Glomalean Fungi from the Ordovician

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Fossilized fungal hyphae and spores from the Ordovician of Wisconsin (with an age of about 460 million years) strongly resemble modern arbuscular mycorrhizal fungi (Glomales, Zygomycetes). These fossils indicate that Glomales-like fungi were present at a time when the land flora most likely only consisted of plants on the bryophytic level. Thus, these fungi may have played a crucial role in facilitating the colonization of land by plants, and the fossils support molecular estimates of fungal phylogeny that place the origin of the major groups of terrestrial fungi (Ascomycota, Basidiomycota, and Glomales) around 600 million years ago.

Arbuscular mycorrhizal (AM) symbiosis between modern plants and fungi is ubiquitous and contributes to nutrient acquisition by most vascular plants (*1*). It has also been reported for hepatics and hornworts (*2, 3*). Arbuscular mycorrhizae may have played an important role in the success of early terrestrial plants (*4, 5*).

Because of the poor preservation of most fungal structures, it has been difficult to interpret the fossil record of fungi (*6–8*). Hyphae, the vegetative body of fungi, bear few distinctive morphological characters, and organisms as diverse as cyanobacteria, eukaryotic algal groups, or Oomycetes can be easily mistaken for them. The most convincing evidence for the presence of fungi is fungal fossils associated with plants, including arbuscular mycorrhizae (*9, 10*) and ascomycetous or chytrid parasites (*11, 12*). The previously reported oldest fungal fossils originate nearly exclusively from a single site, the Devonian Rhynie Chert [400 million years ago (Ma)] (*9, 10*). Possible earlier ascomycetes were reported from the Silurian of Sweden (*13*).

The fossils reported here were found in the Guttenberg Formation, mid-Ordovician dolomite of Wisconsin, which was deposited in a presumed shallow marine setting between 460 and 455 Ma (*14–16*). The fossilized material consists of entangled, occasionally branching, nonseptate hyphae and spores (Fig. 1, A to C and E to G). The hyphae have a diameter of 3 to 5 μm and bear globose to subglobose terminal spores that are 40 to 95 μm in diameter. These spores are connected to cylindrical to slightly flared (Fig. 1, C and G) or recurved (Fig. 1E) subtending hyphae. No specialized attachment structures are visible. Where spores are not entangled in hyphae, a single hyphal connection per spore can be discerned (Fig. 1, C and E to G). We found no single spore that did not retain its subtending hypha. The spore wall apparently consists of a single layer. In their shape, size, and hyphal connection, the spores resemble those of modern glomalean fungi in the genus *Glomus* (Fig. 1, D and H). Similar loose spore clusters are frequently formed by present-day AM fungi but are unknown from algae, other fungi, or Oomycetes.

Species determination in the Glomales depends on the layer structure of the spore wall (*17*). No such layers could be discerned in the fossils. Layers may have degraded before they were deposited, over time, or during the acid treatment used to dissolve the rock. Our experiments with living extant spores showed that the spore wall layers are acid stable, but for dead extant spores, the loss of wall layers by microbial degradation has been frequently reported (*17*).

Our findings push back the time of the fossil record of glomalean fungi by 55 to 60 million years and also suggest that these fungi were present before the first vascular plants arose. Although hepatics and hornworts lack roots that would allow them to form a "mycorrhiza" in a strict sense, AM-like colonization of their thallus has long been known (*2*). Evidence that a hornwort forms an association with a defined AM fungus was only obtained recently (*3*). We have no evidence that these Ordovician fossil fungi were associated with plants, but fossil evidence for Ordovician land plants (*18*) and the well-documented capability of some extant bryophytes for AM-like interactions suggest this possibility. Alternatively, they may have formed symbioses of the *Geosiphon* type or have been saprobes. *Geosiphon* is a nonmycorrhizal ancestral member of the Glomales, which forms an endosymbiosis with cyanobacteria (*19*) and evolved at approximately the same time as the deeply divergent mycorrhizal lineages (*20*). It also forms spores of the *Glomus* type (*21*).

The fossils reported here support time estimates of fungal phylogeny derived from ribosomal small subunit sequences (*22*) (Fig. 2). Using a modified data set from a previous study (*22*), we included the Glomales-like fungi from the Ordovician and some other recently reported fossils to date their divergences within the tree (*23*). Some of these fossils provide useful calibration points for the tree (Fig. 2, points a and d),

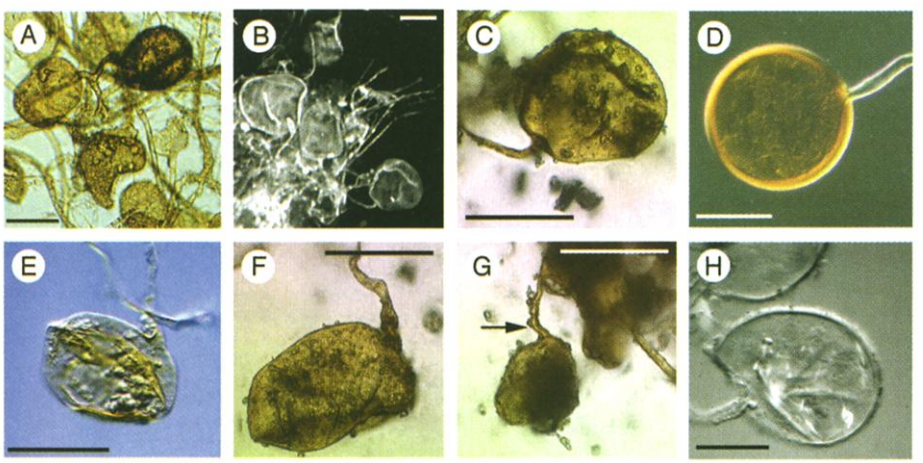

Fig. 1. (**A** to **C** and **E** to **G**) Fossil hyphae and spores from the Ordovician and (**D** and **H**) spores formed by extant glomalean fungi. (A and B) Overviews of the fossilized material. (C, E, F, and G) Fossil spore details. (C) Detail of (B). (D) A spore of present-day *Glomus* sp. S328 with layered wall structure. In (G), the arrow shows walls of a subtending hypha in connection with the spore wall. (H) A spore of present-day *Glomus leptotichum*, a member of the deeply divergent glomalean lineages. Images were obtained by light microscopy (*28*) of the specimens in air (A, C, F, and G), differential interference contrast microscopy of the specimens in polyvinylalcohol-lactoglycerol (D, E, and H), and confocal laser scanning microscopy with the autofluorescence of the material (B). All scale bars are 50 μm.

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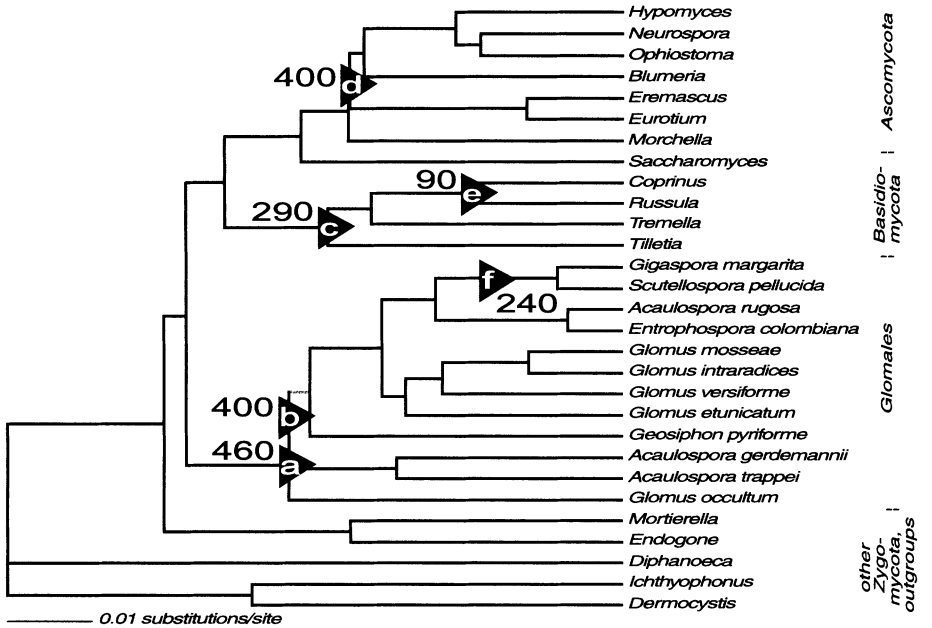

Fig. 2. The phylogenetic tree of the fungi derived from small subunit ribosomal sequences. Letters a to e assign tree branching points to fungal fossils. The respective geological times are given as numbers on the tree. Triangles indicate that all fossils could also have been deposited later in the history of each clade, allowing the origins of the clades to be shifted back in time. Point a indicates the glomalean fossils reported in the present study; b indicates the fossil arbuscular mycorrhizae from the Rhynie Chert (400 Ma) (*9*); c indicates a fossil clamp connection (earliest evidence of Basidiomycetes, 290 Ma) (*29*); d indicates the Ascomycetes from the Rhynie Chert (400 Ma) (*30*); e indicates the gilled mushroom in amber (90 Ma) (*8*); and f indicates the arbuscular mycorrhizae of the Gigasporaceae type from the Triassic (~240 Ma) (*31*). The outgroups were *Diphanoeca grandis* (Choanoflagellate), *Ichtyophonus hoferi*, and *Dermocystis salari* [early divergences of the animals (*32*)]. Branches that are not supported by at least 50% bootstrap analyses are shown in gray.

whereas others apparently were formed after the origin of the clade (Fig. 2, points b, c, and e) (*24*). The assumption that the fossil organisms existed at the time of, or after, the divergence of the deep glomalean lineages (Fig. 2, point a) places the origin of the Ascomycete/Basidiomycete clade at 600 to 620 Ma (*25*), in good agreement with the date of 600 Ma from Berbee and Taylor (*22*). No lineage before the Glomales developed plant symbioses, but numerous later diverging clades did, with global impacts on terrestrial ecosystems.

References and Notes

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16. To eliminate possible problems of modern contamination, we removed 50 cm of the exposed rock before sampling. Samples were taken from the center of the outcrop, contained no weathered surfaces, and had not been penetrated by any modern plant. To eliminate ancient contamination, we used a rock saw to cut off the edges of the samples, which showed no inclusion or any evidence of past penetration. There were no obvious bedding planes in the rocks. If the rocks were contaminated some time after deposition, we would see other more common, similar-sized, resistant organic materials representative of later periods (e.g., vascular plant–type spores or pollen grains, root fragments, and diatoms). Whole samples of rock were subsequently submerged in two baths of concentrated HCl in new plastic containers. Sludge and acid from the first bath were discarded. The sediment from the second bath and occasionally remaining rock were examined under a dissecting microscope to separate the organic matter from the inorganic sediments. Specimens shown in Fig. 1, B and C and E to G, were deposited at the University of California Museum of Palaeontology, Berkeley (UCMP 151983 and 151984).
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23. A subset of 20 taxa from the original data set was combined with newly added sequences from *Mortierella polycephala* and nine glomalean species, among them four representatives of the deeply divergent lineages (*Geosiphon pyriforme*, the dimorphic fungus *Acaulospora gerdemannii Glomus leptotichum*, *A. trappei*, and *Glomus occultum*). A neighbor-joining tree from these data was obtained by the Kimura two-parameter method in PAUP* (*26*) with a gamma shape parameter of 0.5 for among-site variation, which was then normalized under the maximum likelihood criterion with the molecular clock enforced. The maximum likelihood settings were as follows. The transition/transversion ratio (2.17), base frequencies, and the proportion of invariant sites were estimated by maximum likelihood. A gamma shape parameter of 0.5 was assumed for among-site variation.
24. Taylor, Remy, and Hass (*7*) reported an *Allomyces*-like fossil from the Devonian. As the Chytridiomycetes probably originated much earlier than the Ascomycete/Basidiomycete/Glomales clade (*22*, *27*), it would not be useful as a calibration point. It was not included in the sequence alignment, because the 18*S* ribosomal DNA of *Allomyces* causes extremely long branches in phylogenetic trees.
25. This scenario is more compatible with calibration point d in Fig. 2 than assigning the 460-Ma fossils to the separation point of the Glomales/Ascomycete/Basidiomycete clades.
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28. In Fig. 1, C, F, and G were obtained with a Nikon compound microscope equipped with an Image Explorer system (Hitachi Genetic Systems, Alameda, CA) that was used to focus through the specimens and combine the images of the different focusing planes to a composite with a very large depth of field. Figure 1B was obtained with a Molecular Dynamics laser scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA). The excitation wavelength was 530 nm, and the autofluorescence of the specimens above 600 nm was detected. This method allows similar optical sectioning as the method described above, but it also allows sectioning within optically dense areas of the specimen that are not transparent enough for light microscopy.
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30. The earliest Ascomycetes from the Devonian show features [Perithecia or Pseudothecia (*11*)] that are found in the present-day Ascomycete group of Pyrenomycetes and Loculoascomycetes, respectively (*27*). The Pyrenomycetes are the clade of the tree containing *Neurospora*, *Hypomyces*, and *Ophiostoma*. Therefore, these fossils were assigned to the major radiation of Euascomycetes (Fig. 2, point d).
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