Mycoscience 39: 477–480, 1998

Short Communication

Phylogenetic position of an arbuscular mycorrhizal fungus, *Acaulospora gerdemannii*, and its synanamorph *Glomus leptotichum*, based upon 18S rRNA gene sequence

Hiromichi Sawaki, Koya Sugawara and Masanori Saito

National Grassland Research Institute, 768, Senbonmatsu, Nishinasuno, Tochigi, 329–2793, Japan

Accepted for publication 9 October 1998

We examined the phylogenetic position of an arbuscular mycorrhizal fungus which produces two types of spore, *Acaulospora gerdemannii* and *Glomus leptotichum*, based upon the DNA sequence of the 18S rRNA gene. DNA was extracted separately from both *Glomus*-like or *Acaulospora*-like spores and partial 5′-terminus segments of 18S rRNA gene were amplified by the PCR method. Several clones derived from each spore type were sequenced and compared. The sequences from both spore types agreed well, confirming that these morphologically different spores were formed by the same fungus. Nucleotide substitutions were found among several clones, suggesting polymorphism of the rRNA gene in glomalean fungi. Further phylogenetic analysis based upon the whole sequence of the 18S rRNA gene showed that *A. gerdemannii* may be within the order Glomales but is far from the fungi that have been analyzed and probably should be in a new family.

Key Words——18S rRNA; Glomales; PCR; polymorphism; synanamorph.

Arbuscular mycorrhizal fungi belonging to the order Glomales are symbiotic fungi co-existing with various terrestrial plant roots. Since teleomorphs of glomalean fungi are not known, the taxonomy of this group is based upon morphology of presumably asexual spores. Glomales is divided into three families, each of which is comprised of two genera (Morton and Benny, 1990). Early phylogenetic analysis of the nuclear 18S rRNA gene sequence supported the taxonomy, at least to the family level (Simon et al., 1993). Although more than 100 species were reported in Glomales (Walker and Trappe, 1993), only a few species have been examined for their rRNA gene sequences. A more thorough analysis should result in a revised taxonomy based upon morphology and phylogeny.

Acaulospora gerdemannii Schenck & Nicolson, a synanamorph of *Glomus leptotichum* Schenck & Smith, is a glomalean fungus with dimorphic spores (Walker, 1992; Morton et al., 1997). This species was isolated from a semi-natural grassland in Japan and was confirmed to be conspecific with *G. leptotichum* by isolation of single-spore cultures that produced spores of both *A. gerdemannii* and *G. leptotichum* morphs (Murakoshi et al., 1998). The genera *Glomus* and *Acaulospora* are presently placed in different families, the Glomaceae and Acaulosporaceae, respectively, but it is unusual for two different morphs of the same species to belong to different families. Morton et al. (1997) suggested this species may be transferred to Glomaceae or Acaulosporaceae, but the creation of a new monospecific taxon was not discussed. To clarify the phylogenetic position of this species, therefore, we examined its 18S rRNA gene sequence.

Fungal species and culture conditions *Acaulospora gerdemannii* (*G. leptotichum*) MAFF520055, a culture first established as a soil trap culture from a semi-natural grassland in Japan, was used for several single-spore isolations with *Sorghum bicolor* Pers. (Murakoshi et al., 1998). Resultant spores of both *Acaulospora* and *Glomus* morphs were extracted from one of these (voucher specimen no. 96–24) and a further single-spore ramet was grown with *Trifolium repens* L. (voucher specimen no. 97–33). The isolate and their herbarium vouchers are deposited in the Laboratory of Soil Microbiology, the National Grassland Research Institute.

DNA extraction Ten spores from each morph represented by voucher specimen 96–24 and 10 *Acaulospora* morph spores (Voucher 97–33) were collected from sieved pot culture substrate with fine forceps and were washed several times with sterile water by ultrasonication. The spores were crushed in 20 μl of InstaGene matrix (BIO-RAD) with a micropipette tip under a dissecting microscope. DNA was extracted according to the manufacture's protocol. The extracted DNA was further purified by ethanol precipitation and used as a template for the polymerase chain reaction (PCR).

PCR amplification and nucleotide sequencing DNA amplification was executed with Takara ExTaq polymerase and specific primer pairs for partial segments of the 18S rRNA gene: NS1/NS21 for the specimen 96–24,

NS3/NS5, NS4/NS8, and NS3/NS6 for the specimen 97–33 (White et al., 1990; Simon et al., 1992). The reactions were performed in a MJ Research PTC-100 thermal cycler programmed as follows: initial step for denaturation of DNA fragments, 85 s at 94°C; first 14 of 40 cycles, 35 s at 95°C for denaturation, 55 s at 55°C for annealing, and 45 s at 72°C for polymerization; following 11 cycles, polymerization prolonged to 2 min; remaining 15 cycles, polymerization further extended to 3 min; final elongation step, 10 min at 72°C. The amplified fragments were directly ligated with pT7BlueT vector (Novagen) using Ligation-kit No. 2 (Takara). The plasmids were cloned in competent *Escherichia coli* cells of strain NovaBlue (Novagen) and sequenced with AmpliTaq DNA polymerase and universal and reversal M13 sequence primers using an automated DNA sequencer model 373A (Applied Biosystems). The sequences of partial segments amplified with each primer pair adjoined the near full-size 18S rRNA gene of 1785 bps.

DNA sequence and phylogenetic analysis The nucleotide sequences of fragments of about 590 bps derived from spores of both *Glomus* and *Acaulospora* morphs using the NS1/NS21 primer pair were manually aligned and compared. The adjoining 1785 bps nucleotide sequence of *A. gerdemannii* (*G. leptotichum*) 18S rRNA gene was aligned manually with the following published sequences of glomalean and non-glomalean fungi from the nucleotide sequence databases: *Acaulospora rugosa* Morton (accession number Z14005), *Acaulospora spinosa* Walker & Trappe (Z14004), *Entrophospora* sp. (Z14011), *Gigaspora albida* Schenck & Smith (Z14009), *Gigaspora margarita* Becker & Hall (X58726), *Scutellospora dipapillosa* (Walker & Koske) Walker & Sanders (Z14013), *Glomus etunicatum* Becker & Gerdemann (Z14008), and *Glomus versiforme* (Karsten) Berch (X86687); and *Basidiobolus ranarum* Eidam (D29946) (Zygomycetes) and *Saccharomyces cerevisiae* Meyen ex E. C. Hansen (Z75578) (Ascomycetes). In the multi-aligned sequences, deletions and insertions were removed to prevent overestimation of gaps in the following phylogenetic analyses. A data set of 1,500 sites was taken for analyses using the PHYLIP program package, version 3.572 (Felsenstein, 1996). Bootstrap analyses were performed for evaluation of the robustness of phylogenetic topology, with 1,000 random resamplings each for the neighbor-joining (NJ) and UPGMA methods, and 100 resamplings for the maximum-likelihood (ML) method. The data set used for phylogenetic analyses in this study is obtainable from the corresponding author.

Nucleotide Sequences Accession Number The determined nucleotide sequences of the *A. gerdemannii* 18S rRNA gene were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers, AB015052, AB015711, AB015712, AB015713, AB015714, AB015715, AB015716, and AB015717.

Comparison of partial sequence of 18S rRNA from *Glomus* and *Acaulospora* morph spores The NS1/NS21 primer pair amplified DNA fragments of 587–594 bps. Four clones (G1, G2, G3 and G4) from the *Glomus* morph and three clones (A1, A2, and A3) from the *Acaulospora* morph were sequenced, and the determined nucleotide sequences were aligned. Whole sequences of these clones were not shown in this paper, and the sequences will appear in the nucleotide sequence databases. Clone G1 was completely identical to clone A1. In comparison with G1, all other clones contained base substitutions at two to eight sites or a 7 bps insertion at one site in clone A3. Two parts of the sequences are shown in Fig. 1, where dots indicate identical bases with counterpart site of clone A1. The same substitutions from T to C, at sites 213, 222, and 305 bps from the 5′-terminal in clone G1, were found among the independent clones. These substitutions are probably intrinsic, although other substitutions might be artificial mutations caused by PCR amplification.

Some glomalean fungi form their spores inside dead spores of other glomalean fungi (Koske et al., 1986), and there is thus a possibility that, even with single-spore cultures, a spore that is apparently of one species may produce a culture of something different. However, this event is unlikely with our method, since spores were freshly removed from pot cultures, and two generations of single-spore isolation produced the same result. In addition, the sequences obtained from both *Glomus* and *Acaulospora* morphs from our isolate agreed well with each other, confirming that these morphologically different spores were genetically identical and part of the same genet. Nucleotide substitutions were found among the DNA clones, reinforcing previous evidence for polymor-

```
G1 210:TCTTCGGGCGAGTCCCTTGGTG 230   G1 300:ACTTTCGATGG 310
G2 209:....................... 230   G2 299:.......... 309
G3 210:....................... 230   G3 300:.....T..... 310
G4 210:....T........T........ 230   G4 300:.......... 310
A1 210:....................... 230   A1 300:.......... 310
A2 210:....T........T........ 230   A2 300:.......... 310
A3 217:....................... 237   A3 307:.....T..... 317
```

Fig. 1. Partial sequences of rRNA genes independently cloned from *Glomus leptotichum* and *Acaulospora gerdemannii* spores from a single-spore isolate of this dimorphic fungus.
Clones G1, G2, G3, and G4 were from the *Glomus* morph. Clones A1, A2 and A3 were from the *Acaulospora* morph. Shadowed areas indicate that substitution occurred in independent clones.

phism in the rRNA gene of a single species of glomalean fungi (Sanders et al., 1995; Lloyd-Macgilp et al., 1996).

Phylogenetic analysis based on 18S rRNA gene Figure 2 shows phylogenetic trees constructed from 1,500 sites of almost the complete sequence of the 18S rRNA gene. *Acaulospora gerdemannii* (*G. leptotichum*) and other sequence members of the Glomales formed a monophyletic cluster by all methods, reinforcing the view that these fungi all fall within the same ordinal clades. However, the position of *A. gerdemannii* (*G. leptotichum*) was separated from all other glomalean clades. Phylogenetic analysis by all methods indicated that the *A. gerdemannii* (*G. leptotichum*) branch was separated from other Glomales with comparatively high bootstrap percentages: 82.6% in NJ and 79.2% in UPGMA, each with 1,000 replicates, and 76% in ML with 100 replicates. Recently *Geosiphon pyriforme* F. v. Wettstein was found to be an ancestor of the Glomales (Gehrig et al., 1996). We could not clarify the phylogenetic relationship between *G. pyriforme* and *A. gerdemannii* (*G. leptotichum*), because the position of *G. pyriforme* changed depending on the method used. Much more nucleotide information about these organisms is needed to make a strong phylogenetic tree.

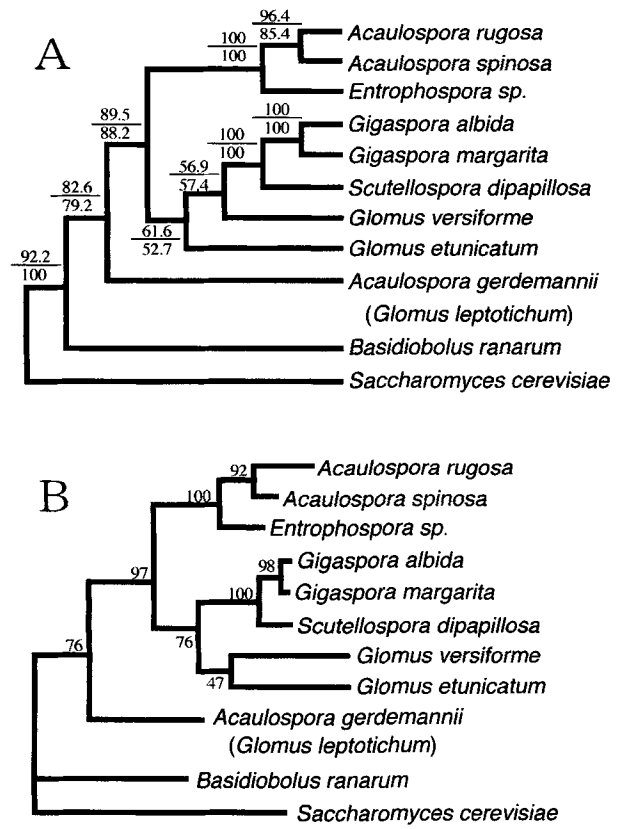

Fig. 2. Phylogenetic relationships among glomalean fungi, *Acaulospora gerdemannii* (*Glomus leptotichum*), *Basidiobolus ranarum* (Zygomycetes), and *Saccharomyces cerevisiae* (Ascomycetes), inferred from 1,500 aligned sites of 18S rRNA gene sequence.
A. Neighbor-joining and UPGMA tree; bootstrap percentages from 1,000 replicates are shown on the respective internal nodes (above percentages by NJ and below by UPGMA). B. Maximum likelihood tree (100 replicates).

The taxonomy of *A. gerdemannii* and *G. leptotichum* has been confused, because of the formation of two different spores morphs (Walker, 1992; Morton et al., 1997). Other than the dimorphic nature in spore production, this fungus has morphological characteristics found in few other known glomalean fungi. For example, the pedicel in the *Acaulospora*-like spore is known only from *Acaulospora nicolsonii* Walker, Reed & Sanders (Walker et al., 1984), but not in any other described *Acaulospora* sp. Thus, both the molecular phylogeny and the morphology of this species suggest that it should be placed in a new family in Glomales. Although Glomales has been classified in Zygomycetes, recent phylogenetic studies showed that this order lies between Zygomycetes and the higher fungi, Ascomycetes and Basidiomycetes (Nagahama et al., 1995; Gehrig et al., 1996; Sugiyama et al., 1996). Sugiyama (1996) suggested that Glomales may be a turning point toward higher fungi from Zygomycetes. Since *A. gerdemannii* (*G. leptotichum*) shows a more complex life cycle than most other glomalean fungi, this and further studies on this species may shed light on the lineage of Glomales with higher fungi.

Acknowledgement——The authors are grateful to Dr. C. Walker for his critical reading and comments on the draft of this paper.

Literature cited

Felsenstein, J. 1996. PHYLIP (Phylogeny Inference Package) version 3.572. Univ. Washington, Seattle, USA.

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

Koske, R. E., Gemma, J. N. and Olexia, P. D. 1986. *Glomus microaggregatum*, a new species in the Endogonaceae. Mycotaxon **26**: 125–132.

Lloyd-Macgilp, S. A., Chambers, S. M., Dodd, J. C., Fitter, A. H., Walker, C. and Young, J. P. W. 1996. Diversity of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi. New Phytol. **133**: 103–111.

Morton, J. B. and Benny, G. L. 1990. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporianeae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. Mycotaxon **37**: 471–491.

Morton, J. B., Bever, J. D., and Pfleger, F. L. 1997. Taxonomy of *Acaulospora gerdemannii* and *Glomus leptotichum*, synanamorphs of an arbuscular mycorrhizal fungus in Glomales. Mycol. Res. **101**: 625–631.

Murakoshi, T., Tojo, M., Walker, C. and Saito, M. 1998. Arbuscular mycorrhizal fungi on adjacent semi-natural grasslands with different vegetation in Japan. Mycoscience **39**: 455–462.

Nagahama, T., Sato, H., Shimazu, M. and Sugiyama, J. 1995. Phylogenetic divergence of the entomophtoralean fungi:

Evidene from nuclear 18S ribosomal RNA gene sequences. Mycologia **87**: 203–209.

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

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

Simon, L. S., Bousquet, J., Levesque, R. C. and Lalonde, M. 1993. Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature **363**: 67–69.

Sugiyama, J. 1996. World of fungi. Kagaku **66**: 318–322. (In Japanese.)

Sugiyama, J., Nagahama, T. and Nishida, H. 1996. Fungal diversity and phylogeny with emphasis on 18S ribosomal sequence divergence. In: Microbial diversity in time and space, (ed. by Colwell, R., Simidu, U. and Ohwada, K.), pp. 41–51. Plenum, New York.

Walker, C. 1992. Systematics and taxonomy of the arbuscular endomycorrhizal fungi (Glomales) – a possible way forward. Agronomie **12**: 887–897.

Walker, C., Reed, L. E. and Sanders, F. E. 1984. *Acaulospora nicolsonii*, a new endogonaceous species from Great Britain. Trans. Br. Mycol. Soc. **83**: 360–364.

Walker, C. and Trappe, J. M. 1993. Names and epithets in the Glomales and Endogonales. Mycol. Res. **97**: 339–344.

White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications, (ed. by Innes, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J.), pp. 315–322. Academic Press, New York.