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Molecular identification and phylogeny of arbuscular mycorrhizal fungi

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Abstract

The fossil record and molecular data show that the evolutionary history of arbuscular mycorrhizal fungi (Glomales) goes back at least to the Ordovician (460 million years ago), coinciding with the colonization of the terrestrial environment by the first land plants. At that time, the land flora only consisted of plants on the bryophytic level. Ribosomal DNA sequences indicate that the diversity within the Glomales on the family and genus level is much higher than previously expected from morphology-based taxonomy. Two deeply divergent lineages were found and described in two new genera, *Archaeospora* and *Paraglomus*, each in its own family. Based on a fast-growing number of available DNA sequences, several systems for molecular identification of the Glomales within roots have been designed and tested in the past few years. These detection methods have opened up entirely new perspectives for studying the ecology of arbuscular mycorrhiza.

A problem for morphology-based taxonomy: One fungus in two different families?

The taxonomy of arbuscular mycorrhizal (AM) fungi has traditionally been based on the morphology of the spores. Families and genera were mainly distinguished by the hyphal attachment and mode of formation of the spore, whereas the substructure of the spore walls played an important role in species identification (Gerdemann and Trappe, 1974; Morton, 1988; Walker, 1992). The resulting three-family structure Glomaceae, Acaulosporaceae, Gigasporaceae (Morton and Benny, 1990) was initially supported by rDNA data (Simon et al., 1993a). However, these data also indicated a large genetic diversity within the genus *Glomus* that was not reflected by the few morphological characters of the spores.

The closest non-mycorrhizal relative to arbuscular mycorrhizal fungi was found to be *Geosiphon pyriforme* (Gehrig et al., 1996). This fungus forms a peculiar symbiosis with cyanobacteria, intracellularly harboring *Nostoc* symbionts. It also produces spores that resemble those of *Glomus* species very closely, even in spore wall ultrastructure (Schüßler et al., 1994). The *Geosiphon* symbiosis, therefore, appeared to mirror a possible ancestor to arbuscular mycorrhiza that could have occurred in terrestrial ecosystems before the origin of land plants.

The discovery of an arbuscular mycorrhizal fungus forming spores that formally belong to two different genera and families was obviously not easily compatible with morphology-based taxonomy. Spores of *Acaulospora gerdemannii* and *Glomus leptotichum* were observed to be formed on the same hypha (Morton et al., 1997). As no evidence could be found that one of these spore types may represent a sexual stage, the two spore types were termed 'synanamorphs'. Molecular biological analysis showed no evidence that they originate from two different organisms (Redecker et al., 2000b). However, their 18S rDNA sequences proved that this dimorphic organism neither belongs to *Glomus*, nor to *Acaulospora*, nor to any of the established families (Sawaki et al., 1998). Instead it belongs to one of several ancestral, deeply divergent lineages within the Glomales (Redecker et al., 2000b).

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Deeply divergent lineages of the Glomales

Ac. gerdemannii/G. leptotichum was not the only AM fungal species found to fall outside the previously known phylogenetic groupings. To date, four other species were reported in two major lineages. Some of these species are dimorphic, one forms only spores of the acaulosporoid type, and others produce only *Glomus*-type spores (Redecker et al., 2000b).

The acaulosporoid spore type of a second dimorphic fungus had been previously described as *Glomus gerdemannii* (Rose et al., 1979). Its actual glomoid morph remained undetected until recently. Spores of both its two synanamorphs are very similar in structure to *Acaulospora gerdemannii/Glomus leptotichum.* rDNA sequences confirmed that they are closely related species. Because of their large phylogenetic distance to all previously known glomalean genera, a new genus was established and the two dimorphic fungi were renamed *Archaeospora leptoticha* and *Ar. gerdemannii*, respectively (Morton and Redecker, 2001).

A third species in this lineage forms rather inconspicuous, small, hyaline spores of the acaulosporoid but not the glomoid type. It was previously known as *Acaulospora trappei*. Ribosomal sequence data showed that it is related to *Ar. leptoticha* and *Ar. gerdemannii* (Redecker et al., 2000b). It was established as the type species of the new genus *Archaeospora* as *Ar. trappei* (Morton and Redecker, 2001).

The molecular data allowed to re-evaluate morphological characters of the acaulosporoid spores of *Archaeospora* that had appeared as mere anomalies before, and had not been assigned great significance. Spores of *Ar. leptoticha* and *Ar. gerdemannii* have inner flexible walls, but these are quite different in their fine structure from the germinal walls of *Acaulospora* species. They do not appear to form a 'germination orb' during germination that allows the germination hypha to penetrate the spore wall. Instead, whenever it was observed, germination appears to occur through the hyphal attachment or its remnants, as in some *Glomus* species. The acaulosporoid spores of *Archaeospora* are separated from the subtending hypha by a short 'pedicel' not found in *Acaulospora* (Morton and Redecker, 2001).

The glomoid spores of *Archaeospora* do not have morphological characters that distinguish them from their distant relatives in *Glomus* sensu stricto (e.g. *G. intraradices*). The same is true for *Glomus occultum* and *Glomus brasilianum*, members of another, deeply divergent lineage which has no close affiliation to *Archaeospora* or *Geosiphon*. *G. occultum* and *G. brasilianum* were placed in the new genus *Paraglomus* in the family Paraglomaceae. However, like *Archaeospora*, *Paraglomus* is distinguished from *Glomus* by rDNA sequences, unique fatty acids and the mycorrhizal morphology. The mycorrhizae of these two genera do not stain at all or only weakly with the standard dyes (Trypan Blue, Acid Fuchsin, etc.) that are routinely used to detect and quantify AM colonization. This is one reason why these fungi have not been extensively studied in their symbiotic interactions. In fact, they may be interesting objects for study, because their large phylogenetic distance to other AM fungi might be correlated with physiological differences. Based on 18S rDNA sequences, *Paraglomus* seems to be the most ancestral lineage within the Glomales (Figure 1; Schwarzott et al., 2001), although there is only low bootstrap support for this.

The relationship of *Geosiphon pyriforme* to the basal mycorrhizal lineages and their branching order has been unclear. The exact branching order of *Geosiphon, Paraglomus*, *Archaeospora* and the clade with the previously known three families was not resolved well (Redecker et al., 2000b). An updated sequence of *Geosiphon* in the databases places this fungus closer to *Archaeospora leptoticha/gerdemanni* (Figure 1; Schwarzott et al., 2001). This finding is quite surprising because currently there are no other data suggesting this relationship except for the small subunit sequence. The sequence signature used to define *Archaeospora* (Morton and Redecker, 2001) is rather similar to the updated *Geosiphon* sequence at the respective site.

Based on the available rDNA data, it appears that *Geosiphon* is derived from mycorrhizal lineages and that its symbiosis with cyanobacteria is not an ancestral precursor to AM. In the long term, the definition of the Glomales will have to be modified to accomodate non-mycorrhizal fungi, because it is likely that the transition from mycorrhizal to other nutrition modes occurred more than once, as has already been shown for ectomycorrhizal fungi (Hibbett et al., 2000).

Other new results of molecular phylogenetics

The genus *Sclerocystis* had been the subject of some taxonomic wrangling. It was unclear whether the formation of complex sporocarps merited a separate genus (Almeida and Schenck, 1990; Wu, 1993). Most

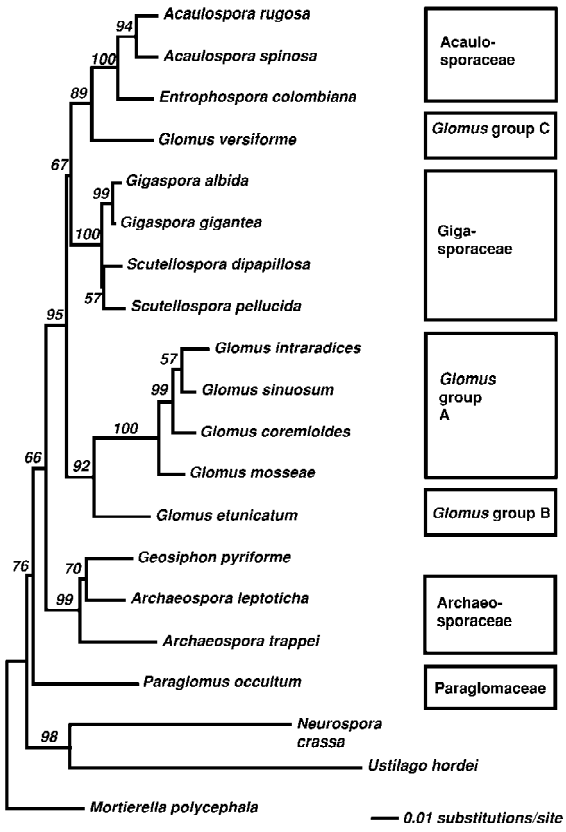

Figure 1. Phylogenetic tree of the Glomales (neighbor-joining analysis of 18S rDNA sequences). Numbers on the nodes denote bootstrap values from 10 000 replicates. Boxes on the right indicate families and the *Glomus* subgroups proposed by Schüßler et al. (2001).

authors agreed that the component spores of the sporocarps showed all characteristics of *Glomus* spores, but as we now know the spore type is formed by several unrelated lineages. There were also several intermediate sporocarp morphologies between *Glomus* and *Sclerocystis*. Using 18S rDNA sequences, Redecker et al. (2000c) resolved the issue and found that the complex sporocarps were formed by members of *Glomus* group A (see Figure 1). From the phylogenetic standpoint, there was no reason anymore to keep *Sclerocystis*. Therefore, the authors placed the last remaining species *S. coremioides* (sensu Almeida & Schenck) into *Glomus*.

Within the Gigasporaceae, *Gigaspora* seems to be the advanced and not the primitive genus. Its few species form a very narrow clade compared to the large variation within *Scutellospora* (Schwarzott et al., 2001). There is even the possibility that *Scutellospora* may be paraphyletic. If this is true, the complex system of germinal walls and the germination shield of *Scutellospora* were lost during evolution of *Gigaspora*.

In previous analyses, *Glomus versiforme* grouped within a weakly supported monophyletic group of *Glomus* species, comprising *G. intraradices, G. mosseae* and *G. etunicatum* (Gehrig et al., 1996; Redecker et al., 2000b; Schüßler, 1999). The recently updated sequence in the databases places it in its own clade with some affinity to the Acaulosporaceae (Figure 1; Schwarzott et al., 2001). Several other *Glomus* species were reported to be in this lineage (Schwarzott et al., 2001).

The fossil history of the Glomales

It has been hypothesized previously that AM fungi played a substantial role in the colonization of terrestrial habitats by early land plants (Pirozynski and Malloch, 1975). The earliest evidence for the AM symbiosis were fossil arbuscules found in specimens of the early land plant *Aglaophyton* from the Devonian Rhynie Chert (400 Myr ago, Remy et al., 1994). Fossil spores and hyphae resembling those of glomalean fungi had been detected in plant material from this site by the 1920s (Kidston and Lang, 1921).

Aglaophyton is thought to be in an intermediate position between bryophytes and vascular plants and was, therefore, already higher evolved than the putative first terrestrial plants (Taylor and Taylor, 1993). Most likely the earliest land plants colonized terrestrial ecosystems in the Ordovician and were similar to today's hornworts and liverworts. Only microfossil evidence has been found for these plants: spores and cellular scraps ('cuticle fragments', Graham, 1993; Gray et al., 1982). Similar microfossils were actually found in connection with hepatic thalli from later epochs and, therefore, confirmed this notion (Edwards et al., 1995). In agreement with fossil findings, molecular and morphological cladistics both support liverworts and hornworts as the oldest branches of the land plant lineage that evolved from green algae (Kenrick and Crane, 1997).

Spores and hyphae strongly resembling today's AM fungi were recently found in 460-million-years-old dolomite rocks from the Ordovician of Wisconsin (Redecker et al., 2000a). This is the earliest evidence for the existence of fungi. The oldest generally accepted fungal fossils found so far had been Ascomycete–like hyphae in arthropod frass from the

Silurian (Sherwood–Pike and Gray, 1985) and the numerous groups of fungi found in the Rhynie Chert (400 Myr ago, Kidston and Lang, 1921; Remy et al., 1994; Taylor et al., 1994, 1999). The Ordovician spores are remarkably similar to today's *Glomus* type spores and have a size of 40–95 μm. Their hyphae are non-septate and 3–5 μm wide.

These findings prove that glomalean fungi were present at a time when the land flora only consisted of plants of the bryophyte grade. Together with the majority of land plants, today's hornworts and liverworts do form associations with AM fungi, although they do not meet all the criteria of AM, because they do not have true roots (Read et al., 2000; Schüßler, 2000). Interestingly, extant mosses do not form AM (Read et al., 2000).

Although a direct connection between those early glomalean fungi and the land plants on the bryophyte level was not demonstrated, their co-occurrence and the proven ability of extant hornworts and liverworts to form an AM symbiosis suggest that they may have been associated. These findings give strong support to the notion that AM fungi were instrumental in the success of early land plants.

New perspectives for ecology: Molecular identification from roots

It has long been recognized as a major problem of ecological studies on AM that the fungi cannot be easily identified within roots. Traditionally, spores were used to determine which species was present and active. However, it is well known that spore formation is highly dependent on physiological parameters and often not correlated with root colonization. Species determination from spores also has many pitfalls for non-experts, when spores are immature, parasitized or lack distinctive morphological characters. Moreover, spore morphology does not reflect mycorrhizal diversity when we consider that various unrelated lineages within the Glomales form very similar spores of the *Glomus* type, and that acaulosporoid spores occur in both Acaulosporaceae and Archaeosporaceae

Therefore, several approaches were taken to develop molecular tools that would allow species identification of AM fungi independent from spore formation. A few authors designed PCR primers from random sequences, most used a systematic approach, comparing sequences of known function. A randomly amplified fragment from *Gl. mosseae* was used to generate specific primers for this species (Lanfranco et al., 1995) and to establish a quantitative PCR system to measure root colonization (Edwards et al., 1997). Most authors, however, used the systematic approach with ribosomal sequences. Provided a sufficient database of sequences and knowledge of phylogeny, this approach allows to tailor the specificity of probes or PCR primers to species, genera or any other level of taxonomy.

Simon et al. (1992) conducted the first pioneering studies of molecular phylogeny and detection of AM fungi. The authors designed the primer VANS1, based on only three 18S subunit sequences from the Glomales, and a set of other primers specific for subgroups of the Glomales. They also proposed an identification system using Single Strand Conformation Polymorphism (SSCP) (Simon et al., 1993b). As more and more sequences became available from a broader taxon sample from the Glomales, it became clear that the VANS1 primer did not amplify from all glomalean fungi (Clapp et al., 1999; Schüßler et al., 2001). Nevertheless, it was used in some studies (Chelius and Triplett, 1999; Clapp et al., 1995; Di Bonito et al., 1995)

Using the group-specific PCR primers for a portion of the 18S rDNA designed by Simon et al. (1993b) and a subtraction hybridization technique to enrich fungal sequences, Clapp et al. (1995) performed the first molecular study of a field population of AM fungi. Their PCR results for *Acaulospora* and *Scutellospora* species were mainly in agreement with spore counts but interestingly there was a strong discrepancy between strong root colonization by *Glomus* and absence of sporulation. An influence of canopy type on this cryptic colonization was also detected. Moreover, concurrent colonization of the same 5 cm root length by all three genera was demonstrated. These results underlined the need to apply molecular methods in order to obtain more reliable data of AM fungal populations in roots.

Other authors targeted different parts of the ribosomal genes, e.g. the large subunit (Kjøller and Rosendahl, 2000; van Tuinen et al., 1998) or the Internal Transcribed Spacers (Redecker, 2000). van Tuinen et al. (1998) employed a nested PCR procedure to circumvent the problem of PCR inhibitors present in many root samples. These authors designed primers specific for four inoculant species to study AM fungal populations in a greenhouse experiment. Root fragments of 1 cm length were analyzed in this study, and again, the pieces were usually colonized by more than

one fungus. In this study, a complex pattern of fungal species interactions was detected. The colonization by the two species from the Gigasporacae was significantly enhanced in combination with certain others, suggesting synergistic interactions among species. A similar approach was used to study AM fungal populations in heavy-metal-polluted soils (Turnau et al., 2001).

A set of five primers targeted at major phylogenetic groups within the Glomales was designed to amplify parts of the 18S ribosomal subunit, the ITS and the 5.8S subunit (Redecker, 2000). These groups also include *Archaeospora* and *Paraglomus*. In addition to the difficulties to detect them by staining, these AM fungi had not been detectable with previously designed molecular tools.

Kjøller and Rosendahl (2000) developed a specific primer for a part of the ribosomal large subunit from members of *Glomus* group A (see Figure 1). This primer was used in a nested PCR reaction. Single Strand Conformation Polymorphism (SSCP) was used by these authors to screen PCR products for sequence differences, which is less labor-intensive than sequencing. Isolates of *G. mosseae* and *G. caledonium* could be differentiated from each other and *G. geosporum* by this approach, and detected side by side in colonized roots. In samples collected from a *Pisum sativum* field study, this method again showed the dicrepancy between spore and root populations of AM fungi. Some sequence types from roots were not found in trap cultures from the respective field site (Kjøller and Rosendahl, 2001). On the other hand, *G. mosseae* was not detected within roots although it was established in trap cultures. One possible explanation for the latter may be a strong seasonal influence on AM colonization of this species, that may remain undetected by spore counting.

Interestingly, Helgason et al. (1998) showed that a putative *G. mosseae* was the most abundant type of AM fungus found in roots in some arable fields in the UK. In fact, these authors reported that the AM species diversity in cultivated soils was strikingly low when compared to a seminatural woodland. On the other hand, *G. mosseae* was not found in the woodland, suggesting that it is better adapted to the conditions in arable land. The authors attributed the differences in AM species composition to recurring soil disturbance by ploughing in the cultivated field sites. The AM population in roots of bluebell (*Hyacynthoides non-scripta*) in the woodland site was characterized by complex seasonal patterns and a significant influence of the canopy type (Helgason et al., 1999). Helgason et al. (1998, 1999) used a variable portion of the 18S small subunit that was amplified in a single step from roots with the PCR primer AM1. This primer discriminates most AM fungal from plant DNAs but also amplifies a wide range of other fungi. The initial screening of the PCR products was performed by RFLPs, before they were sequenced. The proportion of the clones sequenced from the PCR products was assumed to be proportional to root colonization and used to quantify the respective species.

All these studies demonstrate the power of molecular identification methods to elucidate the species composition of active AM fungal communities. So far it has been difficult to obtain alternative sequence markers to the ribosomal subunits. Due to the numerous micro-organisms living on AM fungal spores, the glomalean identity of novel markers is difficult to establish. Even for ribosomal sequences, the steadily growing number of mislabeled sequences in the databases can cause confusion.

Another problem that has been reported with all sequence markers from the rDNA of the Glomales so far, is the variation all these sequences show within spores and morphospecies (Sanders et al., 1995). There is no easy correlation between sequence identity and species identity, as each spore harbours different sequence types. There is no phylogenetic species concept for the Glomales and several aspects of their genetics continue to be a mystery. Until we have obtained more knowledge in these fields, taxa have to be bundled in groups as shown in the studies above, under the assumption that all taxa in the respective group share at least some ecological or physiological characteristics.

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