achieved directly using the GETU1:GETU2 primer pair and root DNA extracts diluted 1/500 and 1/5,000 (Fig. 2, lanes 2, 3). PCR assays using comparable dilutions of root DNA extracts from non-inoculated plants and GETU1:GETU2 yielded no DNA products (Fig. 2, lanes 4, 5). Nested amplification reactions were also tested using diluted corn root extracts and the primer pair ITS1*Kpn*:ITS4*Pst* in the first reaction and the primer pair GETU1:GETU2 in the second reaction (Fig. 2, lanes 6–11). PCRs using ITS1*Kpn*:ITS4*Pst* and dilut-

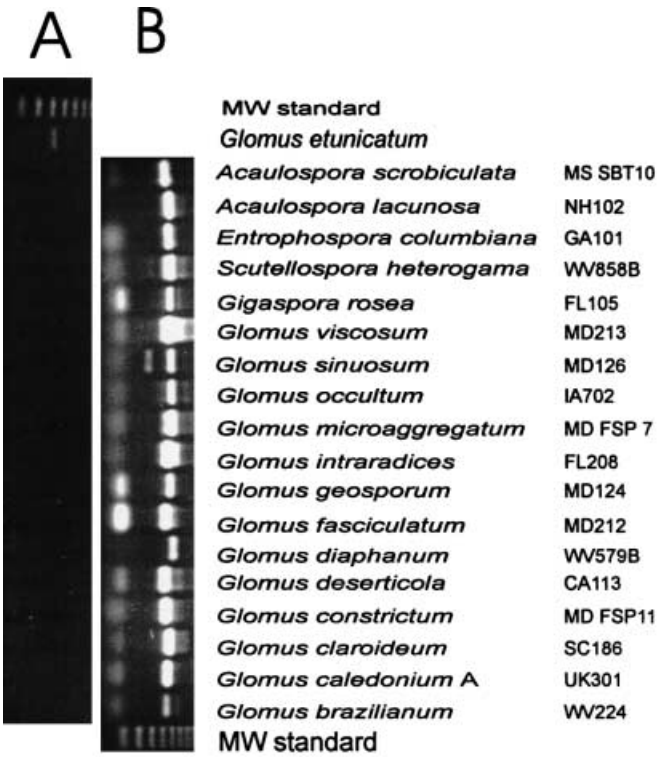

Fig. 1 Amplified products from PCR using genomic DNA preparations from spores of *Glomus etunicatum* and related fungal isolates as templates and **A** *G. etunicatum* specific primers (GETU1 and GETU2) or **B** primers ITS1*Kpn* and ITS4*Pst*. Strain designations are available in the additional documentary material at http://link.springer.de/link/service/journals/00572/index.htm

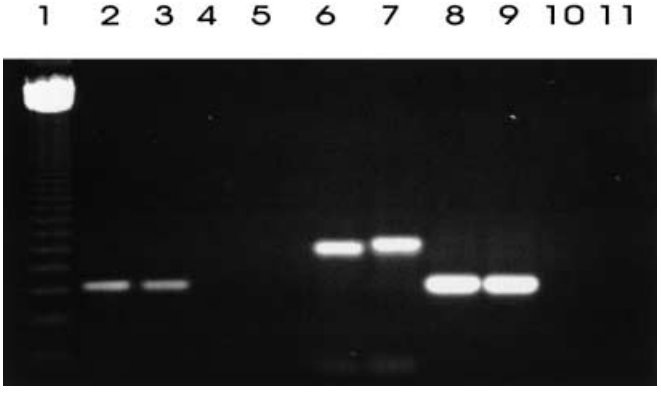

Fig. 2 Comparison of PCR products of rDNA amplified with primer pairs ITS1*Kpn*:ITS4*Pst* or GETU1:GETU2 and DNA preparations from inoculated and non-inoculated corn (*Zea mays* L.) roots. *Lane 1*: 123 bp ladder DNA standard; *lanes 2–5*: PCR products using GETU1:GETU2 and diluted DNA from roots of inoculated (lanes 2, 3) and non-inoculated (lanes 4, 5) corn; *lanes 6, 7*: PCR products using ITS1*Kpn*:ITS4*Pst* and diluted DNA from roots of inoculated (lane 6) and non-inoculated (lane 7) corn; *lanes 8–11*: nested PCR products using GETU1:GETU2 primers to reamplify PCR products from reactions using ITS1*Kpn*:ITS4*Pst* and diluted DNA from roots of inoculated (lanes 8, 9) and non-inoculated (lanes 10, 11) corn. DNA preparations from roots were diluted 1/500 (lanes 2, 4) or 1/5,000 (lanes 3, 5–7) in PCR-grade water. For nested PCRs (lanes 8–11), aliquots of reactions using ITS1*Kpn*:ITS4*Pst* primers were diluted 1/2,000 (lanes 8, 10) or 1/10,000 (lanes 9, 11) in PCR-grade water prior to use as templates for PCRs using GETU1:GETU2 primers

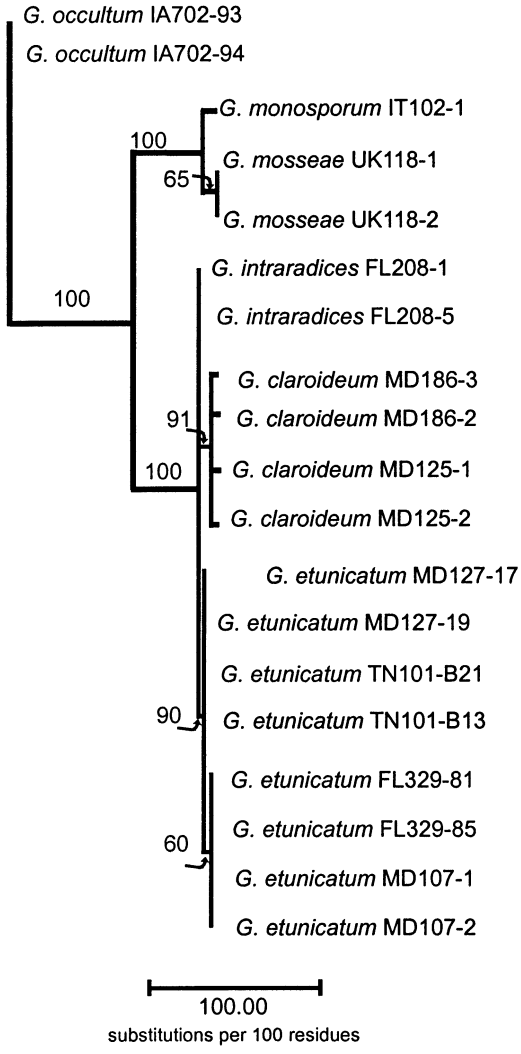

Fig. 3 The most parsimonious tree for 19 *Glomus* rDNA sequences. The tree was inferred from a 277-nucleotide base region spanning the 5.8S and a portion of the ITS2 region, using PAUP 4.0.0d55 with GCG SeqLab. Confidence limits of the branches were estimated by bootstrap analyses as described in Materials and methods; the values above the branch lines indicate the percent this group occurred in 500 bootstrap replicates. *Horizontal lines* are to the scale shown; number of nucleotide substitutions per 100 residues. *Dashed numbers* after strain designations correspond to different clones

ed root extracts from mycorrhizal or non-inoculated roots both yielded products of approximately 550 bp (Fig. 2, lanes 6, 7, respectively). However, when these primary PCR products were diluted and amplified in PCRs using the GETU1:GETU2 primer pair, the expected 400-bp *G. etunicatum* product was only detected when the primary product had been amplified from extracts of mycorrhizal roots (Fig. 2, lanes 8, 9).

Phylogenetic analysis

Using the heuristic search and parsimony approach from PAUP, two full phylogenetic trees of all data are shown

for alignable sequences (Figs. 3, 4). One tree (Fig. 3) was based on 19 *Glomus* sequences spanning 277 bp of the 5.8S and ITS2 regions obtained from 10 different strains representing six currently distinct species. The second tree (Fig. 4) was based on 155 bp regions comprising the 5.8S rDNA gene of 39 sequences of AMF representing three families (Acaulosporaceae, Gigasporaceae, Glomaceae), five genera (*Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, and *Scutellospora)*, and 13 species. Genetic distances between strains are shown on the scale accompanying each tree (Figs. 3, 4) as the number of nucleotide substitutions per 100 residues.

The *Glomus* tree (Fig. 3) shows strong bootstrap support for sequence consistency among different clones and strains of *G. etunicatum* in the 5.8S and ITS2 regions. It also shows the close, yet discreet, relationship of *G. etunicatum* to *G. claroideum* and *G. intraradices*. In contrast, *G. mosseae-monosporum* is solidly distinct from the *G. etunicatum-claroideum-intraradices* branch. The most divergent sequences were obtained from *G. occultum* and included a mix of base substitutions and deletions that occurred primarily in the ITS region, although significant sections of 5.8S were also affected (see the additional documentary material at http://link.springer.de/link/service/journals/00572/index.htm).

In the Glomales tree (Fig. 4), which is based only on nucleotides in the highly conserved 5.8S region, the three main branches obtained in the *Glomus* tree (Fig. 3) are again strongly supported by bootstrap analysis. In addition, the uniquely singular position of the *G. occultum* branch bears the appearance of an outgroup. There is an unusual split juxtaposition of sequences obtained from taxa currently classified in the Acaulosporaceae. Finally, bootstrap analysis of sequences in the Glomales tree (Fig. 4) confirmed the strong, close relationship between the represented taxa from the Gigasporaceae reported previously (Simon et al. 1993b; Simon 1995; Gehrig et al. 1996).

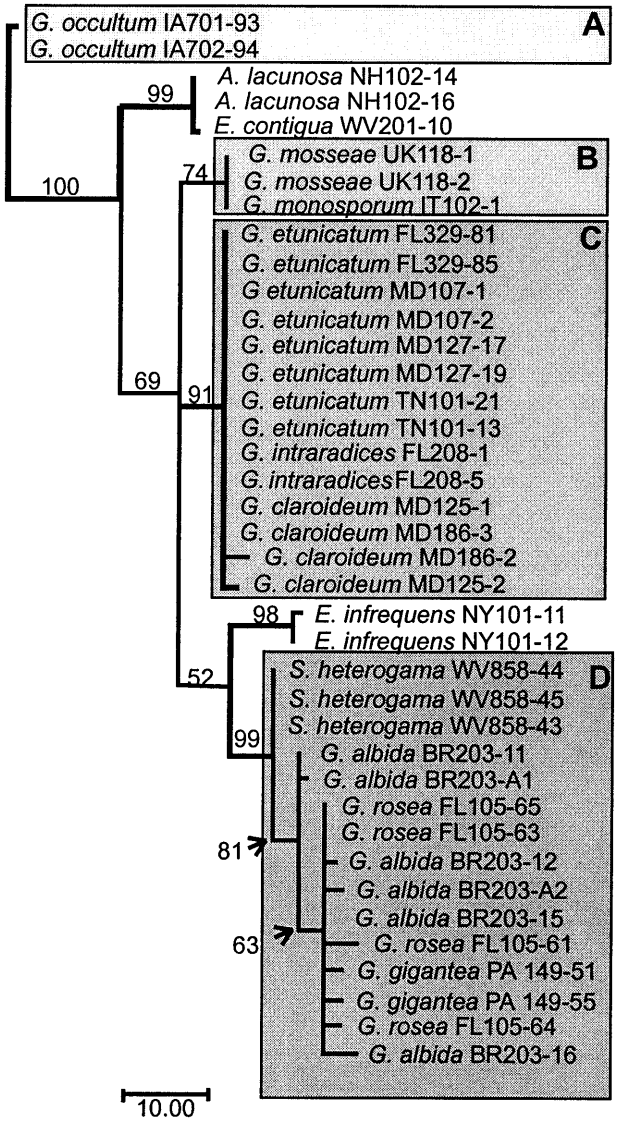

Fig. 4 The most parsimonious tree for 38 rDNA sequences of arbuscular mycorrhizal fungi. The tree was inferred from a 155-nucleotide base region corresponding to the 5.8S rRNA subunit, using PAUP 4.0.0d55 with GCG SeqLab. Confidence limits of the branches were estimated by bootstrap analyses as described in Materials and methods; the values above the branch lines indicate the percent this group occurred in 100 bootstrap replicates. *Bold lines* indicate branches with at least 95% confidence indices. *Horizontal lines* are to the scale shown; number of nucleotide substitutions per 100 residues. *Box A* corresponds to the ancestral group of *Glomus* species described by Redecker et al. 2000; *box B* corresponds to the *G. mosseae* cluster; *box C* corresponds to the *G. etunicatum* cluster; and *box D* corresponds to the Gigasporaceae. Acaulosporaceae are not affiliated with a single position in this tree. *Dashed numbers* after strain designations correspond to different clones

Discussion

In this study, the ITS and 5.8S rDNA regions of several glomalean isolates were amplified by PCR. These PCR amplification products were cloned and sequenced, and sequences were aligned. Regions of potential uniqueness for *G. etunicatum*-specific PCR primers were deduced from these alignments. The selection of GETU1 and GETU2 was based on a comparison of ITS and 5.8S rDNA region sequences of *G. etunicatum* with those of *G. intraradices*, *G. claroideum*, *G. mosseae*, *G. occultum*, *Gigaspora rosea*, *S. heterogama*, *A. lacunosa*, and *E. infrequens*. Amplification test results with the primer pair GETU1:GETU2 show that these primers are specific for *G. etunicatum*.

Analysis of the sequence alignments for these isolates (see the additional documentary material at http://link.springer.de/link/service/journals/00572/index.htm) shows that the ITS and 5.8S regions will not provide the specificity necessary for the design of isolate-specific oligonucleotide primers or probes. For isolate specificity, the use of RAPD-PCR followed by cloning, sequencing, and subsequent oligonucleotide selection and testing, as reported (Abbas et al. 1996) for *G. mosseae* and *Gigaspora margarita* (Lanfranco et al. 1995; Zézé et al. 1997), appears to be a more productive approach at present.

The extent of possible intra-genomic sequence heterogeneities to GETU1 and GETU2 was not determined directly for 100 clones as was done with *G. mosseae* in a previous study (Millner et al. 1998), because sequence alignments for MD107, MD127, and TN101 showed a very high degree of intraclonal and interisolate similarity (see the additional documentary material at http://link.springer.de/link/service/journals/00572/index.htm). Sequence data and results from the PCR amplification tests confirm that GETU1 and GETU2 are sufficiently robust to reliably detect and amplify rDNA regions of *G. etunicatum* exclusively, even if some sequence heterogeneity occurs. These results suggest that the GETU1:GETU2 primer pair would reliably detect *G. etunicatum*, even though sequence heterogeneities may yet be shown to occur in isolates not examined in this study. Heterogeneity of ITS sequences in nuclei within a single spore and between spores of an individual isolate may eventually be discovered for *G. etunicatum* as they have been for *G. mosseae* (Sanders et al. 1995; Dodd et al. 1996; Lloyd-MacGilp et al.1996; Trouvelot et al. 1999) and *Gigaspora margarita* (Zézé et al. 1997). However, we have no evidence from our results that such genomic heterogeneity will render the GETU1:GETU2 primer pair incapable of detecting any remaining homogeneous sequence. Based on the results presented here, we conclude that in extracts from colonized roots or directly from extracts of multiple spores, PCR products would contain at least enough of the target TSO sequences for *G. etunicatum* to be reliably detected when PCR includes the GETU1:GETU2 primer pair. Obviously, prior to use of the GETU1:GETU2 primer pair in tests of field-collected roots at various study sites, tests on the spores of *G. etunicatum* (collected at the field sites of interest) should be conducted to verify that the isolate(s) can be detected and are not in some way fundamentally different from those in this study.

The collection of *G. etunicatum* isolates used in this study includes 47.8% of the isolates presently released by INVAM for this taxon, and thus represents a reasonable cross-section of the current genomic material in the collection for this taxonomic species. Not even potential false-positive or false-negative results from PCR assays using the GETU1:GETU2 primer pair with MD125 and FL329 occurred. These two INVAM isolates are morphologically unusual enough to have been initially characterized as *G. etunicatum*-like. Results from PCR assays with the GETU1:GETU2 primer pair show that MD125 is not *G. etunicatum* but FL329 is. Both of these results are consistent with the rDNA sequence similarity data (Figs. 3, 4 and http://link.springer.de/link/service/journals/00572/index.htm). Identification difficulties with other isolates that have one or more atypical morphological characteristics might also be aided by the use of the GETU1:GETU2 primer pair in PCR assays. In contrast to the recently reported primer LETC1670 (Redecker 2000) designed for use with primer ITS4, our GETU1:GETU2 primer pair only generates product with *G. etunicatum* and not with *G. claroideum*, as reported for LETC1670:ITS4.

The utility of the GETU1:GETU2 primer pair for detecting *G. etunicatum* in colonized roots was demonstrated using DNA extracts of corn. However, at present, we have no precise information on the detection sensitivity of GETU1 and GETU2 in PCR amplifications with mycorrhizal roots, except that detection was positive with high dilutions of root extract as template. *G. etunicatum* was readily detected in PCR amplifications containing root extract DNA diluted 1/5,000 and in nested PCR amplifications containing 1/2,000 and 1/10,000 dilutions of the primary PCR product.

Finally, the phylogenetic analysis further supports the independence of *G. etunicatum* despite its close relationship to *G. claroideum* and *G. intraradices* in the 5.8S and ITS rDNA region. In addition, the phylogenetic analysis of 5.8S rDNA Glomales sequences presented here are consistent with some parts of previously published trees for this group of fungi, but different in other parts. Namely, the branch corresponding to the Gigasporaceae is strongly supported here by bootstrap analysis as it was in trees inferred from partial 18S sequence data (Simon et al. 1993b; Simon 1995; Gehrig et al. 1996; Redecker et al. 2000). Our 5.8S-based tree (Fig. 4) shows bootstrap support for *E. infrequens* as a sister group to the Gigasporaceae. This is consistent with the phylogenetic relationship between some members of the Acaulosporaceae, including *E. colombiana*, and Gigasporaceae reported by Redecker et al. (1999) in their 5.8S tree. However, the disjunct relationship of *E. infrequens* to other members of the Acaulosporaceae included in our tree (*E. contingua* WV201 and *A. lacunosa* NH102, Fig. 4) is inconsistent with the reported subgroup (Redecker 2000) comprising *A. lacunosa* and *E. colombiana*. These inconsistencies show the need for a more extensive examination of the 5.8S and 18S regions among other isolates and taxa of Entrophospora and Acaulospora to determine the nature and significance of this degree of sequence disparity in this family.

The *Glomus* group presented in Fig. 4 comprises two clusters, *G. mosseae* and *G. etunicatum-claroideum-intraradices*. This differs from the 18S trees previously published (Redecker et al. 2000) in which *G. intraradices* and *G. mosseae* were inferred as descendents of *G. etunicatum* and contemporaries with each other. The reports indicated that *G. intraradices* was a descendent of *G. mosseae* (Simon et al. 1993b; Simon 1995; Gehrig et al. 1996). The contemporary relationship between *G. mosseae* and *G. etunicatum* and *G. claroideum* is consistent with the 5.8S tree previously reported by Redecker et al. (1999). The different phylogenetic relationships inferred for these taxa depend on which region of rDNA, 18S or 5.8S was used for phylogenetic analysis.

The very strong ancestral branch obtained for *G. occultum* is consistent with the ancestral lineage of this taxon derived from phylogenetic analysis of 18S sequence data recently reported (Redecker et al. 2000).

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