

Ancestral Lineages of Arbuscular Mycorrhizal Fungi (Glomales)

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Using new and existing 18S rRNA sequence data, we show that at least five species of glomalean fungi lie outside the previously defined families and diverged very early in the evolution of that group. These five fungi would have been missed by many previous ecological studies because their sequences are not well matched to available taxon-specific primers and they do not stain well with the standard reagents used for morphological analysis. Based upon spore morphology, these species are currently assigned to *Glomus* and *Acaulospora*, and two of the species are dimorphic, exhibiting spore stages of both genera. This suggests that dimorphic spores are the ancestral state for the order and that one or the other morphology was lost in various lineages. Our analyses also show that *Geosiphon pyriforme*, a symbiont with cyanobacteria, is not necessarily a sister group of the Glomales; instead, it may be derived from mycorrhizal ancestors. © 2000 Academic Press

INTRODUCTION

Arbuscular mycorrhizae (AM)¹ are an important factor in the ecology of vascular plants. The involvement of arbuscular mycorrhizal fungi in nutrient uptake of plants is well established, especially for phosphate nutrition. The great majority of plants require this root symbiosis (Smith and Read, 1997). Recent work has shown that diversity of AM fungi directly affects diversity and competitive interactions among plants (van der Heijden *et al.*, 1998); thus, identification of AM fungi has become important in ecological studies (Helgason *et al.*, 1998; Streitwolf-Engel *et al.*, 1997).

All of the fungi of this ubiquitous root symbiosis are in the monophyletic order Glomales (Gehrig *et al.*, 1996). Molecular studies (Simon *et al.*, 1993) and fossil evidence (Remy *et al.*, 1994; Taylor *et al.*, 1995) suggest

that the Glomales evolved concurrently with land plants and may have been crucial for the colonization of the land by plants (Pirozynski and Malloch, 1975). The entire order is thought to consist of obligate symbionts; no glomalean fungi have ever been successfully cultivated separately from their plant host.

Taxonomy and identification of glomalean fungi has traditionally relied on the morphology of their large (40- to 800- μ m) multinucleate spores. Approximately 150 species of the mycosymbionts have been described (Walker and Trappe, 1993). Six genera in three families have been defined by their mode of spore formation (Morton and Benny, 1990), and this basic three-family structure has been supported by molecular systematic studies (Simon *et al.*, 1993). The order was traditionally placed in the Zygomycota, a phylum that now appears to be a heterogeneous assemblage of highly divergent fungal lineages (Nagahama *et al.*, 1995; Gehrig *et al.*, 1996; O'Donnell *et al.*, 1998), but recent molecular studies and similarities in spore morphology and symbiotic interface indicate that the closest nonmycorrhizal relative of AM fungi is *Geosiphon pyriforme* (Gehrig *et al.*, 1996; Schüssler *et al.*, 1994). This fungus forms a peculiar endosymbiosis with cyanobacteria, which was hypothesized to be the living relic of primitive symbioses that were formed during the earliest stages of land colonization (Gehrig *et al.*, 1996).

Morphology-based identification of the Glomales is of limited use in ecological settings, because spore production is highly dependent on physiological parameters and may not be correlated with root colonization (Merryweather and Fitter, 1998). Morphological analysis of stained roots is typically used to quantify levels of infection and provides some basis for identification at the family level under certain conditions (Merryweather and Fitter, 1998). This latter approach has limited taxonomic resolution, is technically demanding, and has seldom been employed. For these reasons, much effort has been focused on molecular identification based on ribosomal DNA sequences (Simon *et al.*, 1992; Sanders *et al.*, 1995; Redecker *et al.*, 1997; Helgason *et al.*, 1998; van Tuinen *et al.*, 1998).

Specific PCR primers have been developed to amplify

glomalean rDNA directly from colonized roots while eliminating amplification of plant or nonglomalean fungal DNA (Simon *et al.*, 1992; Helgason *et al.*, 1998; van Tuinen *et al.*, 1998). This promises to reveal much about the spatio-temporal structure of glomalean communities in natural ecosystems.

The function of taxon-specific primers and the accuracy of molecular systematics both rely heavily on the density and distribution of the taxon sample. Yet, the number of taxa within the Glomales is large relative to the number that have been sequenced. For that reason we have expanded the sample to include some of the fungal members which have atypical morphological features or behavior, such as the dimorphic fungus with the synanamorphs *Acaulospora gerdemannii* and *Glomus leptotichum* (Morton *et al.*, 1997). It was recently shown by 18S rDNA sequencing that this fungus is distantly related to other members of the Glomales (Sawaki *et al.*, 1998). However, these authors did not address its phylogenetic placement relative to *Geosiphon pyriforme* and did not recognize the dimorphic fungus as part of several deeply divergent lineages in the Glomales, as shown in the present study. Our results demonstrate that the 18S sequence diversity of the Glomales is much greater than previously realized and that the previous taxon sample was inadequate for both primer design and phylogenetic reconstruction.

MATERIALS AND METHODS

Preparation of Crude DNA Extracts

AM fungi were cultivated in pot cultures of *Sorghum sudanense*, *S. vulgare*, or *Zea mays* at the International Collection of Vesicular–Arbuscular and Arbuscular Mycorrhizal Fungi and extracted as previously described (Morton *et al.*, 1993). Voucher specimens were mounted in polyvinylalcohol–lactoglycerol on diagnostic slides (Koske and Tessier, 1983). For PCR, spores were hand-picked with a micropipette. Spore extracts were produced as previously described (Redecker *et al.*, 1997).

Polymerase Chain Reaction

Fragments of the nuclear ribosomal small subunit were amplified by PCR from spores with the set of universal primers NS1 to NS8 (White *et al.*, 1990), with primer NS20 (Gargas and Taylor, 1992), and primers especially designed for species in this study: LOCT670R (AAGGCCATGACGCTTCGC) and ATRP420 (AA-CAATACAGGGCCTTTAC). ITS were amplified with ITS1F (Gardes and Bruns, 1993) and ITS 4 (White *et al.*, 1990). Cycling parameters for NS1 to NS6 and ITS1F were 30 s at 95°C, 30 s at 52°C, and 2 min at 72°C for 5 cycles and then 30 s at 95°C, 30 s at 51°C, and 2 min at 72°C for 30 cycles. The annealing step was performed at 54/53°C for primers NS7/NS8. The reaction mix consisted of 50 µM each nucleotide, 0.2 µM each of primers, 0.1 U/µl *Taq* polymerase (Perkin–

Elmer, Foster City, CA), and the reaction buffer supplied by the manufacturer. The concentration of MgCl₂ was adjusted to 1.5 mM. Specificity of primers was tested as follows. Amplification with VANS1 (Simon *et al.*, 1992) was assessed with NS2 as second primer from 1:100-diluted PCR product from a previous successful amplification with NS1/NS2.

Restriction Analyses

Restriction digests were performed for 6 h at 37°C with *DpnII* and *HinfI* (New England Biolabs, Beverly, MA) and at 65°C with *TaqI* (Boehringer Mannheim, Mannheim, Germany). Fragment patterns were analyzed on agarose gels containing 2% NuSieve (FMC, Rockland, ME) and 1% Ultrapure Agarose (Gibco BRL, Grand Island, NY) in Tris–Acetate Buffer at 150 mA. Gels were stained with ethidium bromide and documented under uv illumination with an Eagle Eye video system (Stratagene, La Jolla, CA).

DNA Sequencing

PCR products were cloned into pCR 2.1 with the Invitrogen TA Cloning Kit (Invitrogen, San Diego, CA) and then sequenced or sequenced directly. A PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Perkin–Elmer) was used. Electrophoresis and data collection were done using an ABI Model 377 DNA Sequencer (Perkin–Elmer) to which a Macintosh Quadra 650 computer was connected. DNA Sequencing Analysis (version 2.01) and Sequence Navigator (version 1.01) were used for processing the raw data. DNA sequences of ITS were always determined from cloned PCR products. DNA sequences were submitted to the EMBL database under the accession nos. shown in Table 1.

Phylogenetic Analyses

An alignment was obtained from the SSU sequences and previously published data based upon secondary structure features (Van de Peer *et al.*, 1998). The alignment was optimized manually. A total of 220 of 1831 positions that were not certain to be in alignment were excluded from the analysis. Parsimony, neighbor-joining, and likelihood analyses were conducted with PAUP* prerelease versions 4.0.d63, 4.0.d64, and 4.0b2 (Swofford, 1999). *Ustilago hordei* (Basidiomycetes, U00973), *Neurospora crassa* (Ascomycetes, X04971), and *Mortierella polycephala* (Zygomycetes, X89436) were used as outgroups. Alternative tree topologies, which constrained either *Glomus* or *Acaulospora* to be monophyletic, were compared to the most-parsimonious tree by the Kishino–Hasegawa test (Kishino and Hasegawa, 1989) as implemented in PAUP*.

RESULTS

Internal transcribed spacer (ITS) sequences were amplified separately from the two different spore types

TABLE 1
Fungal Isolates and rDNA Sequences Used in This Study

Species	Isolate	Synanamorph	Specimen	Type	Clone no.	Sequence length	Accession No.
<i>A. gerdemannii</i> / <i>Gl. leptotichum</i>	NC176	Glomoid		18S		1665	AJ006466
		Glomoid	L7	18S	28.2	741	AJ006794
		Glomoid	L7	18S	21.1	269	AJ006795
		Acaulosporoid	L9	18S	29.3	496	AJ006796
		Acaulosporoid	L9	18S	30.4	547	AJ006797
		Glomoid	L2	ITS	8.4	512	AJ012109
<i>A. gerdemannii</i> / <i>Gl. leptotichum</i>	FL130	Acaulosporoid		18S		448	AJ012110
		Acaulosporoid		18S	48.4	830	AJ006793
		Acaulosporoid		18S/ITS	48.4	914	AJ012201
<i>Gl. gerdemannii</i>	AU215	Acaulosporoid		18S		453	AJ012202
		Acaulosporoid		ITS	47.4	518	AJ012111
<i>A. trappei</i>	NB112			18S		1569	AJ006800
				18S/ITS	45.4	956	AJ243420
<i>A. trappei</i>	AU219			18S		1010	AJ006801
				ITS	12.4	513	AJ243419
<i>Gl. occultum</i>	HA771			18S		1629	AJ006799
				ITS	43.4	440	AJ012113
<i>Gl. occultum</i>	CL700			18S		1073	AJ006798
<i>Gl. brasilianum</i>	WV219			18S		441	AJ012203
				ITS	46.4	496	AJ012112

of the putatively dimorphic fungus *Acaulospora gerdemannii*/*Glomus leptotichum* and the PCR products were cloned. Restriction analysis of the cloned fragments was used to screen for polymorphisms. Clones exhibiting identical restriction patterns with the three enzymes *DpnII* (Fig. 1), *HinfI*, and *TaqI* were found across both spore types. The few polymorphisms that were found in more than one clone were not unique to either spore type. We sequenced the two most distinct clones 8.4 and 9.5 (Fig. 1). A distance matrix of these sequences and ITS from other isolates used in this study are shown in Table 2, in comparison to the ITS variability found within a single isolate of *Gigaspora margarita* (Lanfranco *et al.*, 1999). This comparison shows that, even if these sequences were unique to the *A. gerdemannii* and *Gl. leptotichum* spore types, which

by RFLP analyses does not appear to be true, the variation between the two sequences is within the same range (0.01 to 0.04) as those seen within a single isolate of *Gigaspora margarita* (Table 2). Thus, there is no evidence that the spore types originate from different families or even species. The closest known relative, *Glomus gerdemannii*, is beyond that range (0.07 to 0.08) but within the range seen among other closely related species (e.g., *Gigaspora albida* and *Gigaspora margarita*, Table 2).

These findings were supported by comparison of partial 18S small subunit (ssu) sequences. Sequences from cloned PCR products from each of the spore types (Table 1; clones 28.2, 21.1, 29.3, and 30.4) and a sequence obtained by direct sequencing from the *Glomus* spore type (Table 1; Accession No. AJ006466) grouped closely together in phylogenetic trees (not shown) and showed approximately 0.3% variation.

We determined the near-complete and complete DNA sequences of the ribosomal small subunit of *A. gerdemannii*/*Gl. leptotichum* NC176, *A. trappei* NB112, and *Gl. occultum* HA771. Partial SSU sequences were obtained from a second isolate of each of these species (FL300, AU219, and CL700, respectively), from *Gl. gerdemannii*, and from *Gl. brasilianum* (Table 1). ITS and 5.8S subunit sequences were determined and used to verify the close phylogenetic relationships of two species pairs (Table 2) that were initially recognized as close using the 18S sequences.

Phylogenetic analyses of our SSU sequences and other selected fungal 18S genes from the databases demonstrate that *A. gerdemannii*/*Gl. leptotichum* represents an early divergence within the Glomales (Figs. 2

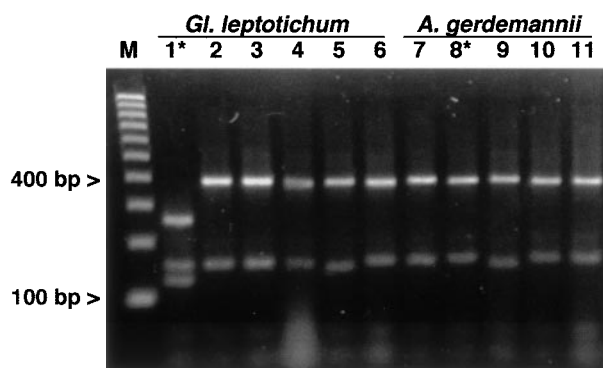


FIG. 1. Restriction analysis of cloned ITS sequences from spores of *Glomus leptotichum* and *Acaulospora gerdemannii*, respectively. The enzyme *DpnII* was used. *Indicates sequenced clones, which most likely represent a wide range of sequence polymorphism.

TABLE 2

Pairwise Distance Matrices of ITS and 5.8S Sequences, Respectively, Calculated by the Kimura Two-Parameter Method in PAUP* (Swofford, 1999) and a Gamma Value of 0.5

(a)	ITS clones								
	9.5	8.4	48.4	47.4	47.5	AU219	NB112	WV215	HA771
<i>A. gerdemannii</i> NC176 clone 9.5	x	0.0132	0.0065	0.0131	0.0132	0.0494	0.05690	0.2332	0.2159
<i>Gl. leptotichum</i> NC176 clone 8.4	0.0213	x	0.0065	0.0132	0.0132	0.0494	0.0569	0.2128	0.2333
<i>A. gerdemannii</i> FL130 clone 48.4	0.0265	0.0207	x	0.0065	0.0065	0.0415	0.0489	0.1974	0.2168
<i>Gl. gerdemannii</i> AU215 clone 47.4	0.0706	0.0817	0.1005	x	0.0000	0.0494	0.0569	0.2128	0.2333
<i>Gl. gerdemannii</i> AU215 clone 47.5	0.0734	0.0768	0.0986	0.0235	x	0.0494	0.0569	0.2128	0.2333
<i>A. trappei</i> AU219	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	x	0.00642	0.1822	0.1709
<i>A. trappei</i> NB112	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	0.1391	x	0.1932	0.1817
<i>Gl. brasilianum</i> WV215	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	x	0.0067
<i>Gl. occultum</i> HA771	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.2077	x

(b)	<i>Gi. albida</i>	gim1	gim2	gim3	gim4
<i>Gi. albida</i>	x				
gim1	0.0709	x			
gim2	0.0643	0.0046	x		
gim3	0.0561	0.0432	0.0375	x	
gim4	0.0510	0.0435	0.0379	0.0144	x

Note. Alignments were prepared manually. Shaded areas indicate conspecificity. (a) above diagonal: 5.8S subunit sequences; below diagonal: ITS sequences from species used in this study. n.a., not alignable; *, *A. trappei* isolates show similarity with *A. gerdemannii* and *G. gerdemannii*, but cannot be aligned reliably. (b) ITS sequences obtained from a multispore preparation of *Gigaspora margarita* (gim1 to gim4; Lanfranco *et al.*, 1999) and *Gigaspora albida* BR203 (AF004707), respectively.

and 3). This dimorphic fungus belongs to neither of the families Glomaceae nor Acaulosporaceae *sensu stricto* as defined by morphological characters (Morton and Benny, 1990) or DNA sequences (Simon *et al.*, 1993, see Fig. 2). Instead, it constitutes an independent deeply divergent lineage. Partial SSU sequences show that *Gl. gerdemannii*, another fungus showing similar spore morphologies and dimorphism, is very closely related to *A. gerdemannii*. This relationship is supported by analysis of highly variable parts of the rDNA: the ITS sequences of the two isolates of *A. gerdemannii*/*Gl. leptotichum* are not alignable to any known taxon, with the exception of *Gl. gerdemannii*, and the 5.8S subunits of both are highly divergent relative to other Glomales (Table 2).

Other early diverging taxa that we found are *Acaulospora trappei*, *Glomus occultum*, and *Glomus brasilianum*, three AM fungi that do not show dimorphism. *Gl. gerdemannii*, *A. gerdemannii*, and *A. trappei* group together on a moderately supported branch (Figs. 2 and 3) but with a considerable phylogenetic distance between *A. trappei* and the others. *Gl. occultum* and *Gl. brasilianum* constitute a separate early branching lineage. Analysis of their ITS and 5.8S sequences confirmed that they are closely related (Table 2). In all cases, isolates of the same species that we tested always grouped together closely, with 100% bootstrap support in 18S-derived phylogenetic trees. Alternative tree topologies that constrained either *Glomus* or *Acaulospora* to be monophyletic were rejected by the Kishino–Hasegawa test at $P = 0.05$.

Together with the previously characterized nonmycorrhizal *Geosiphon pyriforme*, these fungi are separated from the group comprising Glomaceae, Acaulosporaceae, and Gigasporaceae with high support by bootstrap analysis (Figs. 2 and 3). The exact branching order of the deeply divergent groups was not supported by bootstrap support. Trees obtained under the constraint that either *A. gerdemannii*/*A. trappei*, *Ge. pyriforme*, or *Gl. occultum* were the earliest divergence were not significantly different from each other by the Kishino–Hasegawa test.

The unexpectedly high divergence of ribosomal sequences in the glomalean clade poses serious problems for molecular detection methods. Neither of the two previously published glomalean specific primers are well matched to the sequences of the deeply branching AM fungi described in this study. None of the deeply divergent glomalean lineages amplified with the primer VANS1 (Simon *et al.*, 1992). The 5' end of our sequences, which could only be determined from one strand, shows highly altered VANS1 priming sites in these lineages. We also found that, at best, only weak amplification was obtained from spores of these fungi with the AM1 primer (Helgason *et al.*, 1998). This result appears to be caused by one to several mismatches at the 3' end.

DISCUSSION

It has been shown previously that single spores of the Glomales contain divergent rDNA sequences (Table 2;

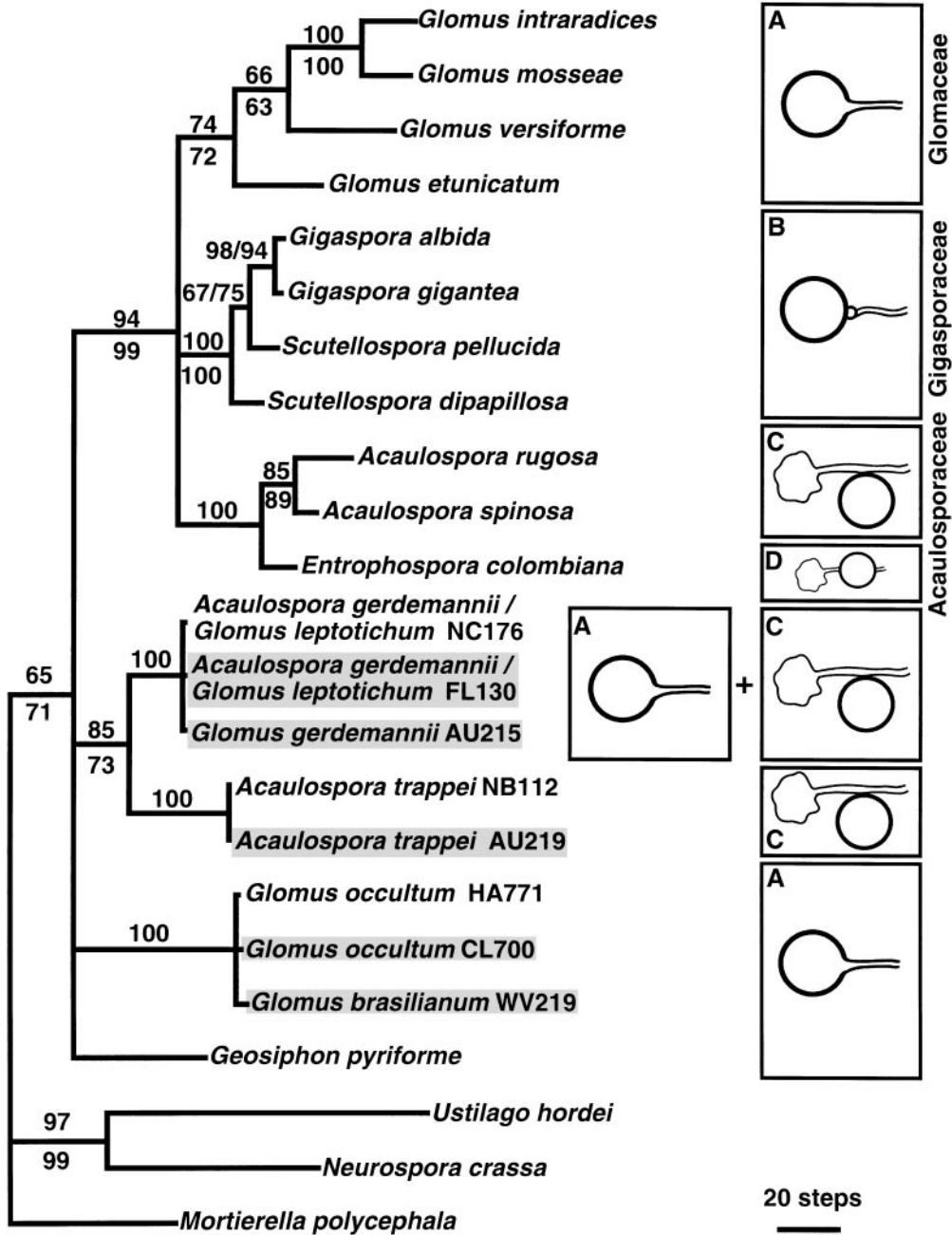


FIG. 2. Phylogenetic tree of arbuscular mycorrhizal fungi obtained from parsimony analysis of 18S SSU sequences. Numbers above the branches indicate bootstrap values of parsimony analysis from 1000 replicates. With this set of taxa, 21 equally parsimonious trees were found; branches supported by less than 60% of bootstrap replicates were collapsed. Numbers below the branches are bootstrap values from the analysis excluding partial sequences (taxa indicated by shading in the tree). In that case, a single most-parsimonious tree was obtained. Length of that tree was 898; consistency index was 0.626. Bootstrap consensus trees from both procedures were identical in topology except for additional taxa. *Ustilago hordei* (Basidiomycetes), *Neurospora crassa* (Ascomycetes), and *Mortierella polycephala* (Zygomycetes) were used as outgroups. Schematical representations in the boxes show the modes of spore formation found in the respective clades. These morphological features have traditionally been used to diagnose glomalean genera and families: (A) spore formation terminally from a swollen hyphal tip; (B) spore formation on bulbous suspensor; (C) sporiferous saccule with laterally borne spore; (D) sporiferous saccule with spore formed within subtending hypha.

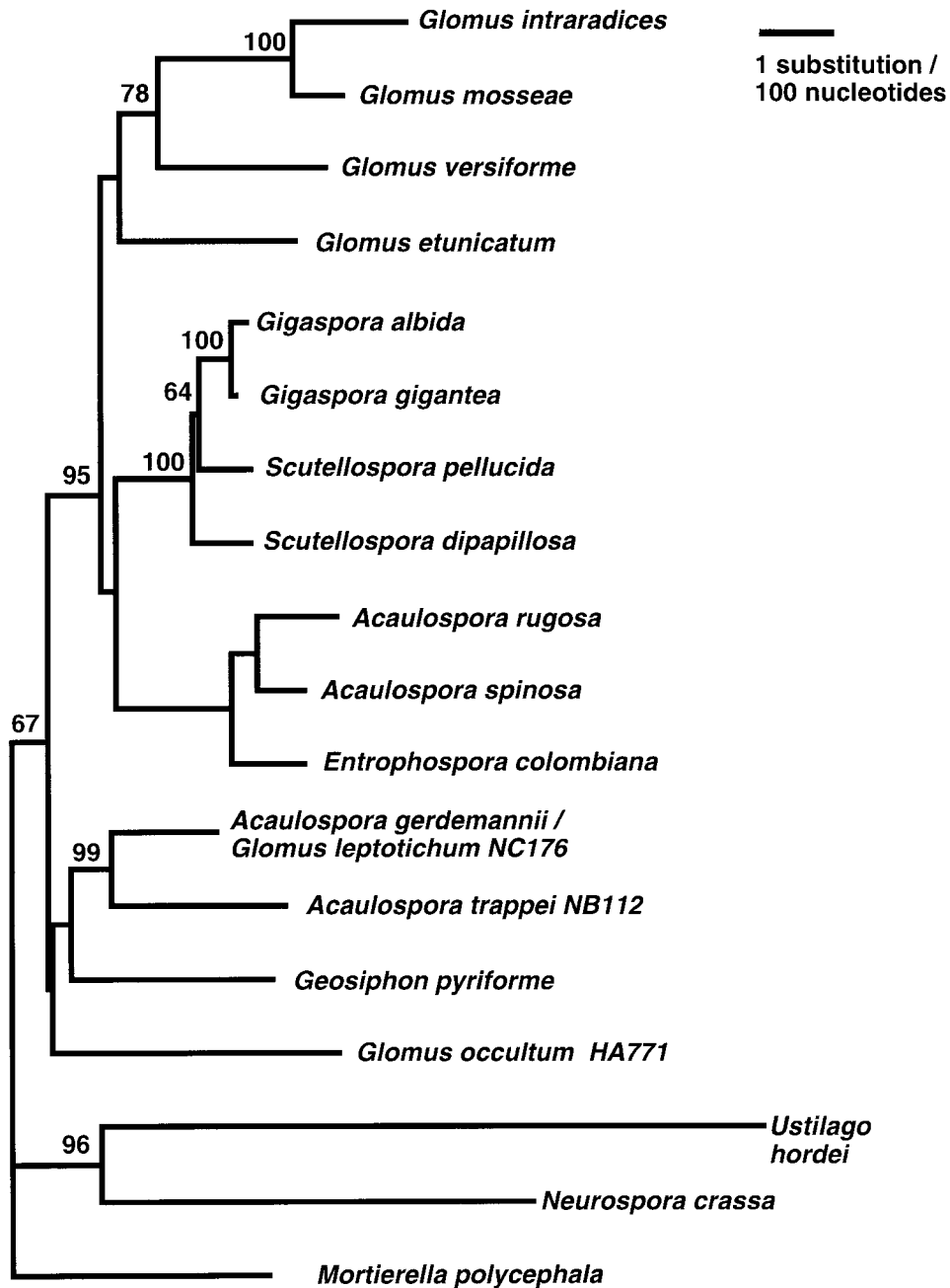


FIG. 3. Phylogenetic tree of the Glomales obtained from distance analysis of 18S SSU sequences. The phylogenetic distances between the branching points of the ancestral lineages *A. gerdemannii*/*A. trappei*/*Gl. gerdemannii*, *Gl. occultum*/*Gl. brasilianum*, and *Ge. pyriforme* are short. Depicted is a neighbor-joining tree obtained by the two-parameter method of Kimura. Bootstrap values above 60% from 1000 replications are shown as numbers on branches. A similar tree was obtained by maximum-likelihood analysis.

Sanders *et al.*, 1995; Lloyd MacGill *et al.*, 1996; Re-decker *et al.*, 1997), which makes it difficult to distinguish closely related AM fungi by sequence comparisons. However, the hypothesis to be tested in this study concerned whether the spore types known as *A. gerdemannii* and *Gl. leptotichum* contain sequences that are divergent to the extent expected for two different genera. The level of divergence that we found is within

the range expected for intraspecific variability (Table 2). Therefore, our findings strongly support previous evidence (Morton *et al.*, 1997; Sawaki *et al.*, 1998) that the two spore types are formed by the same organism.

Our phylogenetic analyses indicate that evolutionary patterns in the Glomales are much more complex than previously thought. The prior notion was that early glomalean fungi were *Glomus*-like and that the forma-

tion of such spores is a primitive trait. This theory appeared to be supported by the fossil record and previous molecular studies (Simon *et al.*, 1993; Taylor *et al.*, 1995). We show that early diverging glomalean lineages comprised fungi-producing spores of both *Glomus* and *Acaulospora* morphotypes and some species are even dimorphic for these spore states. The fossil record is not likely to be helpful, since preserved intracellular fungal structures are needed (Pirozynski and Dalpé, 1989) and the sporiferous saccules of *Acaulospora* may be too fragile for preservation or too rarely formed within roots. The most parsimonious explanation for our data is that the production of both *Acaulospora* and *Glomus* spore types is an ancestral character within the Glomales, and one or the other spore type was lost independently during the evolution of various clades. The dimorphic fungus *A. gerdemannii*/*Gl. leptotichum* would then represent a very interesting "living fossil." An alternative explanation would require that either the *Glomus* or the *Acaulospora* type arose twice by convergence. Our knowledge about the functions of the different modes of spore formation is very restricted; therefore, it is unknown which selection pressures act upon them and whether they represent homologous structures. In any case, the sporiferous saccule of *Acaulospora* does not have the diagnostic value previously assigned to it.

The Glomales, including the newly found lineages and *Ge. pyriforme*, are supported as a monophyletic group by parsimony and neighbor-joining bootstrap analyses (Figs. 2 and 3). However, the bootstrap values for this clade are considerably lower than those in previous studies (Gehrig *et al.*, 1996; Simon *et al.*, 1993), as would be expected when several branches are inserted close to the base of a tree. Hence, the ancestral lineages do not contradict the notion that the AM symbiosis arose only once, but the direction of change with respect to *Ge. pyriforme* is no longer clear. Thus, the *Geosiphon* symbiosis with cyanobacteria either could be the ancestral state prior to mycorrhizal symbiosis, as previously thought (Gehrig *et al.*, 1996), or could be derived from it.

The deep branching of the ancestral lineages implies that the divergence of the glomalean clade occurred earlier than previously thought. Simon *et al.* (1993) estimated that Glomaceae, Gigasporaceae, and Acaulosporaceae diverged 353 to 356 million years ago. Therefore, the deeply divergent lineages, including *Ge. pyriforme*, must have diverged well before that date, which is compatible with the evidence for the appearance of the first land plants, 475 million years ago (Kenrick and Crane, 1997). The earliest known fossil evidence for the AM symbiosis dates back to 400 million years ago, which renders it likely that the first mycorrhizas were formed before that time.

The internodal distances between the newly characterized ancestral lineages and *Ge. pyriforme* are short

(Fig. 3). Therefore, it can be speculated that these branches diverged from one another during a relatively short period of time. This topology would be consistent with a rapid radiation of the Glomales during the colonization of the land.

The phylogenetic placement of *A. gerdemannii* (Morton *et al.*, 1997), *Gl. gerdemannii*, and *A. trappei* relative to that of other *Acaulospora* and *Glomus* species based on 18S genes also has support from morphological features of spores, which contain exceptional features relative to the defined families (Morton and Benny, 1990). Details concerning the spore morphology of these fungi and the necessary changes in taxonomical classification on the genus and family level will be reported elsewhere (Morton and Redecker, in preparation).

The spore morphology of *Gl. occultum* and *Gl. brasilianum* falls within the definition of the genus *Glomus*. In contrast to DNA sequence variation, obvious morphological features are scarce in that genus and it has been suggested before from molecular studies that *Glomus* may be a conglomerate of distantly related organisms (Gehrig *et al.*, 1996; Simon *et al.*, 1993). We show that this discrepancy is even larger than previously thought, which reiterates the necessity and usefulness of molecular phylogenetic studies in the Glomales.

The phylogeny that we found not only explains some morphological anomalies but also helps to interpret anomalies found in fatty acid profiles of glomalean fungi (Bentivenga and Morton, 1996; Graham *et al.*, 1995). *Gl. leptotichum* and *Gl. occultum* are the only fungi of these ancestral lineages tested in those studies, but they contained a unique fatty acid, C16:1 ω 7 *cis*.

All of the fungi in the above-mentioned basal lineages are difficult to detect by root staining procedures. This problem was reported previously for *Gl. occultum* (Morton, 1985) and *A. gerdemannii*/*Gl. leptotichum* (Morton *et al.*, 1997). Intraradical structures stain more weakly with direct blue, trypan blue (Koske and Gemma, 1989), or chlorazol black E (Brundrett *et al.*, 1984) than other *Glomus* or *Acaulospora* species. The only exception among the species tested may be *Gl. brasilianum*, but it stains with variable intensity in plant hosts tested thus far (Morton, unpublished). Therefore, the ecological role of these fungi has been largely ignored in all previous studies based on staining methods. Moreover, the mismatches that these fungi show in the priming sites of VANS1 and AM1 cause problems also with molecular detection.

With our results, the definition of the Glomales as a group can be extended considerably on the molecular level. They also will facilitate detection of these lineages with specific primers or probes and provide additional criteria for defining family and generic groups. Additional work with ancestral glomalean lin-

eages may also provide insight into the transition zone between nonmutualistic and mutualistic fungi.

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