A range-oriented model for ancestral state reconstruction on the embryophyte phylogeny provides a time scale for the evolution of mycorrhizal associations

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A Range-Oriented Model for Ancestral State Reconstruction on the Embryophyte Phylogeny Provides a Time Scale for the Evolution of Mycorrhizal Associations

Abstract

The mycorrhizal symbiosis between soil fungi and the vast majority of land plants is one of the most widespread and ecologically important mutualisms on Earth. This ancient interaction is hypothesized to have facilitated colonization of the terrestrial environment by plants. It has long been assumed that the Glomeromycota, the mycorrhizal symbionts of the majority of terrestrial plants, were the fungal partners of the first terrestrial plants. These Glomeromycota associations were later replaced by mycorrhizal fungi from the phyla Ascomycota and Basidiomycota in several plant lineages. In a few cases, the ability to associate with mycorrhizal fungi was lost entirely. This view was recently challenged by the discovery of mycorrhizal associations with Mucoromycotina (Zygomycota) in many basal lineages of the plant phylogeny, a finding which suggests that these symbionts could also have been involved in the terrestrialization process.

Here, we use a maximum likelihood model for ancestral state reconstruction of mycorrhizal symbionts from the fungal phyla Ascomycota, Basidiomycota, Glomeromycota and Zygomycota on the plant phylogeny to infer the most likely ancestral mycorrhizal symbionts of land plants. To account for the uncertainty in the phylogeny of early land plants, ancestral states were reconstructed using different phylogenetic hypotheses.

The results indicate that the most recent common ancestor of all land plants was associated with Glomeromycota, possibly in a shared state with Mucoromycotina, while the probability of a non-mycorrhizal ancestor is low. Associations with Ascomycota and Basidiomycota evolved in multiple independent instances in land plants and estimated ages for the oldest Basidiomycota associations in liverworts support an early origin of mycorrhizal ecology in Agaricomycetes.

Keywords: *Plants, Mycorrhiza, terrestrialization, Ancestral state reconstruction, Mucoromycotina, Glomeromycota, Basidiomycota, Ascomycota*

INTRODUCTION

Challenges facing Terrestrial Photoautotrophs

The origin of terrestrial plants (Embryophyta) can be traced back to a clade of aquatic Streptophyte algae. Embryophytes are estimated to have diverged from other Streptophyte lineages around 717 Mya (Hedges et al. 2015), to comprise a lineage which would eventually undergo a major ecological transition to terrestrial life. This was not the only and probably not the first transition of photoautotrophic organisms to the terrestrial environments, as terrestrial colonization by cyanobacteria and green algae could have preceded the terrestrialization of embryophytes (Lipnicki 2015; Selosse and Strullu-Derrien 2015). The embryophytes, however, gave rise to the life form dominating our present terrestrial environments. The effect of Embryophyte terrestrialization is anticipated to have been tremendous: carbon could be fixed in unpreceded amounts, while weathering of rocky substrates affected biochemical cycles. This contributed to changes in both atmospheric carbon and oxygen levels which influenced climate and the biosphere (Lenton et al. 2012; Selosse et al. 2015).

Amongst the difficulties photoautotrophs had to overcome in terrestrial environments, were the acquisition of nutrients and moisture from the substrate, while photosynthesizing organs had to remain above the surface (Selosse and Strullu-Derrien 2015). Photoautotrophs could reside to two strategies to respond to desiccation: poïkilohydric or homoiohydric lifestyles (Selosse and Strullu-Derrien 2015). The homoiohydric lifestyle, which is found in the majority of extant embryophytes, often requires a network in the ground to absorb moisture and nutrients. However, the evolution of true roots in Embryophyta lags behind the transfer to the terrestrial environment (Brundrett 2002) and is therefore unlikely to have facilitated the first stages of terrestrialization.

A more plausible scenario can be found in the observation that many terrestrial photoautotrophs engage in a symbiosis withfungal partners. Over 90% of embryophyte families engage in mycorrhizal symbioses (Wang and Qiu 2006), while cyanobacteria and green algae are frequently found in lichenized associations (Lipnicki 2015). The fungal component of the symbiosis, with long hyphal structures, can transfer moisture and nutrients to the photoautotrophic associate,

which provides carbon that has been assimilated in photosynthesis in return (Selosse and Strullu-Derrien 2015).

Indeed, the oldest fossil evidence for mycorrhizal associations between fungi and Embryophyta dates back around 407 Myr. Vesicles, spores, intracellular coils and arbuscule-like structures resembling those of extant mycorrhizal infections where found in the fossils of *Horneophyton lignierin* (Strullu-Derrien et al. 2014). The oldest putative fossil lichens are also from the Lower Devonian: *Winfrenatia*, *Spongiophyton* and *Prototaxites* (Taylor et al. 1997, 2004; Selosse and Strullu-Derrien 2015), but 600 Mya lichen-like fossils from South China suggest that the symbiosis between Fungi and photoautotrophs is much older (Yuan 2005). The first embryophyte fossils are from the Silurian, ca. 470 Ma (Selosse et al. 2015), but these concern spores and debris, making it impossible to find evidence for symbiotic associations around this period.

Even though the oldest Embryophyta fossil predates the oldest fossil evidence of symbiotic interactions, different lines of evidence for a key role of mycorrhiza in terrestrialization are gathering fast. The wide distribution of mycorrhizal associations in extant embryophytes raises the notion that the last common ancestor of Embryophyta was also likely to have been associated with mycorrhizal fungi (Wang and Qiu 2006). This hypothesis finds further support in genomic research, which has shown that genes involved in the formation of arbuscular mycorrhizal infections are present in all major groups of terrestrial plants (Karandashov et al. 2004; Wang et al. 2010) and that genes involved in the mycorrhizal pathway where acquired in a stepwise manner starting as early as the last common ancestor of Chlorophytes and Streptophytes (Delaux et al. 2015). The onset of the pathways for symbiotic interaction was thus likely to predate terrestrialization.

Terrestrial Symbioses from a Fungal Perspective

Inferring the evolutionary history of the symbiotic habit in fungi is a very complex task due to the wide variety of ecological niches these organisms cover, for example as saprophytes, pathogens, mycorrhiza, and lichens (Schoch et al. 2009; Hibbett et al. 2014). One exception can be found in the Glomeromycota, a monophyletic fungal clade of which most members are obligate arbuscular

mycorrhizal fungi (Brundrett 2002). Glomeromycota have coevolved with embryophytes at least since the Early Devonian (Strullu-Derrien et al. 2014) and the great majority of extant plants associate with this clade of arbuscular mycorrhizal fungi (Wang and Qiu 2006). This led to the notion that the Glomeromycota were the ancestral mycorrhizal symbionts of embryophytes. However, recent findings have questioned this hypothesis. The fungal associates of several lineages of liverworts, hornworts and basal vascular plants were found to belong to Mucoromycotina, a fungal clade belonging to the phylum Zygomycota (Bidartondo et al. 2011; Desirò et al. 2013; Rimington et al. 2014). In addition, Rhynie Chert fossils of *Horneophyton lignierin* harbour mycorrhizal structures resembling both Glomeromycota and Mucoromycotina (Strullu-Derrien et al. 2014). These results suggest that Mucoromycotina could have played a much larger role in the process of terrestrialization than previously assumed.

The ancestral symbiotic associations in land plants are assumed to have been replaced in several plant lineages by other types of mycorrhizal associations, such as ectomycorrhiza, ericoid mycorrhiza and orchid mycorrhiza, in multiple independent shifts (Wang and Qiu 2006). These associations involve mycorrhizal fungi from the phyla Basidiomycota and Ascomycota. Within Basidiomycota, the mycorrhizal habit is found in the Agaricomycetes, a clade of fungi in which a wide variety of ecological roles can be found, such as saprotrophs, pathogens, parasites, insect symbionts, lichens and mycorrhiza (Hibbett and Matheny 2009; Hibbett et al. 2014). For the study of the evolution of mycorrhizal interactions, it is important to know when and in which clades the mycorrhizal habit was gained or lost. This is still a subject of debate, as results from various ancestral state studies are contradictory, supporting either the theory of a mycorrhizal ancestor of all Agaricomycetes with many reversals to free living conditions, or the theory of ancestral saprophytes with many independent origins of mycorrhizal habit (Hibbett and Matheny 2009; Floudas et al. 2012).

Hibbett & Matheny (2009) used a new approach to resolve the problem by using a relative age principle. They showed that the ancestral state in Agaricomycetes was unlikely to have been ectomycorrhizal, as the estimated ages of potential hosts were considerably younger than the age of

the Agaricomycetes. However, the associations of the basal Agaricomycete lineages *Tulasnella* (Cantharellales) and *Sebacina* (Sebacinales) in liverworts were not taken into account. These associations occur in Jungermanniopsida and Metzgeriopsida (Kottke et al. 2003; Bidartondo and Duckett 2010): a group of liverworts for which divergence time estimates range from 272 Mya (CI: 243–310) to 361 Mya (CI: 322–393) (Cooper et al. 2012). These age estimates suggest that there might have been a much older potential host co-occurring at the time of divergence of Agaricomycetes, around 290 Mya (95% HPD: 222-372Mya) (Floudas et al. 2012; Kohler et al. 2015).

More support for a saprophytic ancestral state in Agaricomycetes comes from genomic research (Floudas et al. 2012), in which ancestral state reconstruction points to a white-rot ancestral mode, coinciding with an expansion of lignin peroxidases and a drop in carbon burial rates at the end of the carboniferous. A drawback of the study is the number of included taxa; a total of 17 Agaricomycete species is not representative for such an ecologically diverse family. Only one mycorrhizal taxon was included, while at least 13 out of 20 Agaricomycete orders contain ectomycorrhizal species (Hibbett et al. 2014). This study did not include the most basal lineages in Agaricomycetes: Cantharellales and Sebacinales. Another factor that has to be taken into account is that many lignin degrading enzymes could have a broader use than carbon acquisition alone (Sinsabaugh and Follstad Shah 2012).

A more recent study (Kohler et al. 2015) shows that enzymes involved in lignin decay originated in the branch leading to Auriculariales. Indeed, these enzymes are absent from Cantharellales and Sebacinales, two orders which both contain mycorrhizal fungi which are associated with liverworts. In liverworts, Jungermanniales species associate with Sebacinales (Kottke et al. 2003; Pressel et al. 2010) and are host specific, while Metzgeriales species associate with Cantharellales and occasionally Sebacinales (Kottke et al. 2003; Pressel et al. 2010; Krause et al. 2011). Given that the divergence time of Cantharellales and other Agaricomycetes, including Sebacinales, is around 290 Ma (Floudas et al. 2012; Kohler et al. 2015) and that the divergence estimates for the hosts

Jungermanniales and Metzgeriales ranges between 243 Ma and 393 Ma (Cooper et al. 2012), this could suggest early coevolution of liverworts and mycorrhizal Agaricomycetes.

The fossil record concerning the ecology of Agaricomycetes points toward an early white-rot mode in the Agaricomycetes. The oldest white-rot fossil dates back 260 Mya (Stubblefield and Taylor 1986; Floudas et al. 2012), which is in accordance with the origin of lignin degrading enzymes in Auriculariales (Kohler et al. 2015). Until recently, the oldest known ectomycorrhizal root fossils were 50 Mya (Lepage et al. 1997), but the discovery of putative ectomycorrhizal structures in bennettitalean roots in upper Triassic Pteridiosperm fossils from permineralized peat from Hopen Island, Svalbard, suggests that ectomycorrhiza could have been present around 225 Mya (Strullu-derrien, unpublished data, 2015 – colloquium, needs verification). Of course, theses fossils do not have to concern Basidiomycota, as ectomycorrhizal fungi are also found in Ascomycota (van der Heijden et al. 2015) and *Endogone lactiflua* (Mucuromycotina) was also found to form ectomycorrhizal structures on *Pinus contorta* (Walker 1985).

A comparable problem of uncertain ancestral ecology is found for the phylum Ascomycota (Schoch et al. 2009), though the concerning symbiosis is the lichen symbiosis rather than the mycorrhizal symbiosis, as over 40% of described Ascomycota species are lichenized (Ekman et al. 2008; Schoch et al. 2009). Support for an ancestral saprobe ecology as well as an ancestral lichenized ecology has been derived from ancestral state reconstructions (Lutzoni et al. 2001; Schoch et al. 2009).

Inferring Ancestral Symbiotic Associations

Tracing mycorrhizal associations as character states on the embryophyte phylogeny is a useful tool to study the evolution of mycorrhizal plant-fungi associations. But a comprehensive ancestral state reconstruction of mycorrhizal associations from the four fungal phyla, which are known to contain mycorrhizal species, on the embryophyte phylogeny is lacking. This is probably due to the uncertainty of the evolutionary relationships of early embryophytes (Cox et al. 2014; Wickett et al. 2014) as well as the absence of an appropriate model for the reconstruction of ancestral mycorrhizal states.

A good phylogenetic hypothesis is key to the understanding the evolutionary changes in the embryophyte lineages. Unfortunately, numerous conflicting phylogenies exist regarding the deep relationships in the embryophyte tree of life. Even with the most recent advances in phylogenomic research, the earliest branching events still remain unresolved (Cox et al. 2014; Wickett et al. 2014). The Marchantiophyta Basal (MB) topology, in which liverworts (Marchantiophyta) split off in the first branching event, followed by mosses (Bryophyta) and in which hornworts (Anthocerotophyta) are sister to vascular plants (Tracheophyta) is commonly used (Clarke et al. 2011; Cox et al. 2014). This view on the phylogeny is changing, as a monophyletic group with liverworts and mosses gains more support (Cox et al. 2014; Wickett et al. 2014). In these recent analyses, the placement of hornworts remains uncertain, sometimes resolved as the most basal split (Anthocerotophyta Basal / AB hypothesis), sister to vascular plants (2x2 hypothesis) or as sister to the clade compromised by liverworts and mosses and thus leaving vascular plants as the most basal split in embryophytes (Tracheophyte Basal / TB hypothesis). In the present study, the problem was resolved by testing the ancestral state reconstruction on the different hypotheses for the phylogenetic relationships of early land plants.

The second problem, regarding the absence of an appropriate model was resolved by constructing a maximum likelihood model for ancestral state reconstruction, comparable with the dispersal-extinction-cladogenesis (DEC) model for geographic range evolution (Ree and Smith 2008), in which dispersal and extinction rates are free parameters. In the model, mycorrhizal associations are interpreted as discrete ranges, as in theory a plant species can be associated with mycorrhizal fungi from one up to four different fungal phyla (Zygomycota, Glomeromycota, Ascomycota and Basidiomycota). A new mycorrhizal association is coded as a dispersal event and the loss of an association is coded as an extinction event.

Plants can also be non-mycorrhizal, a state in which none of these four fungal phyla are included in the range. In contrast with the DEC model, in which a null range implies the extinction of a species, the null range was utilized to represent non-mycorrhizal species. The null range therefore

forms a valid state, from which dispersal to a mycorrhizal state is allowed. Another assumption from biogeography is that the chance of colonizing a new area partly depends on the size of the previously occupied range. This does not have to be the case for mycorrhizal associations, even though low specificity might facilitate dispersal to new mycorrhizal associates.

These prior assumptions lead to a new instantaneous rate matrix for maximum likelihood inference of mycorrhizal associations (Fig. 1), with the null range (Ø) and the fungal clades Ascomycota (A), Basidiomycota (B), Glomeromycota (G), Mucoromycotina (M) and their combined states as possible ranges. All dispersal events have been given an equal probability in this matrix, although shifts between structurally or evolutionary more distant associations are expected to be less likely. Simultaneous events have been excluded, assuming that the loss of gain of a mycorrhizal association does not occur simultaneously with the loss or gain of another association. Finally, unlike in the Biogeographic models, equal range inherence is assumed for both descendants at a speciation event.

To reconstruct ancestral symbiotic relationships, a number of requirements need to be met. Most important is the relative age of the symbionts. A fungal association cannot be reconstructed as ancestral state on the plant phylogeny if the concerning fungal clade is younger than the reconstructed association. This can be resolved by using a stratified approach as used by Ree and Smith (2008), but also by choosing a level of fungal taxonomy of which the divergence time predates the Embryophyta crown group. A second, very important, aspect is the ecology in the fungal clades, as discussed before. A fungal association cannot be reconstructed as mycorrhizal on the embryophyte phylogeny if mycorrhizal ecology did not exist in the concerning fungal clade at that time.

By using the range-oriented model for ancestral symbiotic associations, a new line of evidence is provided to answer the question if mycorrhizal fungi were indeed important in the terrestrialization process and which mycorrhiza were the most likely ancestral associates of Embryophyta: Glomeromycota, Mucoromycotina or both. A second question concerns the estimated time of the first

associations with Ascomycetes and Basidiomycetes, and whether these results agree with current hypotheses regarding the history of fungal ecology within these phyla.

MATERIAL AND METHODS

Database of Host Fungus Associations

A database of fungal sequences with host references was compiled from GenBank (Benson 2004) with Geneious version 8.1.6 (Kearse et al. 2012). Sequences were retrieved by searching for the terms 'Ascomycota', 'Basidiomycota', 'Glomeromycota' and 'Mucuromycotina' in combination with terms 'mycorrhiza', 'mycorrhizal' and 'mycorrhizae'. Sequences were then filtered on those which had a host reference. The database was further extended with data from the MAARJAM database (Öpik et al. 2010). Additional data was extracted from several publications (Kottke et al. 2003; Bidartondo and Duckett 2010; Bidartondo et al. 2011; Krause et al. 2011; Desirò et al. 2013; Rimington et al. 2014).

For all plant hosts in the database, a Genbank query with the R package SeqinR (Charif et al. 2015) was done to determine which plant species had *rbcL* sequence data available in GenBank. This reduced the size of the database to only those plants which could be included in the plant phylogeny and would be used for ancestral state reconstruction. For the non-mycorrhizal mosses (Pressel et al., 2014), 7 species from 7 major moss orders were chosen to represent this group: *Physcomitrella patens* (Funariales), *Sanionia uncinata* (Hypnales), *Nyholmiella obtusifolia* (Orthotrichales), *Syntrichia ruralis* (Pottiales), *Sphagnum palustre* (Sphagnales), *Takakia lepidozioides* (Takakiales) and *Tetraphis pellucida* (Tetraphidales).

For all orders of the Angiosperm Phylogeny Group system (APG III) (Stevens 2012) that were not yet included in the database, an additional literature search was conducted to find fungal associations. For some species only morphological identifications of arbuscular mycorrhizas were found. These were classified as Glomeromycota in the database. Finally, the database was submitted to a quality check. All fungal Ascomycota and Basidiomycota sequences, which belong to putative

mycorrhizal genera (Rinaldi et al. 2008), were accepted as associations. Saprophytes were removed. For sequences which were not identified up to genus level, the original publications were checked to see if the associations concerned putative mycorrhizal fungi, sometimes in combination with GenBank blast searches. In the latter case, the closest hit which was identified up to genus level was used to determine whether the fungus was putative mycorrhizal species.

Plant Phylogeny and Divergence Dating

DNA sequences for host plants were downloaded from Genbank via Geneious version 8.1.6 (Kearse et al. 2012). For 129 species, full or partial chloroplast genomes were available and used to extract *atpB*, *matK*, *psbA*, *rbcL*, *rps4* and rRna16S data. For the other plant species, all sequences from any of these six markers were downloaded. For these sequences, a quality check was conducted to certify that sequences were not too different from conspecific sequences. For all markers, duplicate sequences were aligned per genus and if the pairwise distance was less than 0.03% between species of a genus, the longest sequence was chosen from each species. If pairwise distance exceeded 0.03% within a genus, a heatmap with dendrograms (Warnes et al. 2015) was plotted to verify whether within species distance did not exceed distance between species. If the longest sequence did not differ greatly from the other sequences from the same species, it was accepted. Otherwise another sequence would be chosen which was more similar to other replicates from the same species. For liverworts, hornworts, polypods and lycopods a number of additional species were added to the dataset to reduce the amount of missing markers per group. Accession numbers are listed in the supplementary data (Supplementary Data accession numbers Phylogenetic analysis).

A final quality check for the data was conducted by running maximum likelihood analysis with RaxML-HPC2 on XSEDE, Cipres science gateway (Miller et al. 2010), to obtain a phylogenetic tree. A partitioned model was used for the six different markers *rps4*, *matK*, *psbA*, *atpB*, *rbcL*, rRna16S and rapid bootstrapping with a GTRGAMMA model was conducted with 1000 bootstrap iterations. The resulting 1008 trees were imported in Geneious version 8.1.6 (Kearse et al. 2012) to create a consensus tree using the consensus tree builder. The phylogeny was generally in accordance with the APG III

system (Stevens 2012) up to the order level, except for some branches with low support and the orchid *Cypripedium macranthos* (GenBank accession: KF925434), which was not placed in Orchidaceae.

For Divergence dating, the dataset was reduced to three markers, to decrease the duration of the analysis. The rapidly evolving gene *matK* was not used, as it was more difficult to align. 16S and *atpB* were not used as there were fewer sequences available from basal lineages for these markers. Sequences were aligned with MAFFT v7.164b (Katoh and Standley 2013), using FFT-NS-i Iterative refinement method.

To test the effect of different phylogenetic hypotheses (Clarke et al. 2011; Cox et al. 2014; Wickett et al. 2014) for the deep-time relationships of land plants on the ancestral state reconstructions, divergence dating analysis was conducted with four different topological constraints: Marchantiophyta basal (MB), Anthocerotophyta basal (AB), Tracheophyta basal (TB) and the Liverworts & Mosses versus Hornworts & vascular plants (2x2) hypotheses.

A series of additional phylogenetic constraints according to current insights in plant evolution (Clarke et al. 2011; Cooper et al. 2012; Stevens 2012) was applied to reach convergence faster. Higher level phylogenetic constraints were given to Angiosperms, Gymnosperm, Tracheophyta, Spermatophyta, Anthocerotophyta, Bryophyta (true mosses), Euphyllophyta and Lycopodiopsida. In liverworts, Haplomitriopsida was constrained to be monophyletic, as well as the remaining groups (Fossombroniales, Jungermanniales, Blasiales, Lunulariales, Marchantiales, Metzgeriales, Neohodgsoniales, Pallaviciniales, Pelliales, Pleuroziales, Ptilidiales and Sphaerocarpales) in order to have a basal split between Haplomitriopsida and other liverworts. Within Gymnosperms, Cycadales was constrained as outgroup for all other Gymnosperms, and *Ginkgo* was constrained as basal split of the remaining Gymnosperms. Within the Angiosperms, Amborellales, Nymphaeales, Australobaileyales where constrained as outgroups in the given order. Further constraints within the Angiosperms were Magnoliids, the group containing monocots and eudicots and the latter two groups were both also constrained.

A number of large orders were also constrained to be monophyletic: Alismatales, Anthocerotales, Apiales, Araucariales, Arecales, Asparagales, Asterales, Boraginales, Brassicales, Caryophyllales, Cupressales, Cyatheales, Cycadales, Dioscoreales, Equisetales, Ericales, Fabales, Fagales, Fossombroniales, Gentianales, Gleicheniales, Isoetales, Jungermanniales, Lamiales, Liliales, Lycopodiales, Malpighiales, Malvales, Marattiales, Marchantiales, Marchantiophyta, Metzgeriales, Myrtales, the clade containing Notothyladales and Dendrocerotales, Ophioglossales, Osmundales, Pallaviciniales, Pelliales, Pinales, Pleuroziales, Poales, Polypodiales, Polypodiopsida, Psilotales, Ranunculales, Rosales, Sapindales, Selaginellales and Solanales.

Divergence time estimation was done in BEAST2 (Bouckaert et al. 2014). To date the phylogenetic tree, a series of calibration points were used (Table 1). A lognormal distribution was chosen for each of the calibration points, in order to have a soft maximum constraint. The standard deviation of log transformed distribution was 0.5 for each calibration point to have a relatively flat distribution and the M parameter was modified to ensure that the 95% quantile was below the soft maximum limit of the fossil constraint. No calibrations were placed on higher nodes in the embryophyte phylogeny, as interpretation of the fossil constraints is dependent on the phylogenetic hypothesis of these nodes.

For *rps4* and *psbA* sequences, the general time reversible model with gamma distributed unequal rates across sites and a proportion of invariable sites (GTR+I+G) was selected as best fit using the Akaike Information Criterion in MrModeltest 2.3 (Nylander 2004) in PAUP* 4.0b10 (Swofford 2002). For *rbcL* the size of the dataset did not allow the MrModeltest run to complete and the GTR+I+G model was chosen for this marker.

A Yule prior was chosen for the analysis, with a lognormal relaxed clock model. The MCMC analyses for the MB hypothesis and 2x2 hypothesis were both had a run duration of 200x10^6 generations. Log files were updated every 5000 generations, and trees sampled every 50.000 generations. Log Combiner was used to resample the trees every 200.000 generations with a burnin of

10 percent, to a total of 901 trees. This was necessary to reduce the number of trees prior to creating the maximum clade credibility tree with Tree annotator v2.2.1 (Rambaut and Drummond 2015).

At the time of writing, the analyses for the TB and AB hypotheses are still running, but reaching convergence. For the intermediate results, the AB run had a duration of 84.430x10^6 generations, using the same sampling frequency as for the previous two hypotheses, but with a burnin of 30%, this resulted in 296 trees. For the TB run, the same procedure resulted in 297 trees.

Ancestral State Reconstruction

Ancestral state reconstruction of mycorrhizal associations on the time calibrated embryophyte phylogeny was done utilizing the Biogeobears R package (Matzke 2015). Functions were modified to allow for a valid reversible null space, to accommodate non-mycorrhizal plants in the analysis. The previously described transition matrix for range evolution along the internodes (Fig. 1) replaced the original matrix (Ree and Smith 2008). As there is no need for cladogenesis events within the symbiosis model, the Bayarea-like model from the Biogeobears R package (Matzke 2015) was used. This specific model has no correction for extinction events and assumes no cladogenesis. Before ancestral state reconstruction, some species (*Riccia crystalline*, *Riccia fluitans*, *Ptilidium pulcherrimum*, *Syzygiella geminifolia*, *Anthoceros angustus*, *Phaeoceros proskaueri*, *Isoetes flaccida*, *Selaginella uncinata*, *Mankyua chejuensis*, Angi*opteris angustifolia*, *Dipteris conjugate*, *Lygodium japonicum*, *Anogramma ascensionis*, *Ceratopteris richardii*, *Myriopteris lindheimeri*, *Cystopteris protrusa*, *Amborella trichopoda*, *Cenchrus americanus*, *Ranunculus macranthus*, *Silene flos-cuculi*, *Brucea javanica*, *Trifolium campestre*, *Quercus wutaishanica* and *Quercus mongolica*) had to be dropped from the phylogeny, as they were solely added to strengthen the coverage of markers for some groups, or because inspection of their fungal symbionts revealed uncertain associations.

Diversification Analysis Fungi

For estimates of speciation rates over time in fungi, the smoothed Time Tree of Life was used (Hedges et al. 2015). Both this tree and the TTOL for fungi were downloaded from

http://www.biodiversitycenter.org/ttol. Tips that were not in the fungal TTOL were dropped from the smoothed TTOL to retain a bifurcating phylogeny for fungal taxa only. The number of Glomeromycota and Mucoromycotina taxa in the phylogeny turned out to be too low to draw conclusions. However, both Ascomycota and Basidiomycota were represented by a considerable number of species, allowing for diversification analysis. Diversification analysis was done with BAMM (Rabosky et al. 2013, 2014a; Rabosky 2014; Shi and Rabosky 2015) and BAMMtools (Rabosky et al. 2014b). BAMMtools was first used to estimate priors for the analysis given the fungal tree. BAMM was run for 9.280.000 generations, until ESS values were larger than 200. Event data was sampled every 2000 generations. After analysis 10% of the samples were discarded as burnin, leaving 4188 samples from posterior for analysis. Results were analysed and plotted using BAMMtools.

RESULTS

Database Description

Our final database includes 806 plant species: 25 hornwort species, 7 non-mycorrhizal mosses, 100 liverworts, 545 angiosperms, 79 gymnosperms, 21 lycopods, and 29 polypods species. For these plants species, 144 Ascomycota, 306 Basidiomycota, 450 Glomeromycota, and 27 Mucoromycotina associations were found (Supplementary Data Host Fungus Associations).

Divergence Dating

All divergence dating runs showed convergence, but some ESS values were still low. All major ESS values for the MB and 2x2 hypotheses were higher than 60, ESS values for the other two hypotheses were lower, but these runs were not as long. Age estimates for the crown group Embryophyta varied between the different phylogenetic hypotheses. The oldest estimate, 519 Mya, was found in the phylogeny in which Liverworts form the most basal split (Table 2).

Divergence dating resulted in a few negative nodes in all analyses except for the MB hypothesis. In the 2x2 hypothesis, two negative nodes were found and the AB and TB hypotheses

resulted in three negative nodes. Except for one node in AB with a length of -2.4828616 Myr, all negative branches had a length smaller than 1 Myr. To enable the ancestral state model to run on these phylogenies, these branch lengths were corrected by giving them a length of 0.001 Myr, subtracting the difference in length from the two descendant branches.

Ancestral State Reconstruction

The last common ancestor of all Embryophyta was reconstructed as mycorrhizal (Fig. 3). The most likely state of mycorrhizal association was an association with Glomeromycota only, with a probability of 0.44, 0.54, 0.50 and 0.62 for the MB hypothesis, the 2x2 hypothesis, the AB hypothesis and the TB hypothesis, respectively. The probability for a state with both Glomeromycota and Mucoromycotina varied from 0.24 to 0.27. After that, 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 probability of a non-mycorrhizal ancestral state was at most 0.011 (Fig. 3). Glomeromycota was the most likely associate 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.

We reconstructed several independent switches from Glomeromycota to Mucoromycotina, Ascomycota and Basidiomycota. The oldest nodes which were reconstructed as Basidiomycete and Ascomycete associations were found in liverworts (Fig. 2; Fig. 5; Fig. 6). The 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 nodes associated with Basidiomycota with a probability around 0.6 are estimated to be around 200 Million years old (Fig. 5; Fig. 6). Other switches to Ascomycota and Basidiomycota were found in Pinales, Orchidaceae, Ericales, Malphigiales and Fagales, but more independent range 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.

Diversification & Speciation Rate Fungi

ESS values from the speciation rate analysis were 593.5 (number of shifts) and 214.1 (log likelihood). In Basidiomycota, a shift in speciation rate is found in the branch leading to Agaricomycetes, around 300 Mya (Figure 4). The speciation rate increases for another 125 million years in Agaricomycetes. For Ascomycota, there is a major shift in speciation rate between 500 and 600 Mya - the rate increases further until around 500 Mya and then stays relatively constant (figure 4). For all fungi, there is a sharp increase in speciation rate in the last 50 million years.

DISCUSSION

Terrestrialization and the Ancestral State

Age estimates for crown group Embryophyta in the present study are within the range of the estimates from literature (Table 2; Fig. 7). The divergence of the fungal phyla Ascomycota, Basidiomycota, Glomeromycota and Zygomycota (Hedges et al. 2015) is likely to have predated the divergence date estimates for the Embryophyta crown group in this study (Table 2; Fig. 7). Given these relative clade ages, the ancestral state reconstruction does not violate the concept that an ancestral mycorrhizal state cannot be reconstructed before the divergence of the concerning fungal clade.

Our ancestral state reconstructions support 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 record (Strullu-Derrien et al. 2014) and genomics (Karandashov et al. 2004; Wang et al. 2010; Delaux et al. 2015). The recent discovery of mycorrhizal associations with Mucoromycotina in many early diverging lineages of Embryophyta (Bidartondo et al. 2011; Desirò et al. 2013; Rimington et al. 2014) led to the question what the role of this more basal lineage of Fungi could have

been in the terrestrialization process. This discovery 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* (Strullu-Derrien et al. 2014), 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 actually are and help to resolve the question concerning their role in terrestrialization.

The different hypotheses for the evolution of plants (Cox et al. 2014; Wickett et al. 2014) had very little effect on the results. The main reason for the absence of a clear effect 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 maximum likelihood analysis for the ancestral state thus depends more strongly on the latter two groups.

The Ecological History of Fungal Phyla

Although the divergence of the fungal clades Ascomycota, Mucoromycotina, Glomeromycota and Basidiomycota is likely to have happened before the diversification within Embryophyta, results concerning the history of fungal ecology are not unambiguous. For Mucoromycotina and Glomeromycota the first evidence of mycorrhizal habit is found around 407 Mya in the fossil record (Strullu-Derrien et al. 2014). 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 symbionts (James et al. 2006). For Mucoromycotina more research will be needed on the history of mycorrhizal interactions.

For Ascomycete and Basidiomycete associations, the ecological history is a challenging subject (Lutzoni et al. 2001; Hibbett and Matheny 2009; Schoch et al. 2009; Floudas et al. 2012; Kohler et al. 2015). 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).

Speciation rate analysis for fungi shows a sharp increase in speciation rate in the last 50 Myr. This could be explained by the phenomenon called the 'pull of the present' (Morlon 2014). The diversification rates in Ascomycota and Basidiomycota lineages are interesting when comparing these to the ecological history of the respective phyla. The diversification of Ascomycota happened long before the first Ascomycota mycorrhizal association (Figure 4). This diversification could be explained by the terrestrialization process, in combination with a lichenized symbiosis. A possible lichenized ancestor could have radiated quickly due to the efficient symbiotic interactions between photoautotrophs and fungi. However, the ancestral ecology of Ascomycete lineages is a topic of discussion (Lutzoni et al. 2001; Schoch et al. 2009). Many mycorrhizal associations in Ascomycota are found within Pezizomycetes. Age estimates for this group predate the age of the first associations (Rinaldi et al. 2008; Hedges et al. 2015). The age estimate for the mycorrhizal genus *Tuber* alone is 271 to 140 Ma (Jeandroz et al. 2008), 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 Triassic (Figure 4). 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 orders (Hibbett and Matheny 2009; Floudas et al. 2012). Both the improved carbon acquisition by lignin peroxidases (Floudas et al. 2012) and the mycorrhizal habit can explain the radiation of Agaricomycetes.

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 Jungermanniales (Cooper et al. 2012). Other estimates for this split are 272 Mya (CI: 243–310) (Newton et al. 2006) and 308.7 ± 7.8 Mya (Heinrichs et al. 2007), 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 associates (Pressel et al. 2010) and the fact that this very basal embryophyte lineage is associated with the two most basal lineages of Agaricomycetes: *Sebacina* and *Tulasnella* (Hibbett et al. 2014). Furthermore, putative ectomycorrhizal structures found in bennettitalean roots 225 Mya (Strullu-derrien, unpublished data, 2015 – colloquium, needs verification) coincide with the time at which Basidiomycota, the phylum to which most ectomycorrhizal species belong, is reconstructed as a mycorrhizal symbiont. However, the phylogenetic position of these fossils needs to be verified before any conclusions can be drawn, as they could also concern Mucoromycotina or Ascomycota.

Nevertheless, a mycorrhizal ancestral state in Agaricomycetes does not disagree with the expansion of lignin peroxidases in the branches leading to Auriculariales (Kohler et al. 2015), nor the drop in Carbon Burial at the end of the Carboniferous, as the first divergence events in Agaricomycetes are older than the acquisition of lignin decomposing enzymes (Floudas et al. 2012; Kohler et al. 2015) . The first fossil evidence of white-rot predates the first fossil evidence of ectomycorrhiza, but this fossil evidence is in accordance with the onset of lignin peroxidases in Auriculariales (Kohler et al. 2015).

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 further 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.

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TABLES

Table 1

Fossil calibration points (Clarke et al. 2011). All calibration points were given a lognormal distribution. The S parameter is the standard deviation of the log-transformed distribution. The M parameter was modified in order to let the soft maximum age constraint coincide with the 95% quantile of the lognormal distribution.

Clade (crown)	Split		Min age (hard)	Max age (soft) 95% quantile	S	M
Tracheophyta	Lycopodiophyta	- Polypodiophyta & Spermatophyta	416.0	454.0	0.5	19
Euphyllophyta	Polypodiophyta	- Spermatophyta	388.2	454.0	0.5	33
Spermatophyta	Gymnosperms	- angiosperms	306.2	366.8	0.5	30
Gymnosperm	Cycadales	- other gymnosperms	306.2	366.8	0.5	31
	Ginkgo	- Coniferae	106.7	366.8	0.5	130
Angiospermae	Amborellales	- other Angiosperms	124.0	248.4	0.5	62
Mesangiospermae	Magnoliids	- Monocots & Eudicots	124.0	248.4	0.5	62
	Nymphaeales	- Australobaileyales & Mesangiospermae	124.0	248.4	0.5	62
	Australobaileyales	- Mesangiospermae	124.0	248.4	0.5	62

Table 2

Divergence time estimates for fungal phyla and Embryophyta. These ages where estimated in present study, using the different phylogenetic constraints.

Topology	Monophyletic bryophytes (TB)	Hornwort Basal (AB)	Hornwort Tracheophyte (2x2)	Liverwort Basal (MB)
Crown age Embryophyta	453.59* (95% HPD: 426.2- 497.8)	484.52* (95% HPD: 445.7 - 531.9)	481.45* (95% HPD: 442.0 - 521.0)	519.54* (95% HPD: 462.9 - 576.8)

Q =

	Ø	A	B	G	M	AB	AG	AM	BG	BM	GM	ABG	ABM	AGM	BGM	ABGM
Ø	-	d	d	d	d	0	0	0	0	0	0	0	0	0	0	0
A	e	-	0	0	0	d	d	d	0	0	0	0	0	0	0	0
B	e	0	-	0	0	d	0	0	d	d	0	0	0	0	0	0
G	e	0	0	-	0	0	d	0	d	0	d	0	0	0	0	0
M	e	0	0	0	-	0	0	d	0	d	d	0	0	0	0	0
AB	0	e	e	0	0	-	0	0	0	0	0	d	d	0	0	0
AG	0	e	0	e	0	0	-	0	0	0	0	d	0	d	0	0
AM	0	e	0	0	e	0	0	-	0	0	0	0	d	d	0	0
BG	0	0	e	e	0	0	0	0	-	0	0	d	0	0	d	0
BM	0	0	e	0	e	0	0	0	0	-	0	0	d	0	d	0
GM	0	0	0	e	e	0	0	0	0	0	-	0	0	d	d	0
ABG	0	0	0	0	0	e	e	0	e	0	0	-	0	0	0	d
ABM	0	0	0	0	0	e	0	e	0	e	0	0	-	0	0	d
AGM	0	0	0	0	0	0	e	e	0	0	e	0	0	-	0	d
BGM	0	0	0	0	0	0	0	0	e	e	e	0	0	0	-	d
ABGM	0	0	0	0	0	0	0	0	0	0	0	e	e	e	e	-

Figure 1

The instantaneous matrix to describe the relative probability of state changes along internodes. All possible combinations of mycorrhizal associations with fungi from the phyla Ascomycota (A), Basidiomycota (B), Glomeromycota (G) and Mucoromycotina (M) are included as ranges. The null range (Ø) represents the non-mycorrhizal state.

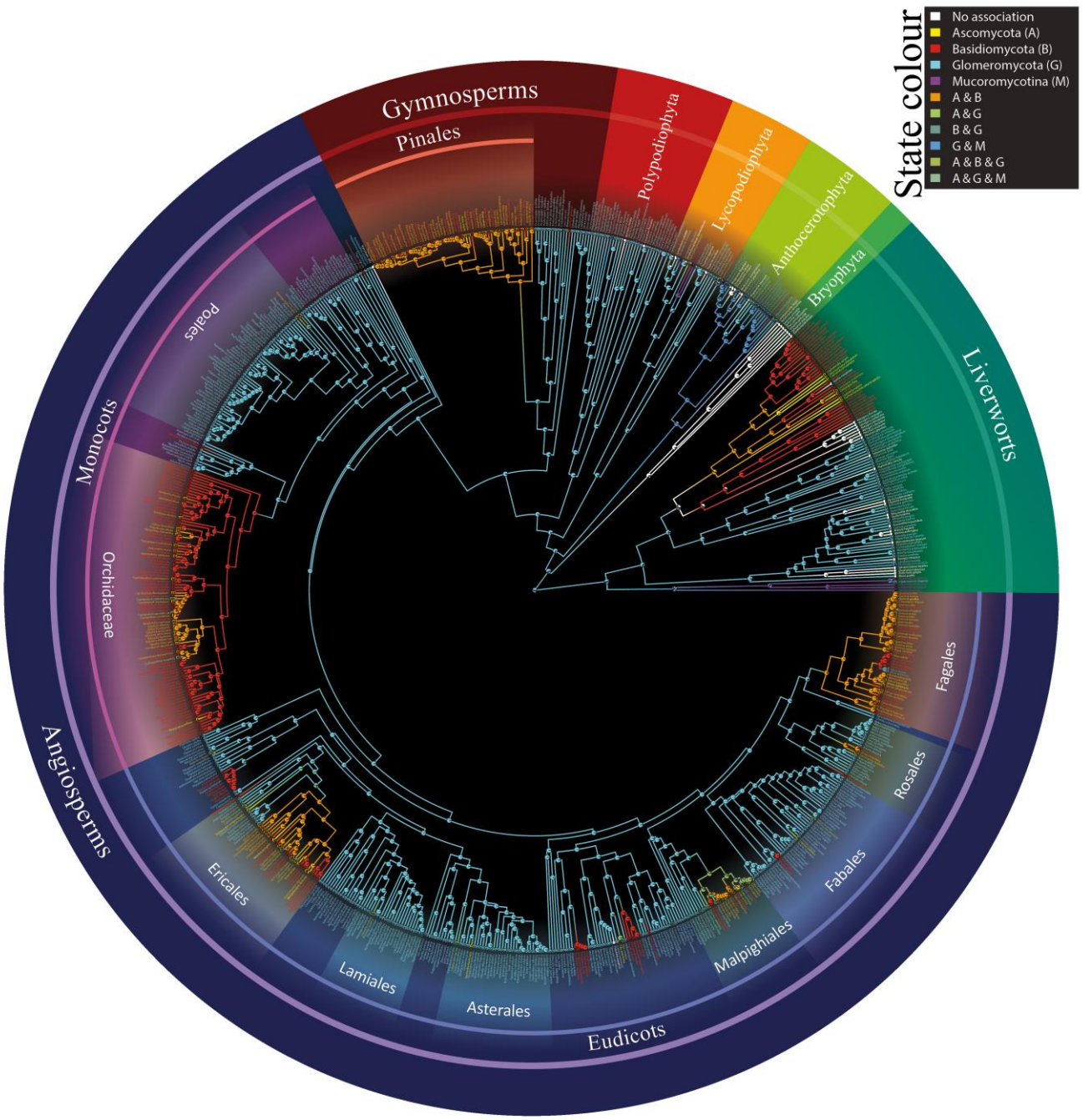

Figure 2

Ancestral state reconstruction of mycorrhizal associations on the plant phylogeny. Letters in the legend represent mycorrhizal associations: (A) Ascomycota; (B) Basidiomycota; (G) Glomeromycota; (M) Mucoromycotina. Combinations of letters represent a combination of mycorrhizal associations. Ancestral nodes are most likely to have been associated with Glomeromycota, suggesting multiple independent switches or 'range' expansions to Ascomycota, Basidiomycota and Mucuromycotina. The earliest reconstructed Ascomycota and Basidiomycota associations were found in liverworts.

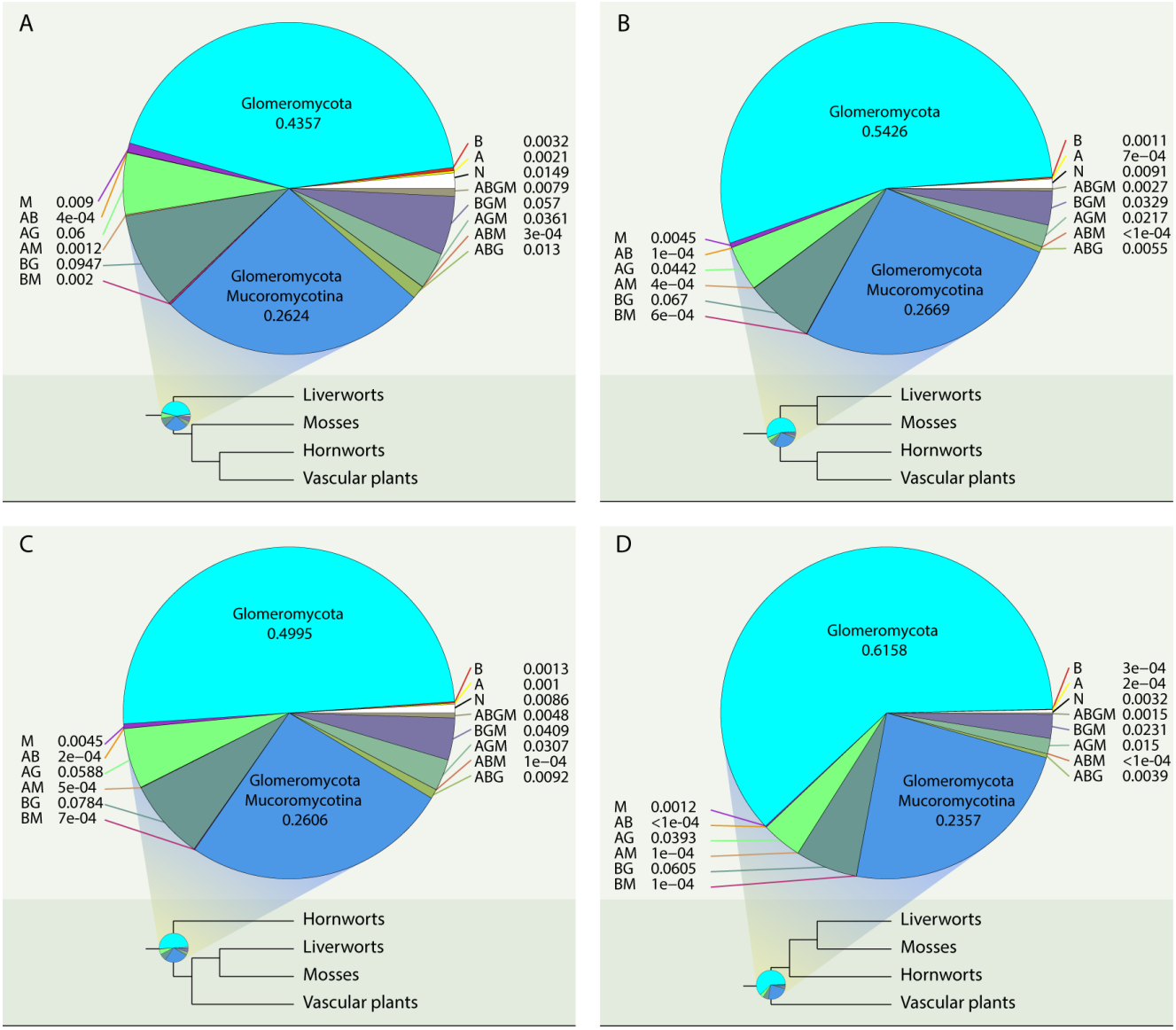

Figure 3

Maximum likelihood ancestral state reconstruction of the mycorrhizal association for the last common ancestor of Embryophyta, given the different phylogenetic constraints. Letters represent mycorrhizal associations: (A) Ascomycota; (B) Basidiomycota; (G) Glomeromycota; (M) Mucoromycotina; (N) Non-mycorrhizal. Combinations of letters represent a combination of mycorrhizal associations. Glomeromycota is the most likely ancestral state, but a combined Glomeromycota and Mucoromycotina state also has a substantial probability. Different hypotheses for the phylogeny of early land plants yield very similar results, although the probability of Glomeromycota is higher in the TB hypothesis (D).

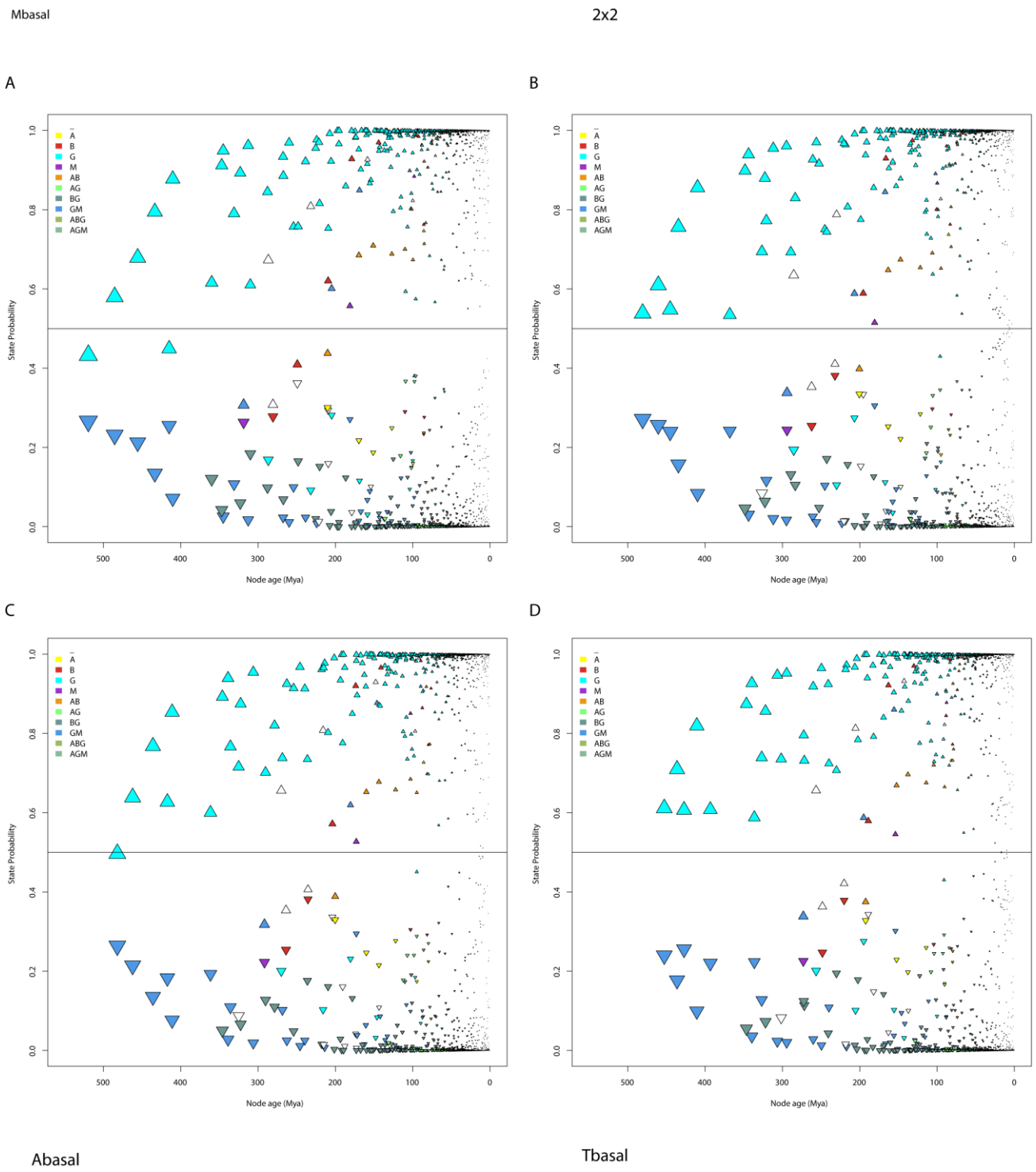

Figure 4

Patterns in probability of mycorrhizal association through time for each of the four hypotheses of early plant phylogeny (with size emphasis on the older associations): (A) MB hypothesis, (B) 2x2 hypothesis, (C) AB hypothesis and (D) TB hypothesis. Letters in the legend represent mycorrhizal associations: (A) Ascomycota; (B) Basidiomycota; (G) Glomeromycota; (M) Mucoromycotina; (_) Non-mycorrhizal. Combinations of letters represent a combination of mycorrhizal associations. All

triangles pointing upward show the state with the highest probability for each node. The triangles pointing downward show the second most probable state. Slight changes in age or probability can be seen between the four phylogenetic hypotheses, but the general pattern remains very similar. Nodes older than 300 Mya are generally reconstructed as Glomeromycota associations, with the combined Glomeromycota and Mucoromycotina state as a second possibility. The first nodes with Basidiomycota as the most likely state ($p>0.5$) appear around 200 Mya.

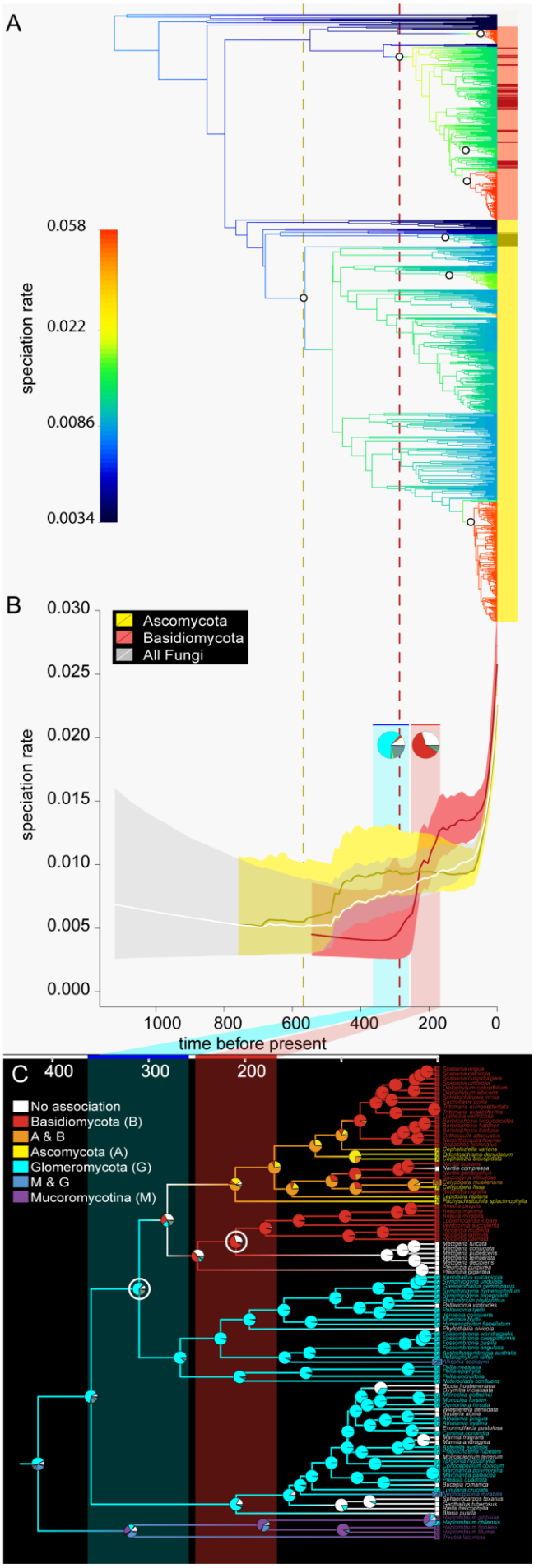

Figure 5

Diversification in Fungal lineages Ascomycota and Basidiomycota compared to the oldest reconstructed occurrences in the ancestral state reconstruction. (A) The fungal time tree of life showing speciation rate as estimated by BAMM and BAMMtools (Rabosky et al., 2013; Rabosky, 2014; Rabosky et al, 2014; Rabosky et al, 2014; Shi & Rabosky, 2015). Circles on branches denote shifts in speciation rate. The colour bar at the tips of the phylogeny shows Ascomycota (yellow) and Basidiomycota (red). Dark red and yellow lines indicate the position of putatively mycorrhizal genera (Rinaldi et al., 2008). The shift in speciation rate in the node leading to Agaricomycetes (A & B, red dashed line) falls in the time interval in which ancestral state reconstruction indicates a switch between Glomeromycota and Basidiomycota in the Liverwort phylogeny. This is the oldest reconstructed Basidiomycota association in the plant phylogeny (C: ancestral state reconstruction of mycorrhizal association; liverworts). The shift in speciation rate in Ascomycota (A & B yellow dashed line) does not coincide with the oldest association of Ascomycota in the plant phylogeny and even predates the most basal split of the Embryophyta phylogeny. (B) Speciation rate in the Ascomycota crown group (yellow), the Basidiomycota crown group (red) and of the entire fungal tree of life (grey) with the 10% through 90% Bayesian credible region of the distribution (shaded areas).

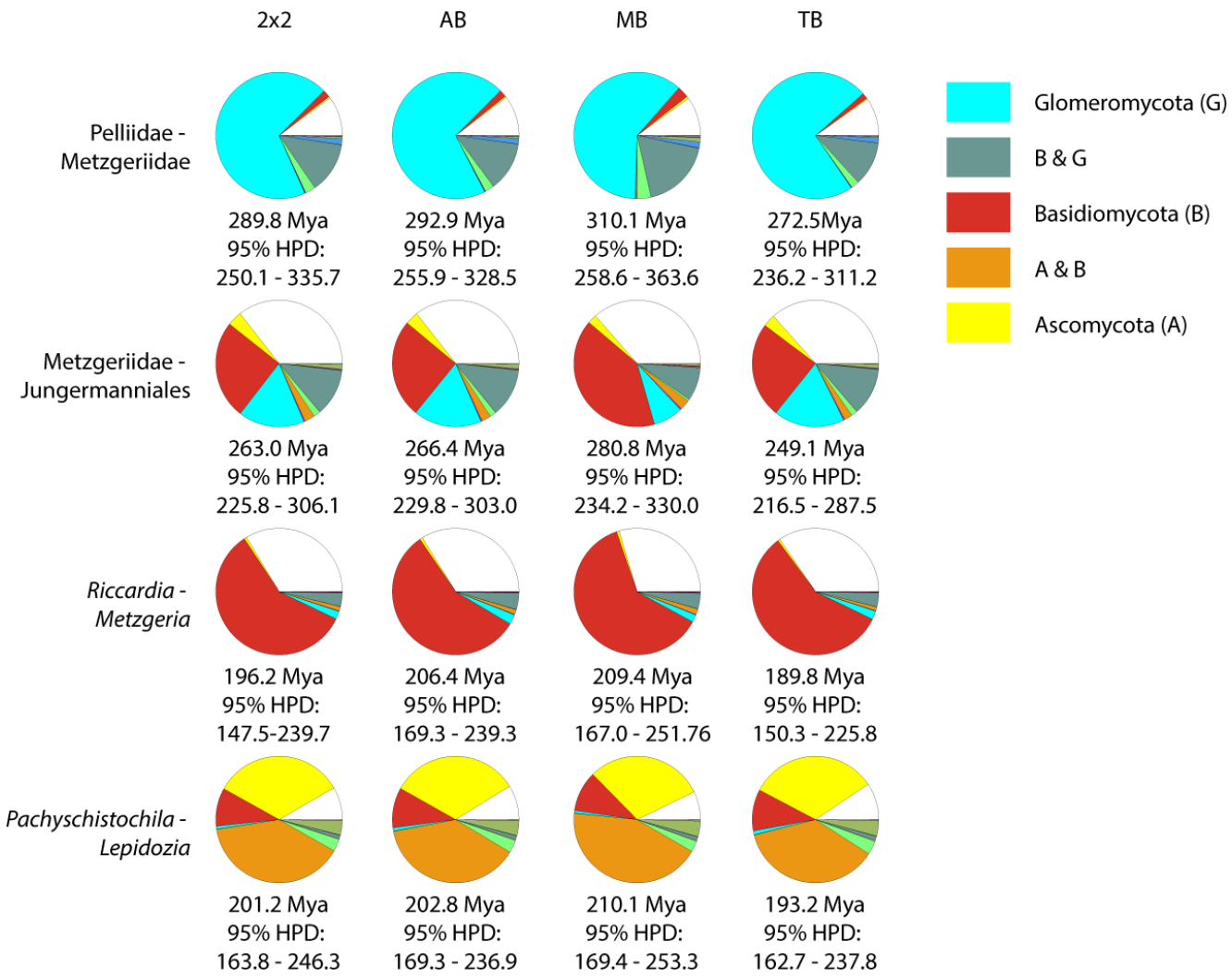

Figure 6

The switch from Glomeromycota associations in liverworts to Basidiomycota and Ascomycota associations, according to ancestral state reconstruction. The different phylogenetic hypotheses are as follows: TB = Tracheophyte basal with Marchantiophyta, Anthocerotophyta and Bryophyta in a monophyletic group; MB = Marchantiophyta as basal split, Bryophyta sister to a monophyletic clade with Anthocerotophyta and Tracheophyta; AB= Anthocerotophyta basal, a monophyletic clade of Marchantiophyta and Bryophyta is sister to Tracheophyta; 2x2 = Marchantiophyta and Bryophyta form a monophyletic clade which is sister to a monophyletic clade compromised by Anthocerotophyta and Tracheophyta. Node ages with their 95% HPD interval are given below each reconstructed node.

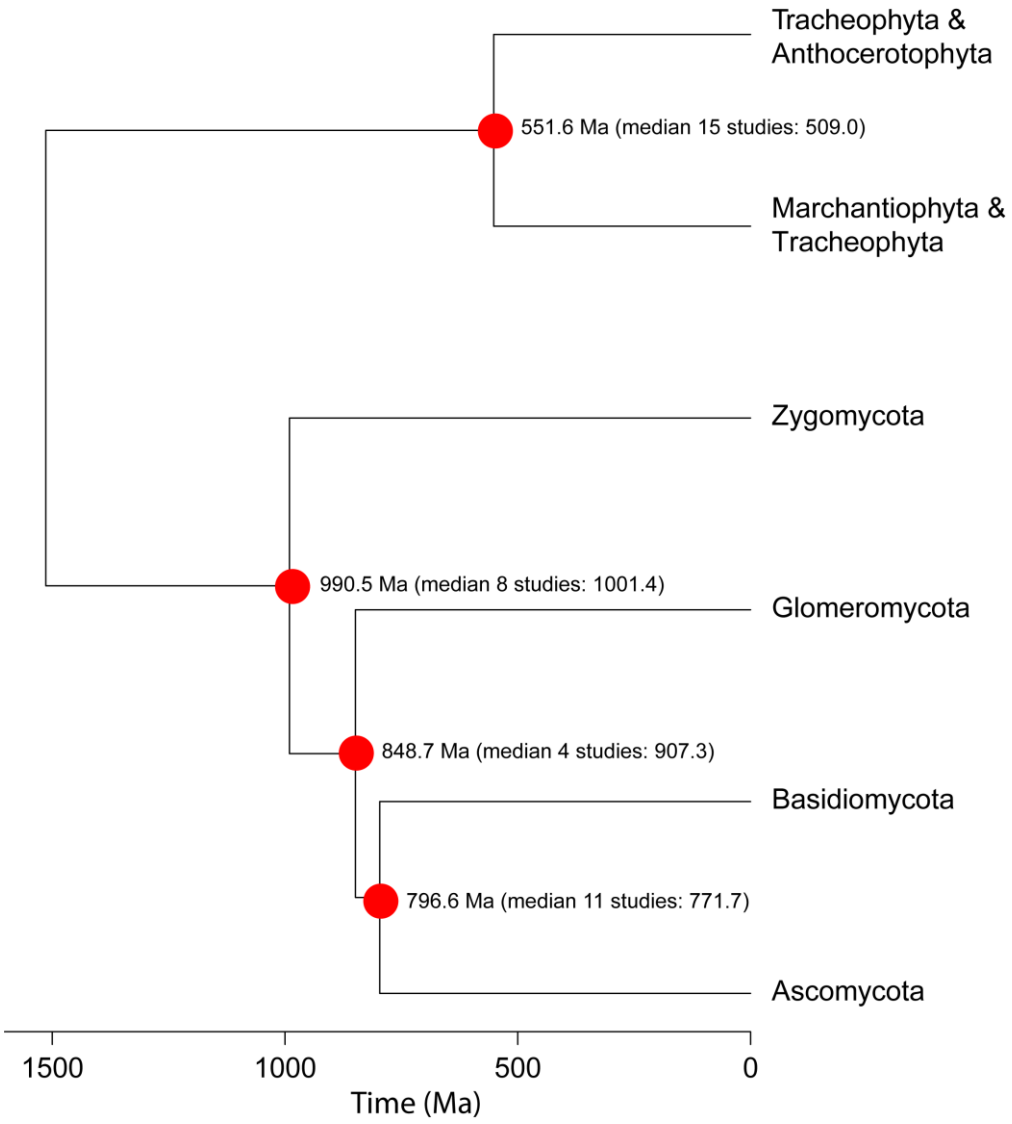

Figure 7

Divergence time estimates for fungal phyla and embryophytes, adapted from the time tree of life (Hedges et al. 2015). The topology of the embryophyte is according to the hypothesis that mosses (Bryophyta) and liverworts (Marchantiophyta) form a monophyletic clade which is sister to the clade containing vascular plants (Tracheophyta) and hornworts (Anthocerotophyta). The crown group age of land plants is estimated to be younger than the divergence of the four fungal phyla which are relevant to this study: Ascomycota, Basidiomycota, Glomeromycota and Zygomycota.