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Molecular phylogeny of the arbuscular mycorrhizal fungi *Glomus sinuosum* and *Sclerocystis coremioides*

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Abstract: We report the first molecular analysis of arbuscular mycorrhizal fungi previously classified in the genus *Sclerocystis*. Fungi in *Sclerocystis* sensu lato were distinguished by formation of complex sporocarps. Most species were transferred to *Glomus*, but both their taxonomic and phylogenetic relations remain the subject of controversy. Phylogenetic analysis of the 18S ribosomal subunit of *G. sinuosum* (= *S. sinuosa)* and *S. coremioides* shows that both species are each other's closest relatives and fall within a monophyletic clade comprising the well-characterized species, *G. mosseae, G. intraradices* and *G. vesiculiferum*, to the exclusion of several other *Glomus* species. This placement indicates that formation of complex sporocarps is an advanced character of some *Glomus* species, but the sporocarpic trait is not sufficiently unique to group these species into a separate genus *Sclerocystis*.

Key Words: Glomales, ribosomal DNA

There has been a long-standing controversy about whether arbuscular mycorrhizal fungi forming sporocarps with spores organized around a central hyphal plexus constitute a separate genus *Sclerocystis* within Glomales. Many other species in the sister genus *Glomus* also produce sporocarps of different degrees of complexity and stability (Morton 1988). However, most were not obligately sporocarpic and did not possess what appeared to be a rather distinctive central hyphal plexus.

The first species discovered, *Sclerocystis coremioides*, was described by Berkeley and Broome (1873). Gerdemann and Trappe (1974) subsequently included four species of *Sclerocystis* in their classification scheme of the Endogonaceae. They commented that only scarce evidence existed for a separation of *Sclerocystis* and *Glomus*. Because of many close similarities, the two genera were grouped into the family Glomaceae (Pirozynski and Dalpé 1989). Morton and Benny (1990) revised the classification scheme to group all arbuscular mycorrhizal fungi, including those in Glomaceae, into a new order Glomales.

Almeida and Schenck (1990) concluded that an unbroken continuum of morphological characters existed between sporocarpic *Glomus* species and all *Sclerocystis* species sensu lato, but excluded *S. coremioides*. As a result, five *Sclerocystis* species were transferred to *Glomus* and only *S. coremioides* remained in the genus *Sclerocystis*.

Wu (1993) hypothesized a model of a smooth evolutionary transition between relatively unorganized, *Glomus*-like sporocarps of *S. rubiformis* and intermediate forms like *S. clavispora, S. liquidambaris* and *S. sinuosa* to *S. coremioides*. He concluded that *S. coremioides* was not unique. This series of transformations led Wu (1993) to reject the changes of Almeida and Schenck (1990) and revert to the previous classification of Gerdemann and Trappe (1974).

Recent molecular studies (Simon et al 1993, Gehrig et al 1996, Redecker et al 2000) suggest that *Glomus* is a polyphyletic conglomerate of distantly related lineages and that some morphological characters previously used to define the genus may not be sufficiently informative. In this context, the phylogeny of sporocarpic species formerly in *Sclerocystis* deserved reexamination using molecular characters to determine whether these fungi constituted a separate lineage or even an ancestral group. In this paper, we sequence the near-complete 18S rDNA from *G. sinuosum* and *S. coremioides*, and analyze the sequences relative to those of other glomalean species using phylogenetic methods.

Methods.—Glomus sinuosum (Gerdemann & Bakshi) Almeida & Schenck, INVAM accession MD126 has been maintained in sudangrass pot cultures of the International Col-

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lection of Vesicular-Arbuscular Mycorrhizal Fungi for over 2 yr. Standard methods were employed (Morton et al 1993), although the culture was maintained continuously with reseeding at 6-mo intervals. Voucher sporocarps were permanently mounted in polyvinylalcohol-lactoglycerol on glass slides (Koske and Tessier 1983), and whole specimens were stored in 0.05% NaN_3 at 4 C. Sporocarps of *S. coremioides* were purchased as "*S. dussii*" from Societé Biorize, Dijon, France. *Sclerocystis dussii* is a synonym of *S. coremioides* (Almeida and Schenck 1990). For PCR, sporocarps were hand-picked with a micropipette. Crude extracts of the sporocarps were produced as described previously (Redecker et al 1997).

Polymerase chain reaction. Overlapping fragments of the nuclear ribosomal small subunit from *G. sinuosum* were amplified by PCR from sporocarp extracts using the universal primers NS1 to NS8 (White et al 1990). For *S. coremioides,* the first third of the 18S rDNA was amplified with primers VANS1 (Simon et al 1992) and NS2. Other parts were amplified with GLOM1070R (CGTAAGGCGCCGAATGAG), GLOM1310 (AATAGCTAGGCYTAACATTG) or GLOM1310R (AAGCTGGCGACCTAACAAT) in various combinations with NS1 to NS8. Details about the primers GLOM1070R, GLOM1310 and GLOM1310R, which are specific for the *Glomus intraradices/mosseae* clade, will be reported elsewhere. Cycling parameters 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 reaction mix consisted of 50 μM of each nucleotide, 0.2 μM each of primers 0.1 U/μL Taq polymerase (Perkin Elmer, Foster City, California) and the reaction buffer supplied by the manufacturer. The concentration of $MgCl_2$ was adjusted to 1.5 mM.

DNA sequencing. Sequences were determined directly from purified PCR products (QIAquick, Quiagen, Hilden, Germany). A PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, California) was used. Electrophoresis and data collection were done on a ABI model 373A DNA Sequencer (Perkin Elmer). DNA Sequencing Analysis (version 2.01) and Sequence Navigator (version 1.01) were used for processing the raw data. Sequences of the 18S subunit were submitted to EMBL as AJ133706 and AJ249715.

Phylogenetic analyses. Two alternative alignments were obtained from the SSU sequences and previously published data. Dataset A comprised the newly reported sequences and 14 other taxa from all three families of the Glomales with *Geosiphon pyriforme* as an outgroup. Because of the broad taxon sampling, 184 positions out of 1723 that could not be aligned with confidence had to be excluded from the analyses, as well as 168 positions that were missing in the *S. coremioides* or *G. vesiculiferum* sequences. *Geosiphon pyriforme* was used as outgroup. Dataset B contained only six taxa from the *Glomus mosseae/intraradices* branch. Only the missing positions mentioned above were excluded (277 out of 1750 characters). The purpose of the second alignment was to allow better differentiation among the *Sclerocystis* species and their closest known relatives by using variable regions of the sequences that had to be excluded in dataset A. *Glomus versiforme* and *G. etunicatum* were used as outgroups. The alignments were adjusted manually.

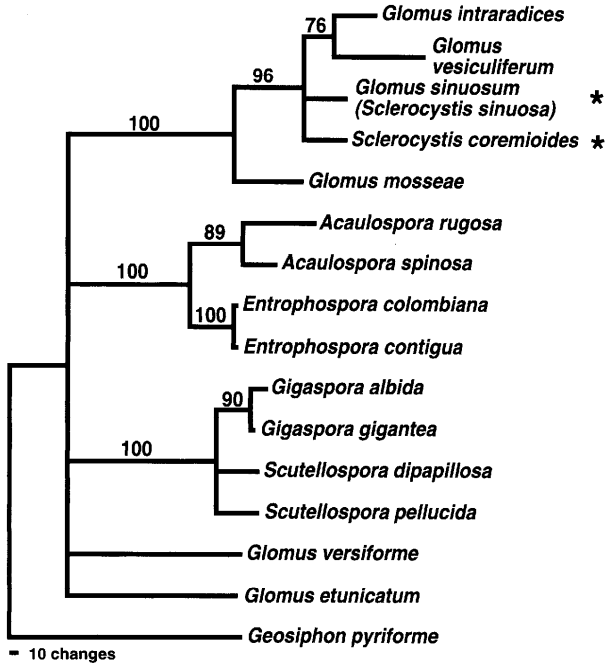

FIG. 1. Parsimony analysis of nuclear 18S rDNA sequences (subset A). Depicted is the bootstrap consensus derived from the single most parsimonious tree (length = 385). Branches with bootstrap values below 60% were collapsed. Numbers on the branches indicate bootstrap values of 1000 replicates. Identical topologies were obtained by distance and maximum likelihood analyses.

The following sequences were obtained from the databases: *Glomus intraradices* (X58725), *Glomus mosseae* (Z14007), *Glomus etunicatum* (Z14008), *Gigaspora albida* (Z14009), *Gigaspora gigantea* (Z14010), *Scutellospora pellucida* (Z14012), *Scutellospora dipapillosa* (Z14013), *Acaulospora spinosa* (Z14004), *Acaulospora rugosa* (Z14005), *Entrophospora colombiana* (Z14006), *Entrophospora contigua* (Z14011), *Glomus versiforme* (X86687), *Geosiphon pyriforme* (X86686). *Glomus vesiculiferum* (L20824). The alignments are available from TreeBASE as S415, M605 and M606. Parsimony, maximum likelihood and distance analyses were carried out with PAUP* prerelease version 4.0.b1 (Swofford 1999).

Results.—The 18S rDNA sequences from *G. sinuosum* and *S. coremioides* were placed with high confidence in a clade of *Glomus* species comprising *G. mosseae, G. vesiculiferum* and *G. intraradices* (FIG. 1). Two nodes with high bootstrap support separated *G. sinuosum/S. coremioides* from peripheral lineages of *Glomus* (e.g., *G. etunicatum/G. versiforme*), and other glomalean genera. Parsimony, maximum likelihood and neighbor-joining analyses of dataset A yielded identical topologies. In all trees *S. coremioides* and *G. sinuosum* were each other's closest relatives.

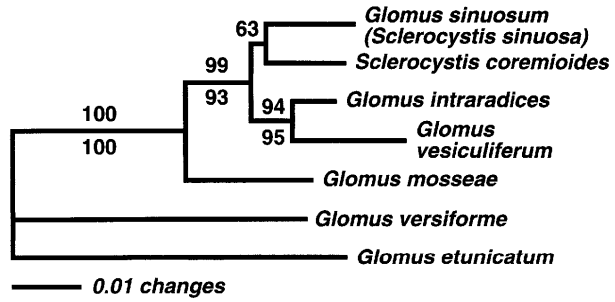

FIG. 2. Neighbor-joining analysis of nuclear 18S rDNA sequences (subset B). Numbers above the branches indicate bootstrap values of 10 000 replicates of neighbor joining, numbers below branches are from 10 000 bootstrap replicates of heuristic searches under the parsimony criterion. Values below 60% are not shown. A monophyletic lineage comprising *S. coremioides* and *G. sinuosum* was only found by neighbor joining analyses. A Branch and Bound search under the maximum likelihood criterion yielded results identical to parsimony analysis.

FIGURE 2 shows the phylogenetic analysis of dataset B. A monophyletic clade of *S. coremioides* and *G. sinuosum* with low bootstrap support of 63% is obtained by neighbor joining but not by parsimony or maximum likelihood analyses.

Discussion.—Almeida and Schenck (1990) considered *S. coremioides* unique and therefore separate from the *Glomus* clade based on four morphological traits: (i) spore formation on separate subtending hyphae rather than from branching sporophores, (ii) a well-defined septum at the same position near the spore base, (iii) arrangement of spores in a hemispherical layer, and (iv) new sporocarps formed from older sporocarps to often fuse into columns. Wu (1993) convincingly showed that these traits were shared to varying degrees by other species in *Sclerocystis* s. l. and that *S. coremioides* was a more recent evolutionary step in a continuous transition of increasing sporocarp complexity. Such an evolutionary progression also had been hypothesized previously by Gerdemann and Trappe (1974) and Morton (1990).

The central issue always has been whether any evolutionary novelty particular to the sporocarpic habit warrants the separation of some taxa into a separate genus, *Sclerocystis,* although they share numerous traits with *Glomus.* Almeida and Schenck (1990) and Wu (1993) share the view that *G. heterosporum, G. dimorphicum* and *G. ambisporum* are transition species. All of the morphological evidence indicates that highly sporocarpic species are an advanced clade of *Glomus.*

The habit of producing less complex organized sporocarps, such as those formed by *G. mosseae,* does not seem to be closely correlated with the phylogeny of glomalean fungi. Sporocarpic species (e.g., *G. mosseae, G. vesiculiferum, G. versiforme*) and species which form spores singly or in aggregates (e.g., *G. intraradices, G. clarum*) are interspersed in phylogenetic trees (see Simon 1996). Other sequence data that indicate a close relationship between sporocarpic and nonsporocarpic pairs of species, such as *G. dimorphicum* and *G. mosseae* (Lloyd MacGilp et al 1996), provide corroborative evidence of this evolutionary trend.

In light of our phylogenetic findings, utilizing both morphological (Morton 1990) and molecular data (this paper), we no longer can justify *Sclerocystis* as a separate genus. By transferring *S. coremioides* into *Glomus,* we bring the efforts of Almeida and Schenck (1990) to their logical conclusion. We also begin the process of revising the genus *Glomus* based on phylogenetic methods that take into account both morphological and molecular traits.

Glomus coremioides (Berk. et Broome) Redecker et Morton, comb. nov.

≡ *S. coremioides* Berk. & Broome, J. Linn. Soc. London 14:137, 1875.

= *Xenomyces* Cesati, *Atti R. Accad. Sci. Fisiche e Math. Napoli* 8:26, 1879.

= *Ackermannia* Patouillard, *Bull. Soc. Mycol. France* 18: 180, 1902.

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