Mycologia, 98(6), 2006, pp. 885–895.

Phylogeny of the Glomeromycota (arbuscular mycorrhizal fungi): recent developments and new gene markers

Dirk Redecker[1]
Philipp Raab
Institute of Botany, University of Basel, Hebelstaße 1, CH-4056 Basel, Switzerland

Abstract: The fungal symbionts of arbuscular mycorrhiza form a monophyletic group in the true Fungi, the phylum Glomeromycota. Fewer than 200 described species currently are included in this group. The only member of this clade known to form a different type of symbiosis is *Geosiphon pyriformis*, which associates with cyanobacteria. Because none of these fungi has been cultivated without their plant hosts or cyanobacterial partners, progress in obtaining multigene phylogenies has been slow and the nuclear-encoded ribosomal RNA genes have remained the only widely accessible molecular markers. rDNA phylogenies have revealed considerable polyphyly of some glomeromycotan genera that has been used to reassess taxonomic concepts. Environmental studies using phylogenetic methods for molecular identification have recovered an amazing diversity of unknown phylotypes, suggesting considerable cryptic species diversity. Protein gene sequences that have become available recently have challenged the rDNA-supported sister group relationship of the Glomeromycota with Asco/Basidiomycota. However the number of taxa analyzed with these new markers is still too small to provide a comprehensive picture of intraphylum relationships. We use nuclear-encoded rDNA and rpb1 protein gene sequences to reassess the phylogeny of the Glomeromycota and discuss possible implications.

Key words: arbuscular mycorrhiza, evolutionary history, ribosomal RNA genes, RNA polymerase genes

INTRODUCTION

Arbuscular mycorrhiza is a widespread mutualistic symbiosis between land plants and fungi of the phylum Glomeromycota. The association is essential for plant ecosystem function because the great majority of plant species depend on it for mineral nutrient uptake. This task is efficiently performed by the extensive extraradical mycelium of the fungal symbionts. Within root cells arbuscular mycorrhizal (AM) fungi form typical tree-like structures, the arbuscules (FIG. 1B), or hyphal coils. Some also produce storage organs, termed vesicles (FIG. 1C). Although the different fungal genera were reported to show some differences in the morphology of their intraradical structures (Merryweather and Fitter 1998) it is generally not feasible to identify species with these characters.

Glomeromycotan fungi produce relatively large (40–800 μm) spores with layered walls, containing several hundreds to thousands of nuclei (Becard and Pfeffer 1993). Spores may be formed singly (FIG. 1A, D), in clusters or aggregated in so-called sporocarps (FIG. 1E, Gerdemann and Trappe 1974). Similar to most Zygomycota, the hyphae of glomeromycotan fungi lack regular septation.

The phylum Glomeromycota comprises about 200 described morphospecies that traditionally have been distinguished by features of the spore wall. The way the spore is formed on the hypha (''mode of spore formation'') has been important to circumscribe genera and families, and the layered structure of the spore walls is used to distinguish species (Morton 1988). Walker (1983) established the concept of ''murographs'' to describe and compare the layered structure of the spore walls more easily. Morton (1995) and Stürmer and Morton (1997, 1999) included considerations of the spore development to group these wall components hierarchically into complexes linked by ontogeny.

There is no evidence that the Glomeromycota reproduce sexually. A study reporting the formation of sexual zygospores by *Gigaspora* (Tommerup and Sivasithamparam 1990) was not confirmed. Studies using molecular marker genes have detected no genetic recombination or only low levels (Kuhn et al 2001). Therefore it is assumed generally that the spores are formed asexually. There are conflicting reports on the question of whether the nuclei in the mycelium and spores of one organism are genetically identical (Hijri and Sanders 2005, Kuhn et al 2001, Pawlowska and Taylor 2004).

Glomeromycotan fungi are obligate symbionts. Progress in obtaining new marker genes has been slow because their spores usually contain numerous other microorganisms, including fungi from other phyla (Hijri et al 2002). Axenic fungal biomass can be obtained only from cultures on transformed plant

Accepted for publication 27 September 2006.
[1] Corresponding author. E-mail: dirk.redecker@unibas.ch

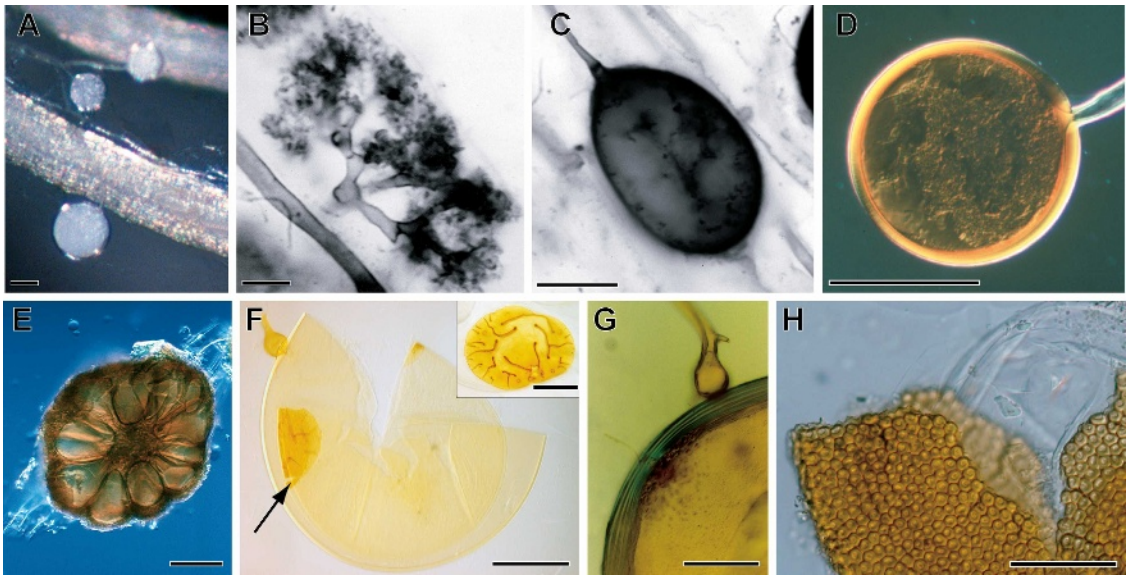


FIG. 1. Some characteristic morphological features of glomeromycotan fungi. A. Colonized roots of *Plantago media* with hyphae and spores of *Glomus clarum*. B. Arbuscule of *Glomus mosseae* stained with chlorazol black. C. Vesicle of *Glomus mosseae*. D. Spore of *Glomus* sp. S328 showing the hyphal attachment. E. Section of a sporocarp of *Glomus sinuosum* with spores grouped around a hyphal plexus and covered by a layer of hyphae. F. Spore of *Scutellospora cerradensis*, showing bulbous sporogenous cell and inner flexible walls with germination shield (arrow). Inset: germination shield of *S. scutata* in face view. G. Germinating spore of *Gigaspora decipiens* with sporogenous cell, warty germination layer and germination hypha. H. Spore of *Acaulospora denticulata* with tooth-like wall ornamentations and inner germinal walls. Spores in D, E, F, G and H were embedded in polyvinylalcohol lactoglycerol and in F, G and H they were cracked under the cover slip. Images courtesy of Kerstin Wex (B, C), Fritz Oehl (F) and the American Society for the Advancement of Science (D). Bars = 100 µm (A, E, F, G), 50 µm (D, H), 5 µm (B, C).

roots, but only a small number of species are available in culture.

The presence of multiple, slightly differing variants of the nuclear-encoded ribosomal RNA genes (rDNA) within single spores is well established (Lanfranco et al 1999, Sanders et al 1995). A similar phenomenon was reported for some protein genes (Kuhn et al 2001) but not others (Stukenbrock and Rosendahl 2005). The rDNA heterogeneity causes problems when closely related species or isolates of the same species are to be distinguished. It was shown recently that the mitochondrial large ribosomal subunit gene does not show this variation (Raab et al 2005), suggesting that mitochondrial genes might be useful as molecular markers in the future.

Due to the problems outlined above there is currently no molecular species concept for glomeromycotan fungi. Nevertheless molecular markers have proven to be highly useful to characterize the diversity of AM fungi in the field and have revealed an unexpectedly high diversity of phylotypes in some settings. Some of these studies indicate that the number of 200 described morphospecies might be a strong underestimation of the true diversity of the Glomeromycota (Husband et al 2002, Vandenkoornhuyse et al 2002).

Before 1974 most arbuscular mycorrhizal fungi were in the genus *Endogone* until Gerdemann and Trappe (1974) placed them in four different genera in the order Endogonales (*Glomus*, *Sclerocystis*, *Gigaspora*, *Acaulospora*). Morton and Benny (1990) established a new order ''Glomales'' in the Zygomycota, comprising six genera. Since then evidence has accumulated supporting the view that arbuscular mycorrhizal fungi are distinct from other Zygomycota. They do not seem to form characteristic zygospores, and in all cases when the nutritional mode has been elucidated they form mutualistic symbioses. Based on their rDNA phylogeny AM fungi are the sister group of Asco- and Basidiomycota and not monophyletic with any part of the Zygomycota. Therefore the ''Glomales'' was raised to the rank of a phylum Glomeromycota (Schüßler et al 2001). In the same study the grammatically incorrect order name ''Glomales'' was corrected to ''Glomerales'' and several new orders were established. It must be emphasized however that ''Glomales'' *sensu* Morton and Benny (1990) is synonymous with the Glomeromycota *sensu* Schüßler et al (2001) and ''Glomerales'' *sensu* Schüßler et al (2001) comprises a smaller subset of these taxa.

In general molecular phylogenies have shown that

glomeromycotan diversity at the phylum and genus level is much higher than expected through microscopic observation of spore morphology (Redecker et al 2000b, Schwarzott et al 2001). Some of the morphological characters that were used previously to delimit genera and families probably have evolved multiple times independently.

Ten genera of the Glomeromycota currently are distinguished:

(i) *Glomus* is the largest genus in the phylum, with more than 70 morphospecies. Spores, typically with layered wall structure, are formed by budding from a hyphal tip. The sporogenic hypha (or ''subtending hypha'') often remains attached to the mature spore (FIG. 1D). The spores germinate through this hyphal attachment or the remains of it. This glomoid mode of spore formation is symplesiomorphic and occurs in several distinct lineages, namely *Glomus*, *Paraglomus*, *Archaeospora*, *Pacispora*, *Diversispora* and *Geosiphon*. Some of these genera were separated from *Glomus* based on molecular phylogenetic data. Clades of *Glomus* are distinguished as *Glomus* group A, B and C (Schwarzott et al 2001). Groups A and B form a monophyletic clade (FIG. 2A). The largest diversity of all *Glomus* lineages has been found in group A, which dominates AM fungal communities in many field settings (Helgason et al 1998, Öpik et al 2003, Vandenkoornhuyse et al 2002)

Some species forming *Glomus*-type spores in complex sporocarps (FIG. 1E) were placed previously in the genus *Sclerocystis*. Almeida and Schenck (1990) transferred all but one of these species to *Glomus*, based on morphological considerations. Molecular analysis of the last remaining species of this genus (the type species *S. coremioides*) showed that it is nested within a clade of other well characterized *Glomus* species, therefore this species also was transferred to *Glomus* (Redecker et al 2000c).

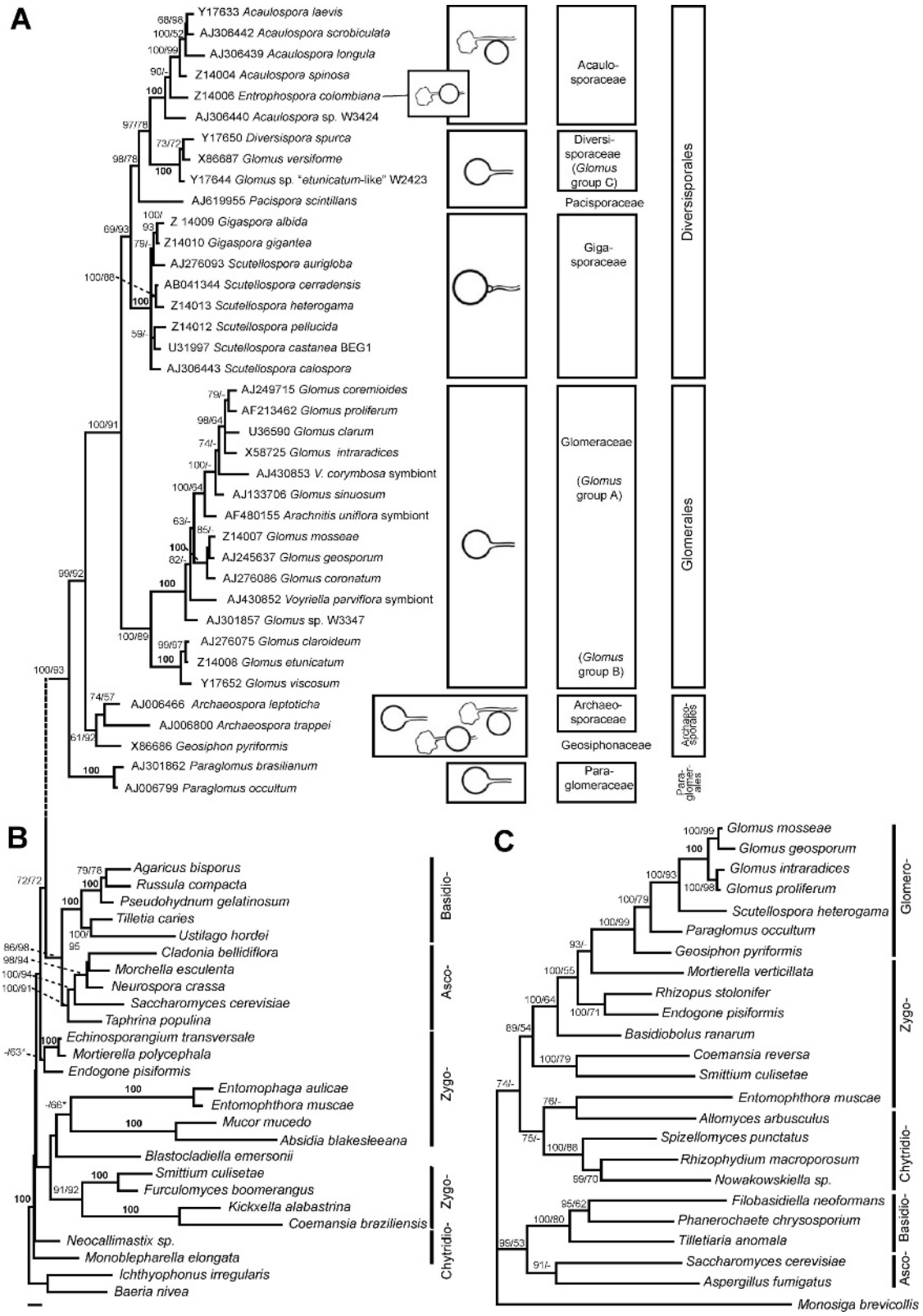
(ii and iii) *Gigaspora* and *Scutellospora* are closely related genera in the family Gigasporaceae. Spores are formed on a bulbous sporogeneous cell and germinate through a newly formed opening in the spore wall. The two genera do not form vesicles within roots, and the extraradical mycelium bears so-called auxiliary cells of unknown function. In contrast to *Gigaspora*, species of *Scutellospora* possess flexible inner spore walls, which are present permanently in mature spores (''germinal walls'' and a ''germination shield'', FIG. 1F). These inner germinal walls and the germination shield are involved in the germination process. The flexible walls are not naturally pigmented but certain sublayers often can be stained with Melzer's reagent. *Gigaspora* germinates through the spore wall after a papillate layer has formed on the inside of the spore wall around the point of penetration (FIG. 1G). Note that this warty layer structure also has been referred to as ''germinal wall'' by some authors (Spain et al 1989), a usage of the term we will not employ here.

(iv and v) The diagnostic feature that was used for the family Acaulosporaceae, comprising the genera *Acaulospora* and *Entrophospora*, is the formation of spores next to a ''sporiferous saccule''. This saccule collapses during spore maturation and eventually disappears. Flexible inner walls (germinal walls) are found in all members of the family. During spore germination a ''germination orb'' is produced on the inner walls, a membraneous structure that is instrumental in penetrating the outer spore wall.

The two genera of the Acaulosporaceae are distinguished by the position of the sporiferous saccule. It is produced laterally in *Acaulospora* and formed within the subtending hypha in *Entrophospora*. The *Entrophospora* mode of spore formation occurs in several different clades. One, exemplified by *E. colombiana*, groups in a clade with *Acaulospora* (FIG. 2A). *E. schenckii* is closely related to *Archaeospora trappei* (Redecker and Morton unpubl). The phylogenetic position of *E. infrequens*, the type species of the genus, is unclear because rDNA sequences from several unrelated glomeromycotan lineages were reported to occur within its spores (Rodriguez et al 2001).

(vi) The genus *Pacispora* was established recently for AM fungal species forming spores in the same way *Glomus* typically does but having flexible inner walls and a germination orb (Oehl and Sieverding 2004). It was reported to be basal to the Gigasporaceae in rDNA phylogenies (Walker et al 2004). The genus name *Gerdemannia* published for the same group only a few weeks later is an illegitimate name based on the publication date (Walker and Schüßler 2004).

(vii) Another clade of *Glomus* species, referred to as *Glomus* group C, is more closely related to the Acaulosporaceae than to *Glomus* groups A and B, based on rDNA phylogenies (Schwarzott et al 2001). One species of it has been described in a new genus *Diversispora* as *D. spurca*, mainly based on ribosomal sequence signatures (Walker and Schüßler 2004).


A
Y17633 Acaulospora laevis
AJ306442 Acaulospora scrobiculata
AJ306439 Acaulospora longula
Z14004 Acaulospora spinosa
Z14006 Entrophospora colombiana
AJ306440 Acaulospora sp. W3424
Y17650 Diversispora spurca
X86687 Glomus versiforme
Y17644 Glomus sp. "etunicatum-like" W2423
AJ619955 Pacispora scintillans
Z 14009 Gigaspora albida
Z14010 Gigaspora gigantea
AJ276093 Scutellospora auriglobo
AB041344 Scutellospora cerradensis
Z14013 Scutellospora heterogama
Z14012 Scutellospora pellucida
U31997 Scutellospora castanea BEG1
AJ306443 Scutellospora calospora
AJ249715 Glomus coremioides
AF213462 Glomus proliferum
U36590 Glomus clarum
X58725 Glomus intraradices
AJ430853 V. corymbosa symbiont
AJ133706 Glomus sinuosum
AF480155 Arachnitis uniflora symbiont
Z14007 Glomus mosseae
AJ245637 Glomus geosporum
AJ276086 Glomus coronatum
AJ430852 Voyriella parviflora symbiont
AJ301857 Glomus sp. W3347
AJ276075 Glomus claroideum
Z14008 Glomus etunicatum
Y17652 Glomus viscosum
AJ006466 Archaeospora leptoticha
AJ006800 Archaeospora trappei
X86686 Geosiphon pyriformis
AJ301862 Paraglomus brasilianum
AJ006799 Paraglomus occultum
Acaulo-sporaceae
Diversi-sporaceae (Glomus group C)
Pacisporaceae
Giga-sporaceae
Glomeraceae
(Glomus group A)
(Glomus group B)
Archaeo-sporaceae
Geosiphonaceae
Para-glomeraceae
Diversisporales
Glomerales
Archaeo-sporales
Para-glomer-ales
B
Agaricus bisporus
Russula compacta
Pseudohydnum gelatinosum
Tilletia caries
Ustilago hordei
Cladonia bellidiflora
Morchella esculenta
Neurospora crassa
Saccharomyces cerevisiae
Taphrina populina
Echinosporangium transversale
Mortierella polycephala
Endogone pisiformis
Entomophaga aulicae
Entomophthora muscae
Mucor mucedo
Absidia blakesleeana
Blastocladiella emersonii
Smittium culisetae
Furculomyces boomerangus
Kickxella alabastrina
Coemansia braziliensis
Neocallimastix sp.
Monoblepharella elongata
Ichthyophonus irregularis
Baeria nivea
Basidio-
Asco-
Zygo-
Zygo-
Chytridio-
C
Glomus mosseae
Glomus geosporum
Glomus intraradices
Glomus proliferum
Scutellospora heterogama
Paraglomus occultum
Geosiphon pyriformis
Mortierella verticillata
Rhizopus stolonifer
Endogone pisiformis
Basidiobolus ranarum
Coemansia reversa
Smittium culisetae
Entomophthora muscae
Allomyces arbusculus
Spizellomyces punctatus
Rhizophydium macroporosum
Nowakowskiella sp.
Filobasidiella neoformans
Phanerochaete chrysosporium
Tilletiaria anomala
Saccharomyces cerevisiae
Aspergillus fumigatus
Monosiga brevicollis
Glomero-
Zygo-
Chytridio-
Basidio-
Asco-

(viii) The genus *Archaeospora* was established for some AM fungi deeply divergent within the phylum in rDNA phylogenies (Sawaki et al 1998, Redecker et al 2000b, Morton and Redecker 2001). *A. leptoticha* and *A. gerdemannii* are dimorphic, producing both glomoid and acaulosporoid spores. A glomoid morph of *A. trappei* was reported only recently (Spain 2003). Most isolates produce both types of spores at the same time. The acaulosporoid spores of *Archaeospora* show several characters distinct from *Acaulospora*. The sporiferous saccule is separated from the hyphae by a short stalk (a ''pedicel''), the flexible inner walls are much thicker in *A. leptoticha* and *A. gerdemannii*, do not stain with Melzer's reagent and do not appear to be involved in the germination process.

(ix) *Geosiphon pyriformis* is the only member of the phylum that is known to engage in a different type of symbiosis (Schüßler et al 1994). It forms an endocytosis containing the cyanobacterium *Nostoc punctiforme*, harboring these photobionts in fungal bladders up to 2 mm large. It forms glomoid spores and first was identified as a basal relative of AM fungi by rDNA phylogeny (Gehrig et al 1996). The exact phylogenetic relationship of *Geosiphon* relative to *Paraglomus*, *Archaeospora* and the clade with the previously known three families was not resolved well in earlier studies (Redecker et al 2000b). An updated sequence of *Geosiphon* in the databases now places this fungus closer to *Archaeospora leptoticha/gerdemannii* (Redecker 2002, Schwarzott et al 2001).

(x) *Paraglomus* species form small, hyaline spores that do not show light microscopic characters that would distinguish them from those of *Glomus* species. However rDNA phylogenies clearly showed that *Paraglomus* is not related to other *Glomus* species and is basal to the phylum (Redecker et al 2000b). *Paraglomus* and *Archaeospora* share the characteristic that their intraradical structures consistently stain weakly or not at all in the standard procedure using acidic stains (trypan blue, direct blue) which is commonly used to view mycorrhizal colonization. The basal position of *Archaeospora* and *Paraglomus* is supported by unique fatty acids not found in other glomeromycotan fungi (Graham et al 1995).

Above the genus level the family Glomaceae was erected by Pirozynski and Dalpé (1989). Gigasporaceae and Acaulosporaceae were established by Morton and Benny (1990). Morton and Redecker (2001) described new families Archaeosporaceae and Paraglomaceae to comprise deeply divergent lineages of AM fungi. Schüßler et al (2001) divided the new phylum Glomeromycota into the orders Glomerales, Diversisporales, Archaeosporales and Paraglomerales. Together with the corresponding new genus *Diversispora*, the family Diversisporaceae was erected (Walker and Schüßler, 2004).

In contrast to evolutionary trends in spore morphology, differences among glomeromycotan lineages with regard to symbiotic behavior or other properties have been more difficult to address. Different preferences with regard to nutrient translocation and increase of root pathogen resistance have been reported (Klironomos 2000). The foraging of the extraradical mycelium has been compared among some species of *Glomus* (Jansa et al 2005). Soil chemistry, agricultural practice and other parameters were shown to have an influence on certain sets of taxa (Helgason et al 1998, Hijri et al 2006). Ecological preferences have been reported in several molecular field studies of AMF diversity. For instance legume roots and nodules appear to contain different AM fungal taxa than other plants under the same conditions (Scheublin et al 2004).

Differences in hyphal architecture and growth patterns have been observed between Glomeraceae and Gigasporaceae. Gigasporaceae can colonize only roots from germinating spores and do not form anastomoses (cross-links) among hyphae. If their hyphae are injured the main hypha is repaired. Glomeraceae are able to colonize roots from myceli-

←

FIG. 2. Phylogenetic trees of Glomeromycota and other fungi. A. Bayesian tree based on 1539 bp of small subunit rDNA sequences. *Neurospora crassa* was used as outgroup. B. Neighbor joining tree derived from maximum likelihood distances, based on 1419 bp of small subunit rDNA sequences. The glomeromycotan clade in the tree was replaced by tree A, which had an extended taxon sampling within the Glomeromycota, minus outgroup. Asterisk indicates clades that were not recovered in Bayesian analysis. In particular the Mucorales grouped with Asco/Basidiomycota in the Bayesian tree. In both A and B the trees had an identical overall topology with trees obtained by maximum likelihood analysis. C. Bayesian tree obtained from protein sequences of rpb1. Numbers on the nodes indicate Bayesian posterior probabilities/bootstrap values from 1000 replicates of neighbor joining (A, B) and Bayesian posterior probabilities/bootstrap values of 100 replicates of parsimony bootstrap with a PAM250-derived step matrix (C). Boldface ''100'' indicates both values were 100%.

um fragments or colonized root pieces; they form extensive anastomoses in their mycelium and they repair injured hyphae by forming a network of anastomoses instead of repairing the main hyphal axis (de la Providencia et al 2005).

The first molecular phylogeny of AM fungi was reported by Simon et al (1993), using ribosomal small subunit (SSU) sequences. These authors addressed the phylogenetic relationships among the three families known at that time and attempted to date their divergence by a molecular clock analysis. Morphological characters and fatty acid methyl ester profiles were evaluated phylogenetically by Bentivenga and Morton (1996). Most of the later phylogenetic studies used SSU sequences, which are still the only gene with a broad taxon sample in the group.

Marker genes other than ribosomal RNA became available in the past few years, but their impact on classification has been limited because some of the results were difficult to interpret. For instance phylogenies based on alpha and beta tubulin gene sequences were obscured by multiple paralogues (Corradi et al 2004). A multigene phylogeny of fungi including basal lineages used nuclear ribosomal small, large subunit and 5.8R subunit, tef, rpb1 and rpb2 sequences and placed the Glomeromycota as a sister group of Asco- and Basidiomycota (James et al 2006).

The aim of this article is to provide an overview of previous studies on glomeromycotan phylogeny and discuss newly available data in the context of the AFTOL project.

MATERIALS AND METHODS

Ribosomal SSU sequences used here were obtained from the public databases. rpb1 sequences were obtained by the procedure outlined (supplementary material) (database accession numbers AM284973-AM284982) or originate from the AFTOL database (http://ocid.nacse.org/research/aftol/data.php). The sequences were aligned in PAUP*4.0 b10 (Swofford 2001). Alignments and trees were submitted to TreeBASE under the study accession number S1614 and the matrix accession numbers M2898 and M2899.

Two datasets of ribosomal SSU sequences were used. Parameters for maximum likelihood analysis and Bayesian analysis were estimated with MrModeltest (Nylander 2004) or Modeltest 3.5 (Posada 2004). The GTR+I+G model was determined to be appropriate for both nucleotide datasets.

After exclusion of regions of ambiguous alignment dataset A comprised 1539 positions of a representative set of glomeromycotan taxa and *Neurospora crassa* as outgroup. Bayesian analysis was performed with four chains over 800 000 generations in MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003). Trees were sampled every 200 generations and a 50% consensus tree was constructed after the exclusion of the first 10% of trees from the first stage of the run (burn-in). A maximum likelihood heuristic search was performed in PAUP*4.0b10 (Swofford 2001), as well as distance analysis by neighbor joining and 1000 replicates of bootstrap analysis. Distances for neighbor joining were obtained by the Kimura 3-parameter method. The proportion of invariable characters and the gamma shape parameter for this analysis were determined with Modeltest.

Dataset B comprised 1419 bp from representatives of all fungal phyla as well as *Baeria nivea* and *Ichthyophonus irregularis* as outgroups. This dataset was analyzed to elucidate the phylogenetic position of the Glomeromycota among other fungal clades. It was analyzed by the same methods as described above. A total of 4 859 000 generations of Bayesian analysis were performed, trees were sampled every 500 generations and 10% were discarded as burn-in.

A protein dataset of 444 amino acids was compiled by translating nucleotide sequences into peptide sequences. Apparent stop codons, most likely the result of sequencing errors, were treated as missing data. In a few instances a possible frame-shift in adjacent amino acids was caused, thus these amino acids were replaced by X. The amino acid dataset was analyzed by these methods: (i) parsimony analysis in PAUP* with 1000 replicates of bootstrapping, using a step matrix based on the PAM250 similarity matrix adapted for PAUP by R.K. Kuzoff. (ii) Bayesian analysis over 1 000 000 generations using a mixed model. Trees were sampled every 500 generations and a 50% consensus was calculated after discarding the first 10% of trees.

RESULTS AND DISCUSSION

The phylogenetic relationship of the Glomeromycota to other fungal phyla.—The Glomeromycota is supported consistently as a monophyletic group in phylogenetic analyses of rDNA and protein genes (FIG. 2B, Berbee and Taylor 2000, Schüßler et al 2001, Helgason et al 2003, James et al 2006). Ribosomal RNA analyses place these species as a sister group of Asco- and Basidiomycota, although not always with strong support. This ''symbiomycota'' clade (Tehler et al 2003) would be characterized by the ability to form mutualistic symbioses with plants or algae that is not normally encountered in fungi outside this clade (an exception is ectomycorrhiza-forming species of the zygomycete *Endogone*, Warcup 1990). A large proportion of land plant-associated species generally are found in this clade. Most fungal groups in the ''symbiomycota'' also frequently form hyphal anastomoses, a trait that is rare or absent in most lineages of Zygo- and Chytridiomycota. However it also is obvious that some lineages of Zygomycota show strong evidence of long-branch attraction in rRNA phylogenies (FIG. 1B), which may exaggerate their separation from Mortierellales, Endogonales, chytrids and possibly Glomeromycota.

Evidence for a concurrent origin of this clade with early land plants 500 000 000–400 000 000 y ago was presented, based on the phylogeny and fossil findings (Redecker et al 2000a), whereas other studies suggested that the Glomeromycota lineage could be older than land plants (1 000 000 000–1 200 000 000 y ago) and might have associated with algae (Heckman et al 2001). However a radiation of major glomeromycotan lineages long before land plants seems unlikely considering that all major lineages in the phylum comprise AM symbionts. An independent adoption of the mycorrhizal way of living by all of these lineages does not seem very parsimonious.

Ribosomal SSU phylogenies (FIG. 2A) also indicate that *Geosiphon pyriformis*, the only known glomeromycotan fungus living in symbiosis with cyanobacteria, is derived from early mycorrhizal lineages. In SSU phylogenetic trees *Geosiphon* is closely related to *Archaeospora* (FIG. 2A), and in some phylogenies it groups even between the two major clades of this genus (Schwarzott et al 2001). In the rpb1 phylogeny *Geosiphon* branches off earlier than *Paraglomus* (FIG. 2C). To conclusively address this relationship with rpb1, data are needed from *Archaeospora*, the AM fungal lineage that is most closely related to *Geosiphon*. Up to now no protein gene sequences have been reported from *Archaeospora*. In addition there is still some possibility that *Geosiphon* forms AM in addition to its unique cyanobacterial symbiosis and that its mycorrhizae have not been detected yet.

It is interesting to note on the other hand that the mycorrhizal status of a large proportion of described glomeromycotan morphospecies in fact has not been confirmed because these fungi have never been obtained in single-species culture. If saprophytes occur in the Glomeromycota it can be assumed that they are derived from mycorrhizal ancestors.

The rpb1 phylogeny presents strong support for a monophyletic Glomeromycota but not for the symbiomycotan clade. This finding is in agreement with analyses of elongation factor and actin genes. The three protein genes indicate a relationship of the Glomeromycota with zygomycotan lineages, in particular the Mortierellales (FIG. 2C, Helgason et al 2003). In contrast, based on tubulin sequences, the Glomeromycota group with the chytrids. The tubulin phylogenies however show a strong separation between fast-evolving (Basidio-, Ascomycota, Entomophthorales, Mucorales and others) and slowly evolving lineages (Glomero-, Chytridiomycota), which can be expected to cause problems in phylogenetic reconstruction.

Phylogenetic relationships within the Glomeromycota.—A SSU-based phylogeny of representatives of the major groups within the Glomeromycota is depicted (FIG. 2A). This gene and the adjacent highly variable internal transcribed spacers still offer the broadest range of taxa, including many unidentified species from environmental studies. The database of ribosomal large subunit sequences obtained from spores has grown considerably in the past few years but has been used less frequently in field studies.

The intraspecies variability of nuclear ribosomal genes complicates distinguishing closely related species but is not an issue at the generic or family level. One study reporting the occurrence of strongly diverging sequences within single spores (Hijri et al 1999) has been shown to be due to contamination by Ascomycota (Redecker et al 1999). Other reports suggesting a sequence continuum among morphologically well separated species (Clapp et al 2001) have been criticized and the results might be due to problems with the culturing of the fungi (Schüßler et al 2003).

Protein genes are available for relatively few taxa, but key taxa are still missing from all datasets. In addition analyses of tubulin genes showed strong problems with paralogous gene copies found in the Glomeromycota (Corradi et al 2004). Therefore it still is not feasible to address comprehensively the intraphylum phylogeny using protein genes and only the SSU phylogeny will be discussed here.

The genus *Glomus* as it was defined before any molecular data were available (e.g. Morton and Benny 1990) later was shown to be polyphyletic (Redecker et al 2000b, Schwarzott et al 2001, Walker et al 2004). The polyphyly was resolved by separating several lineages into the new genera *Archaeospora*, *Paraglomus*, *Diversispora* and *Pacispora* (Morton and Redecker 2001, Walker et al 2004, Walker and Schüßler 2004), but only one species of *Glomus* group C (*G. spurcum*) has been transferred to the new genus *Diversispora*.

The descriptions of both *Paraglomus* and *Diversispora* have presented problems because their separation from *Glomus* is well supported in rDNA phylogenies (and biochemical characters in *Paraglomus*) but morphological characters to distinguish them are scarce. The mycorrhizae of *Paraglomus* and *Archaeospora* share the consistently weak staining behavior with acidic dyes, whereas intraradical structures of *Glomus* group C/*Diversispora* occasionally stain faintly. All *Paraglomus* species currently are known produce small hyaline spores, whereas *Glomus* group C members show a variety of spore morphologies, even including an isolate fitting the morphological description of *Glomus etunicatum*, a species that normally is thought to belong to *Glomus* group B (Schwarzott et al 2001). Walker and Schüßler (2004)

transferred *Glomus spurcum* to the new genus *Diversispora* (grammatically incorrectly as ''*Diversispora spurcum*''), using an inner flexible wall as a distinguishing character. The presence of this feature in *G. spurcum* however is controversial (Kennedy et al 1999) and it might not be present in other members of the clade.

There is some evidence that one isolate of *Acaulospora* (W3424) groups basally to *Entrophospora colombiana* in the monophyletic Acaulosporaceae (Walker et al 2004), but this is not supported in all analyses (FIG. 2 A). Therefore the monophyly of *Acaulospora* as currently defined is not strongly rejected.

It has been disputed whether *Scutellospora* or *Gigaspora* are derived genera within the family Gigasporaceae. *Gigaspora* spores have the same wall structure as early development stages of *Scutellospora*, suggesting a classical Haeckelian recapitulation of phylogeny during ontogeny (Bentivenga et al 1997, Morton 1995). In molecular SSU phylogenies *Gigaspora* appears to be a derived monophyletic clade within the Gigasporaceae. The genus is nested between *Scutellospora* species, rendering the latter paraphyletic. Among *Gigaspora* species the ribosomal RNA sequence variation is low. However phylogenetic analyses do not offer strong support for a paraphyletic genus *Scutellospora* (FIG. 2A) even when more species of *Gigaspora* are included (Schwarzott et al 2001). A derived position of *Gigaspora* would imply the loss of the complex flexible inner walls and the germination shield during the evolution of the genus and a reduction in complexity of the wall structure. The papillate wall layer appearing immediately before germination then could be a relic of the germination shield.

Morton and Benny (1990) divided the order Glomales into the suborders Glomineae (comprising *Sclerocystis*, *Glomus*, *Entrophospora* and *Acaulospora*) and Gigasporineae (*Gigaspora* and *Scutellospora*). The Glomineae was characterized by the formation of vesicles, whereas members of Gigasporineae form auxiliary cells. This concept could not be substantiated by SSU molecular phylogenies. *Acaulospora appendicula* (now *Archaeospora leptoticha*), which was thought to be a transitional species between *Acaulospora* and *Glomus* (Morton and Benny, 1990) later was shown to be in a distinct basal clade (Redecker et al 2000b). Simon et al (1993) presented phylogenetic data supporting an alternative concept uniting Acaulosporaceae and Gigasporaceae.

SSU phylogenies consistently support the Diversisporales clade, comprising Acaulosporaceae, Gigasporaceae, Diversisporaceae and Pacisporaceae (FIG. 2A; Schüßler et al 2001, Walker and Schüßler 2004). Because no rpb1 sequences of *Acaulospora*, *Pacispora* or *Glomus* group C are available the clade cannot be assessed yet with this gene. A combined Bayesian analysis of elongation factor and actin supported the alternative grouping of *Acaulospora laevis* and *Glomus caledonium* but contained several other inconsistencies (Helgason et al 2003). The value of elongation factor sequences to elucidate deep phylogenies recently was questioned because of saturation among deep fungal lineages (Tanabe et al 2004). Alpha and beta tubulin sequences yielded conflicting results on this question (Corradi et al 2004) possibly due to problems with paralogues.

The presence of flexible germinal walls staining with Melzer's reagent is a possible synapomorphy uniting Gigasporaceae, Pacisporaceae and Acaulosporaceae in the Diversisporales. Within the Diversisporales clade the germination shield in *Scutellospora*, the papillate germination wall layer in *Gigaspora* and the germination orb in the Acaulosporaceae and *Pacispora* could be homologous structures. The discovery of *Pacispora*, a genus that shows glomoid spore formation but has germinal walls and a germination orb, further supports this hypothesis as a possible morphological intermediate. Consequently it appears as an early divergent lineage in the Diversisporales clade (FIG. 2A). In the phylogeny presented by Walker et al (2004) *Pacispora* groups together with the Gigasporaceae, whereas it is closer to Acaulosporaceae/*Glomus* group C in FIG. 2A.

The Gigasporaceae shows some unique characteristics in their growth and symbiotic behavior (see introduction) and a characteristic mode of spore formation. These features were suggested to set apart this group from all other glomeromycotan fungi, but they are equally compatible with phylogenies supporting this family as a derived clade of the Diversisporales.

In this context *Glomus* group C either should be expected to be a basal member of the Diversisporales arising before the development of germinal walls or a secondary loss of germinal walls might have occurred in this group. Most SSU analyses place *Glomus* group C close to the Acaulosporaceae, which would be most consistent with the second alternative.

Overall the evolutionary trends within the Glomeromycota suggest that the glomoid type of spore formation is a symplesiomorphy. This is supported by the earliest known fossils of Glomeromycota, which are glomoid spores (Redecker et al 2002). The acaulosporoid and entrophosporoid types of spore formation also arose early in organisms that most likely were dimorphic.

CONCLUSION

Although several lines of currently available evidence support the ''symbiomycota'' concept of a relation-

ship of Asco-, Basido- and Glomeromycota the conflicting results from different genes should incite further analysis. Analyses using different methods of phylogenetic analysis and a broad spectrum of genes clearly are needed. The same is true for the intraphylum relationships, where evolutionary tendencies among the clades can be resolved only with a more complete sampling of key taxa with multiple genes.

ACKNOWLEDGMENTS

The authors thank Tim James and Arthur Schüßler for providing unpublished sequences from the AFTOL project, Fritz Oehl and Kerstin Wex for providing images, the Botanical Institute at the University of Basel for generous support, Zuzana Sykorova for critically reading the manuscript and the Swiss National Science Foundation for financial support through grants 3100 066688.01 and 3100 A0109466.1 to D.R. We also acknowledge support from NSF 0090301, Research Coordination Network: a phylogeny for kingdom Fungi to M. Blackwell, J.W. Spatafora and J.W. Taylor.

LITERATURE CITED

Almeida RT, Schenck NC. 1990. A revision of the genus *Sclerocystis* (Glomaceae, Glomales). Mycologia 82:703–714.

Bécard G, Pfeffer PE. 1993. Status of nuclear division in arbuscular mycorrhizal fungi during in-vitro development. Protoplasma 174:62–68.

Bentivenga SP, Bever JD, Morton JB. 1997. Genetic variation of morphological characters within a single isolate of the endomycorrhizal fungus *Glomus clarum* (Glomaceae). Amer J Bot 84:1211–1216.

———, Morton JB. 1996. Congruence of fatty acid methyl ester profiles and morphological characters of arbuscular mycorrhizal fungi in Gigasporaceae. Proc Natl Acad Sci USA 93:5659–5662.

Berbee ML, Taylor JW. 2000. Fungal molecular evolution: gene trees and geologic time. In: McLaughlin DJ, McLaughlin EG, Lemke PA, eds. The Mycota. New York: Springer Verlag. p 229–246.

Clapp JP, Rodriguez A, Dodd JC. 2001. Inter- and intra-isolate rRNA large subunit variation in *Glomus coronatum* spores. New Phyto 149:539–554.

Corradi N, Hijri M, Fumagalli L, Sanders IR. 2004. Arbuscular mycorrhizal fungi (Glomeromycota) harbour ancient fungal tubulin genes that resemble those of the chytrids (Chytridiomycota). Fungal Genet Biol 41:1037–1045.

de la Providencia IE, de Souza FA, Fernandez F, Delmas NS, Declerck S. 2005. Arbuscular mycorrhizal fungi reveal distinct patterns of anastomosis formation and hyphal healing mechanisms between different phylogenic groups. New Phytol 165:261–271.

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

Gerdemann JW, Trappe JM. 1974. Endogonaceae in the Pacific Northwest. Mycol Mem 5:1–76.

Graham JH, Hodge NC, Morton JB. 1995. Fatty acid methyl ester profiles for characterization of glomalean fungi and their endomycorrhizae. Appl Environ Microbiol 61:58–64.

Heckman DS, Geiser DM, Eidell BR, Stauffer RL, Kardos NL, Hedges SB. 2001. Molecular evidence for the early colonization of land by fungi and plants. Science 293: 1129–1133.

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW. 1998. Ploughing up the wood-wide web? Nature 394: 431.

———, Watson IJ, Young JPW. 2003. Phylogeny of the Glomerales and Diversisporales (Fungi: Glomeromycota) from actin and elongation factor 1-alpha sequences. FEMS Microbiol Lett 229:127–132.

Hijri I, Sykorova Z, Oehl F, Ineichen K, Mäder P, Wiemken A, Redecker D. 2006. Communities of arbuscular mycorrhizal fungi in arable soils are not necessarily low in diversity. Mol Ecol 15:2277–2289.

Hijri M, Hosny M, van Tuinen D, Dulieu H. 1999. Intraspecific ITS polymorphism in *Scutellospora castanea* (Glomales, Zygomycota) is structured within multinucleate spores. Fung Genet Biol 26:141–151.

———, Redecker D, Macdonald-Comber Petetot JA, Voigt K, Wöstemeyer J, Sanders IR. 2002. Identification and isolation of two Ascomycete fungi from spores of the arbuscular mycorrhizal fungus *Scutellospora*. Appl Environ Microbiol 68:4567–4573.

———, Sanders IR. 2005. Low gene copy number shows that arbuscular mycorrhizal fungi inherit genetically different nuclei. Nature 433:160–163.

Husband R, Herre EA, Turner SL, Gallery R, Young JPW. 2002. Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. Mol Ecol 11:2669–2678.

James TY, Kauff F, Schoch C, Matheny PB, Hofstetter V, Cox C, Celio G, Gueidan C, Fraker E, Miadlikowska J, Lumbsch HT, Rauhut A, Reeb V, Arnold AE, Amtoft A, Stajich JE, Hosaka K, Sung G-H, Johnson D, O'Rourke B, Crockett M, Binder M, Curtis JM, Slot JC, Wang Z, Wilson AW, Schüßler A, Longcore JE, O'Donnell K, Mozley-Standridge S, Porter D, Letcher PM, Powell MJ, Taylor JW, White MM, Griffith GW, Davies DR, Humber RA, Morton JB, Sugiyama J, Rossman AY, Rogers JD, Pfister DH, Hewitt D, Hansen K, Hambleton S, Shoemaker RA, Kohlmeyer J, Volkmann-Kohlmeyer B, Spotts RA, Serdani M, Crous PW, Hughes KW, Matsuura K, Langer E, Langer G, Untereiner WA, Lücking R, Büdel B, Geiser DM, Aptroot A, Diederich P, Schmitt I, Schultz M, Yahr R, Hibbett D, Lutzoni F, McLaughlin D, Spatafora J, Vilgalys R. 2006. Reconstructing the early evolution of the fungi using a six gene phylogeny. Nature 443:818–822.

Jansa J, Mozafar A, Frossard E. 2005. Phosphorus acquisition

strategies within arbuscular mycorrhizal fungal community of a single field site. Plant Soil 276:163–176.

Kennedy LJ, Stutz JC, Morton JB. 1999. *Glomus eburneum* and *G. luteum*, two new species of arbuscular mycorrhizal fungi, with emendation of *G. spurcum*. Mycologia 91:1083–1093.

Klironomos JN. 2000. Host-specificity and functional diversity among arbuscular mycorrhizal fungi. In: Bell CR, Brylinsky M, Johnson-Green P, eds. Microbial biosystems: new frontiers, 8th International Symposium on Microbial Ecology. Halifax, Canada: Atlantic C Soc Microb Ecol. p 845–851.

Kuhn G, Hijri M, Sanders IR. 2001. Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. Nature 414:745–748.

Lanfranco L, Delpero M, Bonfante P. 1999. Intrasporal variability of ribosomal sequences in the endomycorrhizal fungus *Gigaspora margarita*. Mol Ecol 8:37–45.

Merryweather J, Fitter A. 1998. The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta*: I. Diversity of fungal taxa. New Phytol 138:117–129.

Morton JB. 1988. Taxonomy of VA mycorrhizal fungi: classification, nomenclature and identification. Mycotaxon 32:267–324.

———. 1995. Taxonomic and phylogenetic divergence among five *Scutellospora* species based on comparative developmental sequences. Mycologia 87:127–137.

———, Benny GL. 1990. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order Glomales and Gigasporineae and two new families Acaulosporaceae and Gigasporaceae with an emendation of Glomaceae. Mycotaxon 37:471–491.

———, Redecker D. 2001. Two new families of Glomales, Archaeosporaceae and Paraglomaceae, with two new genera *Archaeospora* and *Paraglomus*, based on concordant molecular and morphological characters. Mycologia 93:181–195.

Nylander JAA. 2004. MrModeltest 2.0 Evolutionary Biology Centre, Uppsala University: Uppsala, Sweden.

Oehl F, Sieverding E. 2004. *Pacispora*, a new vesicular arbuscular mycorrhizal fungal genus in the Glomeromycetes. J App Bot 78:72–82.

Öpik M, Moora M, Liira J, Koljalg U, Zobel M, Sen R. 2003. Divergent arbuscular mycorrhizal fungal communities colonize roots of *Pulsatilla* spp. in boreal Scots pine forest and grassland soils. New Phytol 160:581–593.

Pawlowska T, Taylor JW. 2004. Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. Nature 427:733–737.

Pirozynski KA, Dalpé Y. 1989. Geological history of the Glomaceae, with particular reference to mycorrhizal symbiosis. Symbiosis 7:1–36.

Posada D. 2004. Modeltest 3.5 Facultad de Biologia, Universidad de Vigo: Vigo, Spain.

Raab P, Brennwald A, Redecker D. 2005. Mitochondrial large ribosomal subunit sequences are homogeneous within isolates of *Glomus* (arbuscular mycorrhizal fungi, Glomeromycota). Mycol Res 109:1315–1322.

Redecker D. 2002. Molecular identification and phylogeny of arbuscular mycorrhizal fungi. Plant Soil 244:67–73.

———, Hijri M, Dulieu H, Sanders IR. 1999. Phylogenetic analysis of a dataset of fungal 5.8S rDNA sequences shows that highly divergent copies of internal transcribed spacers reported from *Scutellospora castanea* are of Ascomycete origin. Fung Genet Biol 28:238–244.

———, Kodner R, Graham LE. 2000a. Glomalean fungi from the Ordovician. Science 289:1920–1921.

———, ———, ———. 2002. *Palaeoglomus* grayi from the Ordovician. Mycotaxon 84:33–37.

———, Morton JB, Bruns TD. 2000b. Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). Mol Phylogen Evol 14:276–284.

———, ———, ———. 2000c. Molecular phylogeny of the arbuscular mycorrhizal fungi *Glomus sinuosum* and *Sclerocystis coremioides*. Mycologia 92:282–285.

Rodriguez A, Dougall T, Dodd JC, Clapp JC. 2001. The large subunit ribosomal RNA genes of *Entrophospora infrequens* comprise sequences related to two different glomalean families. New Phytol 152:159–167.

Rokas A, Krüger D, Carroll SB. 2005. Animal evolution and the molecular signature of radiations compressed in time. Science 310:1933–1938.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

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

Sawaki H, Sugawara K, Saito M. 1998. Phylogenetic position of an arbuscular mycorrhizal fungus, *Acaulospora gerdemannii*, and its synanamorph, *Glomus leptotichum*, based upon 18s rRNA gene sequence. Mycoscience 39: 477–480.

Scheublin TR, Ridgway KP, Young JPW, van der Heijden MGA. 2004. Nonlegumes, legumes and root nodules harbor different arbuscular mycorrhizal fungal communities. Appl Environ Microbiol 70:6240–6246.

Schüßler A, Mollenhauer D, Schnepf E, Kluge M. 1994. *Geosiphon pyriforme*, an endosymbiotic association of fungus and cyanobacteria—the spore structure resembles that of arbuscular mycorrhizal (AM) fungi. Bot Acta 107:36–45.

———, Schwarzott D, Walker C. 2003. Glomeromycota rRNA genes—the diversity of myths? Mycorrhiza 13: 233–236.

———, ———, ———. 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105:1413–1421.

Schwarzott D, Walker C, Schüßler A. 2001. *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (Glomales), is non-monophyletic. Mol Phylogen Evol 21:190–197.

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

Spain JL. 2003. Emendation of *Archaeospora* and of its

type species, *Archaeospora trappei*. Mycotaxon 87:109–112.

———, Sieverding E, Schenck NC. 1989. *Gigaspora ramisporophora*: a new species with novel sporophores from Brazil. Mycotaxon 34:667–677.

Stukenbrock EH, Rosendahl S. 2005. Development and amplification of multiple co-dominant genetic markers from single spores of arbuscular mycorrhizal fungi by nested multiplex PCR. Fung Genet Biol 42:73–80.

Stürmer SL, Morton JB. 1997. Developmental patterns defining morphological characters in spores of four species in *Glomus*. Mycologia 89:72–81.

———, ———. 1999. Taxonomic reinterpretation of morphological characters in Acaulosporaceae based on developmental patterns. Mycologia 91:849–857.

Swofford DL. 2001. PAUP*: phylogenetic analysis using parsimony (*and other methods) Sinauer Associates: Sunderland, Massachusetts.

Tanabe Y, Saikawa M, Watanabe MM, Sugiyama J. 2004. Molecular phylogeny of Zygomycota based on EF-1 alpha and RPB1 sequences: limitations and utility of alternative markers to rDNA. Mol Phylogen Evol 30: 438–449.

Tehler A, Little D, Farris J. 2003. The full-length phylogenetic tree from 1551 ribosomal sequences of chitinous fungi. Mycol Res 107:901–916.

Tommerup IC, Sivasithamparam K. 1990. Zygospores and asexual spores of *Gigaspora decipiens*, an arbuscular mycorrhizal fungus. Mycol Res 94:897–900.

Vandenkoornhuyse P, Husband R, Daniell TJ, Watson IJ, Duck JM, Fitter AH, Young JPW. 2002. Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. Mol Ecol 11: 1555–1564.

Walker C. 1983. Taxonomic concepts in the Endogonaceae: spore wall characteristics in species descriptions. Mycotaxon 18:443–455.

———. 1992. Systematics and taxonomy of the arbuscular mycorrhizal fungi (Glomales)—a possible way forward. Agronomie 12:887–897.

———, Blaszkowski JDS, Schüßler A. 2004. *Gerdemannia* gen. nov., a genus separated from *Glomus*, and Gerdemanniaceae fam. nov., a new family in the Glomeromycota. Mycol Res 108:707–718.

———, Schüßler A. 2004. Nomenclatural clarifications and new taxa in the Glomeromycota. Mycol Res 108:981–982.

Warcup JH. 1990. Taxonomy, culture and mycorrhizal associations of some zygosporic Endogonaceae. Mycol Res 94:173–178.

SUPPLEMENTARY MATERIAL

Crude extracts from spores were obtained by the method of Redecker et al (1997) and used for a nested PCR procedure. In the first step primers RPB1-PR1F (GTMCCTCCACCRCCTGTTCGTC) and RPB1-PR3R (CTCAGCACTACGACCAGCAGAGTCA) were used. PCR cycling parameters were 3 min at 95 C, 30 cycles of 1 min at 95 C, 1 min at 56 C and 4 min at 72 C for 30 cycles, followed by 5 min at 72 C. The PCR product was diluted 1:10. In the second step of the nested PCR, primers RPB1-PR2F and RPB1-PR3R were used under the same cycling conditions.

Additional primers listed below were used to sequence the PCR fragment of an approximate length of 1560 bp: RPB1-PR2F: CCTGTTCGTCCSAGTATTCAAATG, RPB1-PR4F: GGTAAAGAAGGACGTCTTCGTGGA, RPB1-PR5: ACCTTGTTGTGCGGTRCCAACCGT, RPB1-PR6F: TCGGACAATTTRCGTCATTCTACTC, RPB1-PR7: GGTAYRTGCATATTCATTTCATC, RPB1-PR8: CCATATAAGTTGCRCAATGA, RPB1-PR9F: CAACTTTCCGTCTTAATCTCTCC.

PCR products were ligated into Topo pcr 4.0 (Invitrogen, Basel, Switzerland) and used to transform competent *E. coli* Topo10 cells (Invitrogen). Inserts were sequenced in both directions with an Applied Biosystems Prism DyeTerminator 3.1 kit on an ABI 310 capillary sequencer (Applied Biosystems, Foster City, California). Sequences were aligned and corrected in BioEdit (Hall 1999) and submitted to the EMBL database under the accession numbers AM284973-AM284982.