

# The dawn of symbiosis between plants and fungi

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**The colonization of land by plants relied on fundamental biological innovations, among which was symbiosis with fungi to enhance nutrient uptake. Here we present evidence that several species representing the earliest groups of land plants are symbiotic with fungi of the Mucoromycotina. This finding brings up the possibility that terrestrialization was facilitated by these fungi rather than, as conventionally proposed, by members of the Glomeromycota. Since the 1970s it has been assumed, largely from the observation that vascular plant fossils of the early Devonian (400 Ma) show arbuscule-like structures, that fungi of the Glomeromycota were the earliest to form mycorrhizas, and evolutionary trees have, until now, placed Glomeromycota as the oldest known lineage of endomycorrhizal fungi. Our observation that *Endogone*-like fungi are widely associated with the earliest branching land plants, and give way to glomeromycotan fungi in later lineages, raises the new hypothesis that members of the Mucoromycotina rather than the Glomeromycota enabled the establishment and growth of early land colonists.**

**Keywords:** ecology; evolution; palaeobiology; mutualism; mycorrhizal; liverwort

## 1. INTRODUCTION

The idea that symbiosis with arbuscular mycorrhizal fungi (Glomeromycota) allowed rootless early plants to invade poorly developed primaevial soils [1] and go on to transform the biosphere has gained increasingly widespread acceptance [2–5], helped by some fossil support [6,7] and the absence of any plausible alternatives. In other words, arbuscular mycorrhizal fungi are the most common fungi forming endomycorrhizal (i.e. intracellular) mutualisms with today's vascular plants and these fungi are also the most ancient

endomycorrhizal fungi known to date. Thus, the assumption that Glomeromycota fungi were involved in the colonization of land by plants has not been contested, arbuscular mycorrhizas are regarded as 'the mother of plant root endosymbioses' [8], and methods that only detect Glomeromycota are routinely used in molecular ecological studies of mycorrhizal plants.

Curiously, analyses of the earliest branching lineages of land plants (i.e. liverworts, Marchantiophyta [9]), have so far revealed only symbioses with the most recently branched lineage of the Glomeromycota (i.e. Glomerales) or with even more recent lineages of Basidiomycota and Ascomycota [10]. Because the plant taxon sampling available in previous studies was severely limited and some of the plant–fungal symbiotic morphologies observed in the earliest branching lineage of liverworts (i.e. Haplomitriopsida [11]) are considered unusual [12,13], we investigated the associations of diverse 'lower' plants to test whether they are symbiotic with other ancient fungal lineages.

## 2. MATERIAL AND METHODS

### (a) *Specimens*

At each of 25 sites, we collected one to two colonies (less than 5 cm diameter) of each species of lower plant. Each collection was subsampled within a week by removing 5–10 thalli or stems. These were cleaned with forceps and rinsed in distilled water. Fungal fruitbodies were obtained from Oregon State University Herbarium. Plant vouchers are in the herbarium of the Natural History Museum. Plant nomenclature follows the latest scheme [14].

### (b) *Fungal detection*

Specimens were prepared for scanning and transmission electron microscopy as detailed elsewhere [12,13]. For molecular analysis, a healthy colonized apical 2–3 mm length of plant or a 1–2 mm inner fragment of fungal fruitbody was placed in 300 µl of lysis buffer, stored at –80°C, and subsequently used for a genomic DNA extraction as described elsewhere [15] with a purification step using GeneClean (QBioGene). Glomeromycota nrDNA primers [16,17] failed to amplify most samples, thus we used the fungal nuclear ribosomal small subunit NS1/EF3 primer set [18,19] for all samples, and the translation elongation factor EF1-983f/EF-2218r primer set [20] and nuclear ribosomal internal transcribed spacer+large subunit ITS1F/LR7 primer set [18,21] for representative non-Glomeromycota samples. These regions were amplified (JumpStart, Sigma), cloned (TOPO TA, Invitrogen) and four to eight clones sequenced (BigDye v. 3.1 on ABI3730 Genetic Analyzer, Applied Biosystems). Representative DNA sequences are deposited in GenBank (JF414136–JF414235).

### (c) *Phylogenetics*

Maximum-parsimony analyses were performed using PAUP\* v. 4.0b10 [22] for heuristic searches with 1000 replicates and tree-bisection-reconnection branch swapping holding 100 trees with multrees. Support was assessed via non-parametric bootstrapping using 1000 replicates. A source of uncertainty for the hypothesis introduced in this study is the placement of the Mucoromycotina and Glomeromycota. A multi-gene phylogeny of fungi indicates that the Mucoromycotina represents a deeper branch than the Glomeromycota [23]. While other analyses support this placement [24], some alternatives also have support [25,26]. To address this issue, we re-analysed the dataset from James *et al.* [23] by reducing the number of dikarya to 10 taxa each and increasing the Glomeromycota to six and the Mucoromycotina to nine taxa. Eucytrids were the outgroup. See the electronic supplementary material, table S3 for a list of taxa. Nucleotide datasets (18S rDNA, 26S rDNA, 5.8S rDNA) and protein datasets (*TEF*, *RPB1*, *RPB2*) were aligned with multiple sequence alignment based on fast Fourier transform [27] using the G-INS-i algorithm and ambiguous regions were excluded in GENEIOUS PRO [28] resulting in a combined dataset with 5229 positions. We applied an independent substitution model to each region. The 18S and 26S rDNA were fitted with GTR+I+G models, 5.8 S rDNA with GTR+G, and proteins with JTT+G. Phylogenies were estimated with maximum likelihood (ML) and Bayesian relaxed clock (BRC). The ML was performed with RAxML [29]. Non-parametric bootstrap was calculated by re-running 100 replicates; for

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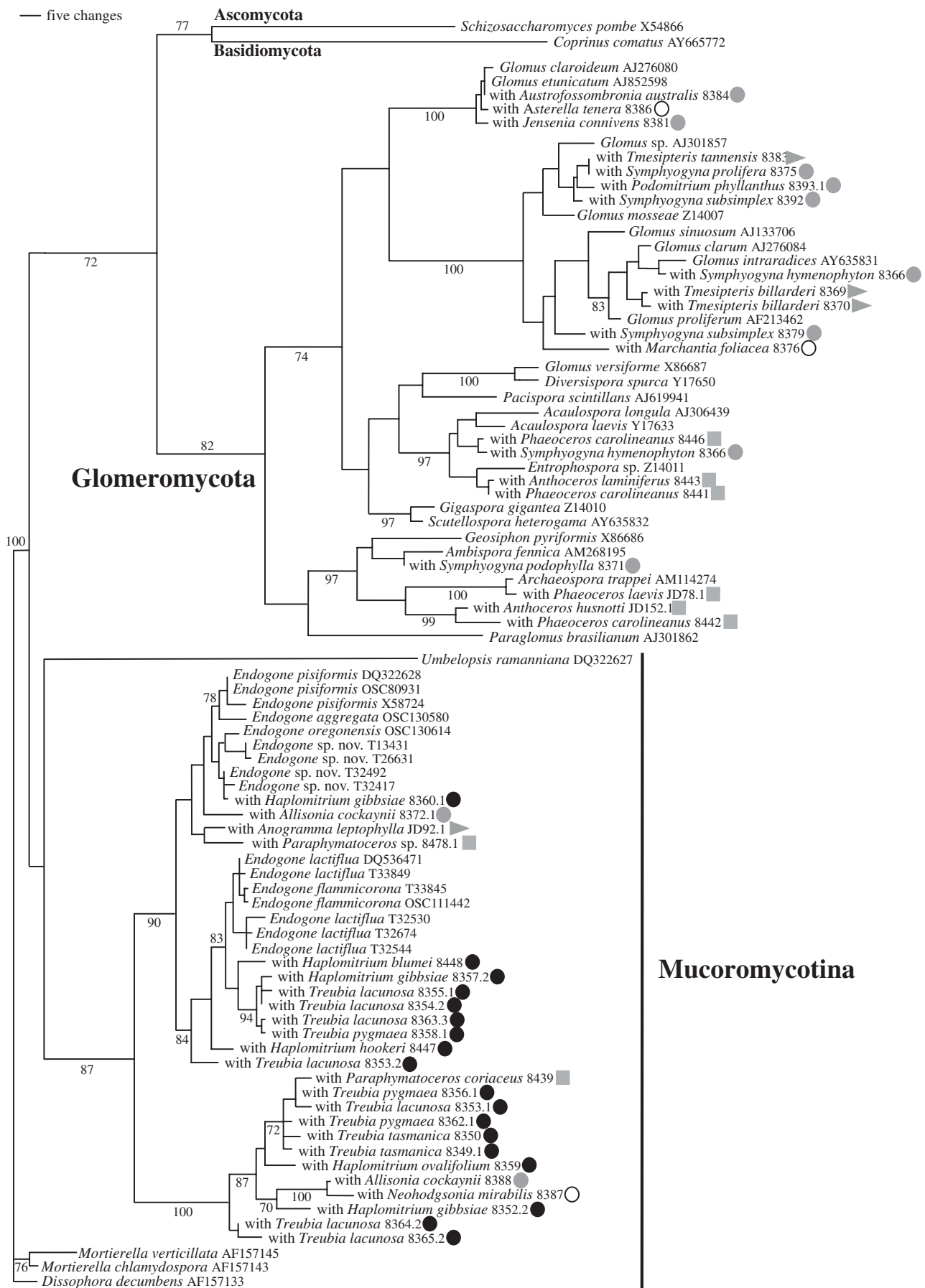


Figure 1. Phylogenetic placement of fungi detected in basal (black filled circles), simple (grey filled circles) and complex thalloid liverworts (open circles), hornworts (squares) and ferns (right-facing triangles) within the Glomeromycota and Mucoromycotina using a full-length fungal nuclear ribosomal small subunit gene maximum-parsimony tree.

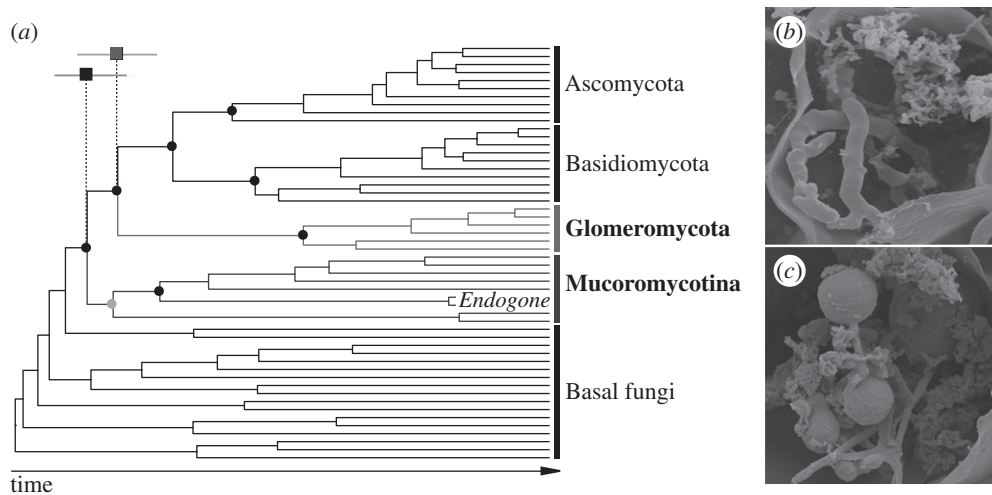


Figure 2. Topology, relative chronology and morphology of the fungal lineages detected. (a) Maximum clade credibility topology inferred using a Bayesian relaxed clock analysis of six genes. Black dots represent support for relevant lineages (greater than or equal to 90% bootstrap and a posterior probability of 1.0). The grey dot indicates 65% bootstrap and a posterior probability of 0.99. The 95% confidence intervals of the relative age estimates for lineage origins are shown. (b) Scanning electron micrograph of a Glomeromycota-containing plant cell. (c) A Mucoromycotina-containing plant cell. See the electronic supplementary material, table S1 for all the symbioses investigated.

performance reasons GTR+I+G was assigned to nucleotide and JTT+G to protein data (electronic supplementary material, figure S2). The BRC with BEAST [30] used an uncorrelated lognormal relaxed clock and Yule speciation. The root was fixed to an arbitrary ‘age’ of 1000. Two independent runs of  $1 \times 10^7$  generations converged to the same likelihood. Trees sampled during the last  $8 \times 10^6$  generations were combined for a maximum clade credibility tree (electronic supplementary material, figure S3).

### 3. RESULTS AND DISCUSSION

Findings from molecular ecology and electron microscopy reveal that at least six species of liverworts from the earliest diverging clade of land plants (Haplomitriopsida), one species of simple thalloid liverwort (Jungermanniopsida), one species of complex thalloid liverwort (Marchantiopsida), two species of hornwort and one species of fern associate with *Endogone*-like fungi (Mucoromycotina) rather than with the Glomeromycota (figure 1; electronic supplementary material, figure S1 and table S1). The plants sampled originate mainly from Gondwanan locations in Tasmania and New Zealand, but also from Malaysia and Europe. By contrast, a range of more derived thalloid liverworts, hornworts and ferns associate with all major groups of typical glomeromycotan fungi (figure 1 and electronic supplementary material, table S1).

Glomeromycotan fungi are intracellular and they form trunk hyphae (3–6  $\mu\text{m}$ ), fine arbuscules (0.8–1.5  $\mu\text{m}$ ) and vesicles (20–30  $\mu\text{m}$ ). Fungi in the Mucoromycotina form thick-walled spores in extracellular mucilage and their intracellular colonization is characterized by fine coils (0.8–1.5  $\mu\text{m}$ ) and thin-walled swellings (8–12  $\mu\text{m}$ ) (figure 2*b,c* and electronic supplementary material, table S2). Notably, the fungal symbiosis of *Treubia* (Haplomitriopsida) resembles most closely the distinctive inter- and extracellular colonization of the Devonian fossil plant *Nothia* [31].

A six-gene phylogenetic analysis provides support for the origin of Mucoromycotina predating that of Glomeromycota (figure 2*a*), so members of the Mucoromycotina may be better candidates for facilitating plant

terrestrialization during the Mid-Ordovician (475 Ma), concomitant with the divergence of liverworts [32]. The fossil data necessary to date accurately the diversification events in the fungi are currently lacking, which prevents a test of this hypothesis using absolute molecular clock data. Relaxed molecular clock analysis did not detect a relative age difference between the origin of the Glomeromycota and *Endogone*, but in the absence of internal calibration points and with limited taxon sampling for Endogonales such results must be treated with caution [33]. Even when a difference in relative age is taken into account, both fungal lineages may still predate the origin of land plants, as suggested for the Glomeromycota [34]. Nonetheless, the observation that *Endogone*-like fungi could have been the earliest endomycorrhizal fungi suggests the need to reconsider the process whereby plants were enabled to colonize the terrestrial environment, a key event in the history of life on the Earth.

The Endogonales are remarkably understudied fungi and are unrepresented or poorly included in the world’s fungal reference DNA sequence, culture and herbarium collections. As most other fungi, they lack a fossil record. Unlike Glomeromycota, some *Endogone* spp. are saprobic, culturable and sexual. Understanding their evolutionary and ecological significance is complicated because intracellular colonization by Mucoromycotina in lower plants is largely indistinguishable by light microscopy from that produced by glomeromycotan fungi in *Paris*-type endomycorrhizas. Because the DNA of fungi in the Mucoromycotina is not detected by Glomeromycota-specific molecular approaches, common and widespread intracellular symbioses involving plants and Mucoromycotina are likely to have gone routinely undetected until now.

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