Mycorrhizal symbiosis with Mucoromycotina facilitated the terrestrialisation of land plants

# Authors

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# Abstract

The mycorrhizal symbiosis between soil fungi and land plants is one of the most widespread and ecologically important mutualisms on earth. It has long been hypothesized that the Glomeromycota, the mycorrhizal symbionts of the majority of plants, facilitated colonization of the terrestrial environment by plants in the Ordovician. This view was recently challenged by the discovery of mycorrhizal associations with Mucoromycotina in several early diverging lineages of land plants, suggesting involvement of these fungi in the terrestrialisation process. However, competing, plausible hypotheses for the order of divergence of the land plants result in different reconstructed scenarios for the build up of repertoires of mycorrhizal symbiosis. Utilizing a large species-level database of mycorrhizal associations and a Bayesian approach to state transition dynamics we here show that the recruitment of Mucoromycotina is the primary transition from the initial state of no mycorrhizal association. We further found that transitions between different combinations of either or both of Mucoromycotina and Glomeromycota occur at high rates and found similar promiscuity among combinations that include either or both of Glomeromycota and Ascomycota with a nearly fixed association with Basidiomycota: gains and losses of association with the latter occur at exceedingly low rates. Our results demonstrate that the most likely scenario whereby symbiosis between land plants and mycorrhizae was built up involved terrestrialisation mediated by Mucoromycotina.

Land plants diverged from aquatic algae in the Neoproterozoic1 as a lineage that would eventually undergo the ecological transition to terrestrial life. This transition altered the global climate and the biosphere through biotic chemical weathering of rocks, carbon fixation, and an increase in atmospheric oxygen levels2,3. Terrestrial life requires plants to extract nutrients and moisture from the substrate while photosynthesizing organs remain above the surface. As the evolution of roots lagged behind the transfer to the terrestrial environment4, these could not have facilitated the first stages of terrestrialisation. A likely early adaptation to overcome this challenge would have been symbiosis with a fungal partner5,6. In these mycorrhizal associations, the fungal partner can absorb moisture and nutrients with long hyphal structures and pass these on to the plant in return for sugars produced by photosynthesis in the leaves.

Mycorrhizal symbiosis is found in over 90% of extant land plant families7, which form associations with members of four different fungal phyla: Glomeromycota, Basidiomycota, Ascomycota and Zygomycota (the latter of which represented by Mucoromycotina8,9). The majority of land plants associate with arbuscular mycorrhizal fungi from the phylum Glomeromycota, while other types of mycorrhizal associations, such as ectomycorrhiza, ericoid mycorrhiza and orchid mycorrhiza, belong to the Basidiomycota or Ascomycota8. The Glomeromycota are a fungal phylum of mostly obligate mycorrhizal fungi10. Fossil evidence suggests that this group has coevolved with land plants for at least 407 Myr, as vesicles, spores, intracellular coils, and arbuscule-like structures resembling extant mycorrhizal infections were found in fossils of *Horneophyton* *lignierin*11 in the Rhynie chert deposits. Further support for ancient origin of these interactions comes from genomics, as genes involved in the formation of arbuscular mycorrhizal infections are homologues and were acquired in a stepwise manner, with potentiation starting as early as the last common ancestor of Charophytes and Embryophytes12–14.

Within Ascomycota and Basidiomycota, inferring the origin of the mycorrhizal habit is complicated by the many independent origins of a wide variety of nutritional modes, such as saprophytes, parasites, insect symbionts, pathogens, mycorrhiza and lichens15–18. In Basidiomycota, mycorrhizal habit is found in 11-15 out of 20 Agaricomycete orders15, including the two most basal lineages Sebacinales and Cantharellales. Whether or not mycorrhizal habit in the approximately 246 Myr old1 Agaricomycete clade was ancestral or has multiple independent origins is a topic of discussion17–23. Within Ascomycota, over 40% of species are lichenized16,24, and mycorrhizal habit occurs in fewer genera than in Basidiomycota25. However, the age estimate for the mycorrhizal genus *Tuber* alone is between 271 and 140 Ma26. Potential hosts for mycorrhizal members of Ascomycota are found in liverworts (Metzgeridae and Jungermanniales), gymnosperms (Pinales), and several flowering plant lineages (Orchidaceae and several clades of Eudicots)7,27. Amongst these, the oldest potential hosts are members of the liverworts, in a clade whose age is estimated between 243 and 393 Mya28–30, thus suggesting that the onset of the mycorrhizal habit in Ascomycota and Basidiomycota was around this time27,31–36. However, the oldest fossil evidence consists of relatively young mycorrhizal root fossils dating 50 Mya37.

The preponderance of the evidence till recently has led to wide acceptance of the view that Glomeromycota were the ancestral mycorrhizal symbionts of land plants38,39. The ancestral symbiosis was assumed to have been replaced in several plant lineages by other types of mycorrhizal associations in multiple independent shifts7. However, the recent discovery that many members of early diverging lineages of land plants, including liverworts, hornworts, and basal vascular plants, engage in mycorrhizal symbiosis with Mucoromycotina (Zygomycota), challenged this notion and suggest that Mucoromycotina could also have facilitated terrestrialisation9,38,40,41. Subsequent to this discovery, Rhynie chert fossils where re-evaluated, revealing mycorrhizal infections resembling both Glomeromycota and Mucoromycotina11. Furthermore, it is unknown whether the molecular pathways involved in the formation of mycorrhizal infections with Mucoromycotina are the same as those involved in the symbiosis with Glomeromycota.

Given that the four fungal phyla diverged prior to the divergence of land plants1, it is possible to treat mycorrhizal association with these phyla in different combinations as character states on the plant phylogeny and analyse transition dynamics between these states in a Bayesian phylogenetic comparative context. Database description. Ancestral state reconstruction-Rutger Taking into account the uncertainty of the evolutionary relationships of early embryophytes42,43, we assessed the probability of all possible combinations of mycorrhizal associations for the most recent common ancestor of land plants.

# Results

Ancestral state reconstruction indicates that the most recent common ancestor of all Embryophyta was mycorrhizal (Fig. 3). The different hypotheses for the evolution of plants42,43 had very little effect on the results. The probability of a non-mycorrhizal ancestral state was at most 0.011 (Fig. 3). The most likely state of mycorrhizal association was an association with Glomeromycota only, with a probability between 0.44 and 0.62, depending on the topology of the land plant phylogeny. The probability that the last common ancestor was associated with Mucoromycotina as well as Glomeromycota varied between 0.24 and 0.27. After these two most likely states, highest probabilities were assigned to Glomeromycota in combination with Ascomycota, Basidiomycota, or both Basidiomycota and Mucoromycotina (in order of probability), but all of these three states had a probability lower than 0.095 (Fig. 3). The Glomeromycota were the most likely associates in all nodes older than 300 Myr (Fig. 4) and Mucoromycotina and Glomeromycota as a combined state was the second most likely state in these nodes.

The oldest switches to Ascomycota or Basidiomycota were found in liverworts (Fig. 2; Fig. 5; Fig. 6). A shift between Glomeromycota and Basidiomycota happened along the branches leading to Metzgeriidae and Jungermanniidae, and could have involved two independent shifts or one shift (Fig. 5). The first node associated with Basidiomycota with a probability higher than 0.6 has age estimate of around 200 Million years (Fig. 5; Fig. 6). Other independent switches to Ascomycota and Basidiomycota are found in Pinales, Orchidaceae, Ericales, Malphigiales and Fagales, but more independent shifts to Ascomycota and Basidiomycota associations can be found (Fig. 2). The long branch leading to Pinales, makes it difficult to precisely estimate the time of the switch to the ectomycorrhizal Ascomycota and Basidiomycota in this group.

# Discussion

Ancestral state reconstruction supports the hypothesis that the terrestrialization of land by plants was facilitated by symbiotic interactions with fungi. This result is in accordance with evidence from the fossil record11 and genomics12–14. The recent discovery of mycorrhizal associations with Mucoromycotina in many early diverging lineages of Embryophyta38,40,41 is reflected by a substantial probability of an association with Mucoromycotina in the most basal nodes, but this state is always found in combination with Glomeromycota and the probability of a state with only Glomeromycota is still highest for these nodes (Figure 3). Around 407 Mya, Glomeromycota and Mucoromycotina were associated with *Horneophyton ligneri*11, suggesting that our current model underestimates the presence of Mucoromycotina in the older nodes. Future sequencing for Mucoromycotina would be the way find out how widespread these previously overlooked fungi are and help to resolve the question concerning their role in terrestrialization.

Evidence for mycorrhizal habit found in our ancestral state reconstruction predates evidence from the fossil record by 46-112 million years, depending on the phylogenetic hypothesis for early land plants. However, no conflict is expected for Glomeromycota fungal ecology, as most members of this phylum are obligate mycorrhizal symbionts23. For Mucoromycotina more research will be needed on the history of mycorrhizal interactions. For Ascomycete and Basidiomycete associations, the ecological history is a challenging subject16–19,48. The switch to Basidiomycota associations in liverworts was likely to have happened between 300 and 200 Mya while age estimates for the oldest Ascomycete association vary between 190 and 210 Mya (Figure 5). Many mycorrhizal associations in Ascomycota are found within Pezizomycetes. Age estimates for this group predate the age of the first associations1,25. The age estimate for the mycorrhizal genus *Tuber* alone is 271 to 140 Ma26, suggesting that Ascomycota associations could have been present in Embryophyta lineages before the reconstructed associations. For Basidiomycota, the age estimates for the first mycorrhizal associations coincide with the radiation of Agaricomycetes in the Triassic1.

This result challenges the notion that the last common ancestor of Agaricomycetes was a saprophyte and that the mycorrhizal habit evolved independently in many Agaricomycete orders17,18. On the other hand, there is a possibility of extinct mycorrhizal lineages co-occurring with the ancestor of Agaricomycetes. Habitat preferences same in liverworts-leading to similar mycorrhiza. These conflicting results could also be caused by overestimation of the age of liverworts. However, an extensive study on divergence dates in liverworts, based on nine liverwort fossils reveals even older estimates: 361 Mya for the divergence of Metzgeriidae and Jungermanniales28. Other estimates for this split are 272 Mya (CI: 243–310)29 and 308.7 ± 7.8 Mya30, suggesting that the age of the first Basidiomycota associations in this study might even be conservative. Further support for the reliability of the ancestral state reconstruction is found in the high degree of host specificity between liverworts and their Basidiomycota associates27 and the fact that this very basal embryophyte lineage is associated with the two most basal lineages of Agaricomycetes: *Sebacina* and *Tulasnella*15. Nevertheless, a mycorrhizal ancestral state in Agaricomycetes does not disagree with the expansion of lignin peroxidases in the branches leading to Auriculariales19, as the first divergence events in Agaricomycetes are older than the acquisition of lignin decomposing enzymes18,19. In order to resolve the question whether or not the Mycorrhizal habit in Agaricomycetes existed around the divergence time of Metzgeriidae and Jungermanniales, further research genomic research on the evolution of these enzymes and the genes involved in the mycorrhizal symbiosis is needed. Combined with evidence from the fossil record, this could help to understand the evolution of the mycorrhizal habit in this group of fungi.

Tracing the evolution of mycorrhizal associations on the plant phylogeny provides us with the probability of the ancestral symbionts, and provide evidence for the time and taxa in which shifts to other types of association occurred. Further applications of the model could include mycorrhizal types, independent rates for different transitions. Futhermore, using this model we could look for correlation of plant traits

The main reason for the absence of an effect of tree topology is the length of the branches leading to crown Bryophyta and crown Anthocerotophyta. Although the most basal splits of these groups are included in the phylogeny (*Takakia* and *Sphagnum* in Bryophyta and *Leiosporoceros* in Anthocerotophyta), the branches leading to the crown nodes of these groups are much longer than those leading to Marchantiophyta and Tracheophyta. The result of ancestral state thus depends more strongly on the latter two groups.

# Material & Method

*Data collection*

Through literature study, we compiled a database of 804 plant species distributed over 86 plant orders and their mycorrhizal status. The database includes 25 hornworts, 7 mosses, 100 liverworts, 543 angiosperms, 79 gymnosperms, 21 lycopods, and 29 polypods species. For these plants species, we found associations with 144 Ascomycota, 306 Basidiomycota, 450 Glomeromycota, and 27 Mucoromycotina (Table S1). We downloaded DNA sequences for host plants from Genbank via Geneious version 8.1.649. For liverworts, hornworts, polypods and lycopods we added a number of additional species to the dataset to reduce the number of missing markers per group, resulting in a total of 825 species for phylogenetic analysis. For 129 species, full or partial chloroplast genomes were available, which we used to extract sequences for *psbA*, *rbcL* and *rps4*. Full or partial *rbcL* sequences were also available for all the other plant species, and we downloaded any available sequences for the other two markers. Accession numbers are listed in the supplementary data (Table S2).

*Phylogenetic analysis and divergence dating*

For each marker, we aligned the sequences with MAFFT v.750 using the FFT-NS-i Iterative refinement method, and then selected the optimal partitioning scheme with Partitionfinder 251,52. Except for the first and second codon position of *psbA*, which were placed in a single partition, all codon positions of the three markers were assigned to separate partitions. The GTR+I+γ model was selected for all partitions using the AICc. We conducted a search for the best tree with multiparametric bootstrap supports using RAxML53. Bootstrapping was stopped automatically after reaching the extended majority rule criterium (MRE). We performed divergence dating with BEAST2 v2.3.254 on a reduced molecular dataset containing the first and second codon positions of *rbcL*, using the maximum likelihood tree as a fixed tree topology and using six fossil calibration points (Table S3). We selected aso as on the agethe - we modifiedWe chose aran To test the effect of different phylogenetic hypotheses42,43,55 for the deep-time relationships of land plants on ancestral state reconstruction, we rooted the likelihood tree according to the different hypotheses and applied our divergence dating protocol to each rooted topology (Fig).

Log Combiner was used to resample the trees every … generations with a burnin of … percent, to a total of … trees. The maximum clade credibility tree was constructed with Tree annotator v2.2.156.

ATxMB burnin 5%; 14251 trees processed. Mean heights

*Comparative analysis and hypothesis tests*

The four major fungal groups to which species belong that participate in various types of mycorrhizal associations were already in existence prior to the diversification of land plants. Therefore, we could treat each distinct repertoire of associations that land plants form with these groups as a discrete state, whose evolutionary transition dynamics we modelled subsequent to two assumptions.

First, because there are qualitative differences between the types of mycorrhizal associations that are formed with some of the different fungal groups (e.g. intracellular versus ectomycorrhizal association), we assumed that the evolutionary adaptations required to enable such associations are not gained (or lost) instantaneously. Hence, we disallowed state shifts that implied multiple, simultaneous gains and losses such that, for example, a change from a state representing a repertoire confined to Glomeromycota to one confined to Mucoromycotina has to pass through an intermediate state where the repertoire is broadened to include both groups. Second, because the respective adaptations that enable different types of mycorrhizal association are likely subject to evolutionary trade-offs such that repertoires of associations cannot expand infinitely we limited any intermediate states to those we observe in nature. For example, simultaneous association with both Glomeromycota and Mucoromycotina does occur in our database of extant taxa, but complete generalism that includes all fungal groups in a single repertoire does not, which is why we allowed the former, but not the latter, as possible ancestral states.

A convenient side effect of these assumptions was that this limited the number of free parameters in the state transition (*Q*) matrix, which otherwise would have undergone a combinatorial explosion had we included all possible permutations in the repertoires of mycorrhizal association as distinct states, which would have impeded convergence in our analyses. To mitigate such proliferation of potentially unneeded, free parameters further, we performed our analyses using Reversible-Jump MCMC, as implemented in BayesTraits’s ‘multistate’ analysis mode. We ran each of our analyses in triplicate for 106 generations, as initial experimentation had demonstrated reasonable convergence in our data under these settings. In cases where we required estimates of marginal likelihoods, i.e. for hypothesis testing by Bayes factor analysis, we approximated these using a stepping stone sampler that we ran for 100 stones, with 200,000 iterations per stone.

Using this approach, we reconstructed the ancestral states for the four different rootings of our phylogeny. However, although such analyses result in estimates for the posterior distribution of states at any given hypothetical node of interest (such as the root), they do not necessarily result in a single, unambiguous scenario for the order in which mycorrhizal associations are acquired, especially not when multiple states are reconstructed with similarly large posterior probabilities at deep nodes (as was the case). Given the number of fungal groups and the differences and similarities among these with respect to the types of mycorrhizal associations they participate in, we expected there to be distinct paths along which repertoires of association have evolved. Interrogation and visualisation of the *Q* matrix showed that, broadly, two such paths appear to exist: one where various permutations of association with Glomeromycota and Mucoromycotina are gained and lost, and another that traverses Ascomycota, Basidiomycota in addition to Glomeromycota. However, which of these paths was taken first was not yet evident.

We therefore constructed explicit hypothesis tests to distinguish between various plausible scenarios. To do so, in addition to the assumptions affecting the *Q* matrix outlined above, we further constrained our analyses to require the absence of any mycorrhizal association on the root node, and then tested which initial gain was best supported by the data. To quantify this, we estimated the marginal likelihood of the model where the root is constrained to have no association but without any additional constraints on the order in which subsequent associations are acquired (beyond the general assumptions already discussed), and compared this with models where, respectively, each of the initial gains of a single fungal group is disallowed. The logic here is that disallowing the initial shift that best fits the data will result in the marginal likelihood that differs most significantly from the less-constrained model.

Lastly, to place the expansion of repertoires of mycorrhizal association on a temporal axis, we placed the ancestral state reconstructions for the scenario where the root node has no mycorrhizal association in bins of 50 Myr to visualise these in a states-through-time plot.

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# Author contributions