Article title: Seed plant families with diverse mycorrhizal states have higher diversification rates

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Short running title: Higher diversification in mycorrhizal diverse plant families

Keywords: diversification rates, mycorrhizal states, seed plants, key innovation, mycorrhizal diversity

Type of article: Letter

Number of words: Abstract (153), Main Text (3727)

Number of references: 43

Number of figures: 4

Number of tables: 0

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Statement of authorship: MIM, TQ and MFP designed the research; GB and MIM organized and curated the original downloaded data for posterior analysis; GB, MIM and TQ analyzed the data; GB made all the figures; all authors wrote the manuscript

Data accessibility statement: All data, R scripts, and documents related to this manuscript are available at [https://github.com/gburin/mycoDiversif]

Summary

Most of plant species have mycorrhizas, which can be classified in four types: Arbuscular (AM), Ecto (EM), Orchid (OM) and Ericoid Mycorrhiza (ER). Since the AM ancestral state, some plant lineages have switched partner (EM, OM and ER) or lost the association (NM). Evolutionary transitions to a novel mycorrhizal state (MS) might allow plant lineages to access new resources, enhancing diversification rates. However, some clades are not restricted to one MS, and this variability might promote diversification. Here, we address the relationship between MS diversity and seed plant diversification. Using the Fungal-root database, which compiles plant species and their MS, we assigned a single MS to each plant family, calculated the MS heterogeneity and estimated their diversification rates using the method-of-moments. Our results showed higher diversification rates in families with mixed MS, and a positive relationship between MS heterogeneity and diversification rates, which suggests that MS lability promotes plant diversification.

Introduction

Understanding the basis of the exceptional plant diversity has been a matter of interest for ecologist and evolutionary biologist since Darwin. Great focus has been placed on estimating plants diversification rates and identifying the factors that could influence them (Eriksson & Bremer 1992; Moore & Donoghue 2007; O'Meara *et al.* 2016; Vamosi *et al.* 2018). The acquisition of novel traits (sometimes referred to as "key innovations"), such as pollination by animals (Eriksson & Bremer 1992) or physiological seed dormancy (Willis *et al.* 2014), have been proposed to promote diversification of plant lineages. This "key innovation" perspective suggests that the acquisition of a novel trait might allow a given lineage to exploit the environment in a significantly different way, potentially resulting in an explosive radiation.

One crucial innovation in plant evolution was the association with soil fungi during land colonization (Pirozynski & Malloch 1975; Selosse & Le Tacon 1998; Strullu-Derrien *et al.* 2018). Before plant colonization, land was hostile, with extreme drought and temperatures, and barren rocky substrate; hence, the association with terrestrial fungi allowed the algae ancestors of plants to successfully colonize the land (Selosse *et al.* 2015). This initial symbiotic association was the prelude of modern mycorrhizas (Feijen *et al.* 2018), the

association between fungi and root plants in which plants transfer carbon to fungi and receive nutrients in turn (Smith & Read 2008). Today, this symbiosis is present in 86% of land plants species (Heijden *et al.* 2015), and based on their structure and function can be classified in four major types: arbuscular mycorrhiza (AM), ectomycorrhiza (EM), orchid mycorrhizal (OM) and ericoid mycorrhizal (ER) (Brundrett 2002).

Ancestral state reconstruction and the fossil record suggest that the ancestor of seed plants probably had AM associations (Redecker *et al.* 2000; Maherali *et al.* 2016). This is the most frequent mycorrhizal type in plants (74% of extant plant species) and is characterized by an association with Glomeromycete fungi (Heijden *et al.* 2015). Between 100 and 200 million years ago, some lineages switched fungal partners to several lineages of Basidiomycetes, forming what is described as the EM associations (Brundrett 2002). The acquisition of EM resulted in new root functional capabilities as freezing tolerance (Lehto *et al.* 2008), which seem related to the dominance of EM angiosperms and gymnosperm in cool forests (Brundrett 2002). Similarly, Orchidaceae and species within the Ericaceae family recruited new fungal lineages and formed OM and ER associations respectively. Orchids associate with fungal families Ceratobasidiaceae, Tulasnellaceae and Sebacinaceae, which in addition to nutrient exchange, promote seed germination which cannot germinate without mycorrhizal support (Rasmussen 2002). Ericoid mycorrhizal associations (ER), on the other hand, involve mainly fungi from Sebacinales and Helotiales and are mostly frequent under acidic and infertile heathland conditions (Perotto *et al.* 2002; Heijden *et al.* 2015). Finally, some lineages have lost their mycorrhizal associations and became non-mycorrhizal (NM). This transition has frequently occurred through an intermediate state of facultative arbuscular mycorrhiza (AM) plants (Maherali *et al.* 2016). Some of NM lineages evolved alternative resource-acquisition strategies (Werner *et al.* 2018) like cluster-roots in Proteaceae (Neumann & Martinoia 2002) or parasitism in Loranthaceae (Wilson & Calvin 2006).

Therefore, since the AM ancestral state some plant lineages have followed different mycorrhizal evolutionary pathways: switching partner (EM, OM and ER) or losing the association (Werner *et al.* 2018). Evolutionary transitions to a novel mycorrhizal state might allow plant lineages to access unexplored ecological resources, facilitating them to colonize environments that were not available before, and possibly enhancing their diversification rates. However, there are lineages in which some species acquire a new mycorrhizal state and at the same time, other species retain the ancestral state (AM) (Brundrett n.d.) increasing the vari-

ability of mycorrhizal states, which might in fact promote diversification of these lineages. Both hypotheses have not been evaluated in plants; however, the few studies available from the fungal perspective suggest that shifts in mycorrhizal associations might affect diversification of involved partners (Sánchez-García & Matheny 2017; Sato *et al.* 2017).

Even though mycorrhizal symbiosis has been pointed out as a key factor in the evolution and diversification of land plants (Brundrett & Tedersoo 2018; Feijen *et al.* 2018) this has not been evaluated before. In this study we address the following questions: (1) Do the lineages that established derived mycorrhizal associations present higher diversification rates than the ones that retain the ancestral mycorrhizal state? This investigates the idea of a key innovation mechanism of diversification; (2) Is there a relationship between mycorrhizal variability and diversification rates among different plant lineages? This would investigate the idea that evolutionary lability might increase diversification dynamics. To answer these questions, we explored the relationship between the mycorrhizal state and the diversification rates of several seed plant families.

Materials and Methods

Mycorrhizal state database

To obtain information of plant species and their mycorrhizal state, we used the FungalRoot database, a recently published global databank of plant mycorrhizal associations (Soudzilovskaia *et al.* 2019). This database compiles previous lists and surveys of plant species and their mycorrhizal associations, including 36,303 records for 14,870 plant species. Additionally, based on these empirical records and on expert opinion, the authors proposed a list of mycorrhizal status at the plant genus level, which contains 14,541 total genera, from which 12,558 correspond to seed plant genera that together results in information for 295,221 seed plant species.

Recently, Brundrett & Tedersoo (2019) pointed out potential mistakes in mycorrhizal type identification on large databases, and how these misdiagnoses might lead to wrong conclusions. Although their approach used to determine these errors (taxonomic approach; Brundrett (2017)) is controversial (Guillermo Bueno *et al.* 2019; Sun *et al.* 2019) and the proportion of errors they detected in databases is relatively low

(Brundrett & Tedersoo 2019), Brundrett & Tedersoo (2019) are right to point out that caution must be taken when analyzing large databases of plant mycorrhizal status. Therefore, in addition to the main analyses that were conducted using the genus-level list, we evaluated if the results were maintained when using only empirical data, by conducting the same analyses using the species-level list. Additionally, in the species-level list, the authors included remarks for 3,954 plant records (out of 36,303), indicating potential mistakes or misidentification of mycorrhizal associations in the original publication (see details in Table Media 3 in Soudzilovskaia *et al.* (2019)). Then, to test the effect of potential errors in the database, we conducted the analyses (i) excluding and (ii) without excluding plant records that had remarks (see Appendix S1 in Supporting Information). Furthermore, to assess the effect of possible undetected errors in the genus-level dataset, we introduced errors to the mycorrhizal state of 20% of plant species (one order of magnitude higher than the error estimated from Brundrett & Tedersoo (2019)). The results obtained with the error-introduced databases were similar than those derived from original data (Appendix S2).

Family mycorrhizal state and diversity

The genus-level list from FungalRoot database includes information for genera belonging to 392 seed plant families. Before using this list, we prepared the data as follows (i) Typo correction: removed entries with spaces at the end, with double spaces or line breaks, (ii) matched genera to families using the table *Spermatophyta_Genera.csv*, obtained from Zanne et al. (2014), (iii) Used package “taxize” (Chamberlain *et al.* 2019) for R (R Core Team 2019) to fill in for genera without family data and (iv) removed Ferns and Mosses. In the genus-level list, genera were classified as AM, EM, NM, OM, ER, or with multiple mycorrhizal status (i.e. AM-EM, AM-NM). The genera that were classified with multiple mycorrhizal status were classified as MIX, to indicate that these genera presented more than one mycorrhizal state. Instead of using MIX as a separate mycorrhizal status, we divided the species richness of those genera equally into the types that composed the MIX category, e.g. if one genus had 100 species and was classified originally as AM-NM, we would then add 50 species to the AM category and 50 species to the NM category prior to grouping all data per family. We obtained the richness of each genus from The Plant List ([theplantlist.org]), and then calculated the number of species with each mycorrhizal state within each family, discarding those genera

that had unknown mycorrhizal type. Each family was assigned a unique mycorrhizal state (AM, EM, NM, ER or OM) when more than 60% of species sampled belonged to this mycorrhizal state. If no single state were present in more than 60% of species, the family was assigned as "MIX", to indicate no dominance of any mycorrhizal association. Other thresholds for the assignment of family mycorrhizal state were tested and the pattern was similar (50%, 80% and 100%, Appendix S3). However, we excluded from the analyses the families from which all species belonged to genera that were classified as MIX (only 18 families) given that in this case there is no direct information for any species about its specific mycorrhizal state (MS), and using those could strongly bias our results given our methods. Given our methodological choice to assign equal proportions of MS for those genera classified as MIX, that would, by definition, fix the absolute value of diversity index for all those families, and hence possibly remove any signal for a potential association between the diversity index and diversification rates (see below). Given that those genera only make up for the entirety of very few families (only 18 out of 392), we decided to simply remove those families from the main analysis (analyses without removing those families presented similar results and are shown in Appendix S3). To investigate the effect of mycorrhizal diversity in the diversification dynamics we estimated the "Mycorrhizal Type Diversity Index", which is calculated by estimating the heterogeneity of the mycorrhizal states in each family using the shannon diversity index.

Diversification rates

First, to explore the underlying diversification model behind plant seed diversification, we assessed the association between age and richness among seed plant families. Thus, following Sanchez-Reyes et al. (2017), we evaluated the association between stem age and richness, including all seed plant families available (i.e. without removing families lacking information on mycorrhizal states) and correcting for phylogenetic structure and not. Stem group ages of the families were obtained from the dated molecular phylogeny of seed plants of Zanne et al. (2014) and the number of species of each family was obtained from The Plant List ([http://www.theplantlist.org]). No association was found between stem group age and richness, either considering or not phylogenetic structure (R^2 = 0.009; R^2 = 0.007, respectively; Fig.1), suggesting that diversification rates significantly vary among clades (Sánchez-Reyes *et al.* 2017) and justifying further

investigation. Diversification rates for each seed plant family were estimated using the method-of-moments from Magallón & Sanderson (2001) and stem group ages. Because the relative contribution of extinction is unknown, we used two distinct scenarios to characterize the relative extinction rates (ϵ), one with no extinction, ϵ = 0.0 and another with high extinction, ϵ = 0.9. We are aware of more sophisticated and direct methods (e.g. BAMM; Rabosky, (2014)) to investigate the association between trait states and diversification dynamics, but the plant phylogeny is massively under-sampled at the species level. Therefore, we decided to use simpler and less data hungry methods, and to discuss our results in the light of the methods limitations.

The tree used in this study (Zanne et al. 2014) was build using a Maximum Likelihood framework, and therefore consists in a single topology, not allowing phylogenetic uncertainty to be readily incorporated. That said, given the fact that the possible variations in age estimates should, at this temporal scale, not really influence the diversification estimates (rates are calculated using age in a log scale), we believe this has no impact in our results. More importantly, we replicated the same procedure using a different family-level phylogeny (Harris & Davies 2016), allowing some level of phylogenetic uncertainty to be addressed. This study estimates diversification rates using the same source of information for species richness per family (theplantlist.org), but with different values for family ages, therefore rendering these rates slightly different from our estimates. As expected, the results are virtually identical (figures S10, S11 and S12, and table S23) and we show here only the results from the Zanne et al. (2014) study (all the results for the other phylogeny can be seen in the supplemental material).

Phylogenetic signal

The seed plant phylogeny (Zanne *et al.* 2014) was pruned to obtain a family level phylogeny, with one species per family as tips. From this pruned phylogeny we calculated the phylogenetic signal of mycorrhizal traits and diversification rates. For the continuous variables - mycorrhizal diversity index and diversification rates - we calculated phylogenetic signal using Pagel's Lambda (Pagel 1999) using the function phylosig in the package phytools in R (Revell 2012). For the categorical variable, mycorrhizal state, we estimated the phylogenetic signal using the D parameter (Fritz & Purvis 2010) with the function phylo.d in *caper* package in R (Orme *et al.* 2018).

Statistical analysis

As some (but not all) of the mycorrhizal traits and diversification rates showed significant phylogenetic signal (Appendix S4), we evaluated the effect of mycorrhizal associations on diversification rates by both considering and not the phylogenetic structure in the residuals. We tested for potential differences in diversification rates between plant families with different mycorrhizal types using both ANOVA and a phylogenetic ANOVA using the function phylanova from phytools in R. Each mycorrhizal state was used as group and their diversification rates as response variable. Because the mycorrhizal states OR and ER only had one and two family respectively, those were removed from this analysis.

To test for the relationship between mycorrhizal heterogeneity and diversification rates we performed a linear model with raw data, and a PGLS regression in the R package *caper*) (Orme *et al.* 2018) with diversification rates as response variable and mycorrhizal heterogeneity as explanatory variable. For PGLS models we used the lambda value obtained from the previous phylogenetic signal analysis. To further explore the potential confounding effect and the association between mycorrhizal association and diversification dynamics, we performed PGLS regressions to assess the relationship between mycorrhizal diversity index, age and species richness.

Results

The genus-level list from FungalRoot database contained information about mycorrhizal state of 295,221 species that belong to 392 families of seed plants. From these, the families OM (Orchidaceae) and ER (Ericaceae and Diapensiaceae) were excluded due to lack of replication, and 18 MIX families were excluded because of the inability to stablish the proportion of mycorrhizal status within them (see Materials and Methods). Then, we kept 372 families for the analyses. According to our classification, using 60% threshold for mycorrhizal state assignment, 290 families were AM (for example, Amaryllidaceae, Asteraceae and Lamiaceae), 9 were EM (like Fagaceae, Nothofagaceae, Betulaceae and Pinaceae), 46 were NM (such as Brassicaceae, Caryophyllaceae and Juncaginaceae) and 27 were mixed (Fig. 2). Mixed families contain species that retained the ancestral state (AM) and species that present a different mycorrhizal state (EM or NM). There

were three different types of mixed families: 21 mixed families had AM and NM species (such as Amaranthaceae, Cyperaceae and Juncaceae), four had AM and EM species (Casuarinaceae, Hydrocharitaceae and Juglandaceae) and three had AM, EM and NM (Goodeniaceae, Nyctaginaceae and Polygonaceae). The phylogenetic signal strength differs among mycorrhizal types, but all mycorrhizal states are phylogenetically clustered to some extent (Table S21). Likewise, the phylogenetic signal of diversification rates was significantly different from a random structure in r□ = 0.0 and r□ = 0.9 (Table S22). There was a significant difference in diversification rates between the mycorrhizal states, irrespective of the extinction scenario (standard ANOVA: $r_{\epsilon = 0.0}$: F = 7.25, p = 0.013; $r_{\epsilon = 0.9}$: F = 7.35, p = 0.007; Fig. 3a and 3b), which was observed in the ANOVA and in the phylogenetic ANOVA (Tables S12 and S13). The a posteriori analysis of the ANOVA showed that diversification of MIX families was significantly higher than that of AM and NM families (Table S15) and the same tendency is observed when correcting for the phylogenetic structure (Table S14). The ANOVA also showed there was no significant difference in diversification rates between the different types of mixed families ($r_{\epsilon = 0.0}$: F = 0.67, p = 0.51; $r_{\epsilon = 0.9}$: F = 0.97, p = 0.39).

The higher values of mycorrhizal diversity index were found in Nyctaginaceae (1.09), Polygonaceae (0.98) and Rhizophoraceae (0.726), while the lowest value was zero and it was observed in 275 families that have all species in the same mycorrhizal state, like in Pinaceae (EM, n = 255), Araucariaceae (AM, n = 38) and Droseraceae (NM, n = 189). There was a positive correlation between mycorrhizal diversity index and diversification rates, observed with the linear models and with the PGLS, and under the two scenarios of extinction (Figure 4a and 4c). The R^2 are surprisingly high, and together with the p-values of the models, are shown in each panel of Fig. 4. Mycorrhizal diversity index had no correlation with age and a significant but very low correlation with species richness (R^2 = 0.002 and 0.01, respectively; Fig. 4b and 4d).

The additional analyses of adding a mycorrhizal misidentification to 20% of the species, supported our main conclusions, which are the positive association between mycorrhizal diversity index and diversification rates, and MIX families having higher diversification rates (Appendix S2). The additional analyses at the species level also showed a positive association between mycorrhizal diversity index and diversification rates (Tables S5 and S10). Although the ANOVA analysis at the species level showed a similar tendency for most comparisons, it did not show significant differences on diversification rates among different mycorrhizal

states for all thresholds (Fig. S1 and S3, and tables S1 and S2).

Discussion

The association with mycorrhizal fungi has been indicated as a key acquisition in the evolution of plants, nevertheless its effect on plants diversification has not been evaluated before. Here we presented the first attempt to assess the relationship between mycorrhizal associations and diversification rates of plants. Due to the under-sampling of seed plants phylogeny and mycorrhizal state database, we used a simple and conservative approach that allows us to tackle this question.

Our results showed that there was no difference on diversification rates between AM, EM and NM families (Fig. 3; Table S14 and S15). This shows that families that acquired novel mycorrhizal associations (EM and NM) do not have higher diversification rates than families that retained the ancestral state (AM), contrary to what was expected in a scenario of key innovation in mycorrhizal associations as a mechanism of diversification. Thus, regarding our first question, the lineages that established derived mycorrhizal associations do not differ in their diversification rates from AM families. Contrary, our analyses showed that families with mixed mycorrhizal state have higher diversification rates than AM and NM families (Fig. 3, Table S14). Mixed families included three subtypes of mixed: families with AM and NM species, families with AM and EM species and families with AM, EM and NM species; the three subtypes had higher diversification rates and there was no significant difference on rates between them. This shows that regardless of the mycorrhizal states that composed the mixed families, they have the highest diversification rates, suggesting that it is the diversity of mycorrhizal states that promotes diversification rather than a specific mycorrhizal state.

In addition, there was a positive and significant association between mycorrhizal diversity index and diversification rates, which does not depend on our categorical criteria of mycorrhizal state assignment to families. These associations with diversification rates, are observed when correcting or not for the phylogenetic structure, suggesting that the relationship is not due to phylogenetic relatedness between families. Also, the patterns are observed under different scenarios of extinction, and even with ϵ = 0.9, where extinction could have an important role, the relationship is conserved. Given that diversification rates are determined

by age and richness of the family, the effect of those variables could have driven the relationship between mycorrhizal heterogeneity and diversification rates. We observed no significant correlation between mycorrhizal heterogeneity and age; and we see a similar pattern with species richness, although the correlation is significant, but the R^2 is quite low (Fig. 4b, 4d). This supports that mycorrhizal heterogeneity is mainly associated with diversification rates, not with age or richness per se. In addition, these patterns are also observed when we analyzed the species-level data (Appendix S1).

Both results, the ANOVA for family mycorrhizal type and association between mycorrhizal heterogeneity and diversification, suggest that independent of which mycorrhizal state is involved, a higher heterogeneity of mycorrhizal states in a family might promote diversification rates. We interpret mycorrhizal heterogeneity as a result from a higher evolutionary lability of the mycorrhizal states within these families, which has been suggested to promote diversification in other biotic interactions (Hardy & Otto 2014). Each mycorrhizal state provides advantages to plants in certain environments but not in others (Brundrett 2002), thus families that are composed by species with different mycorrhizal states might have been able to switch states in evolutionary time, making them able to evolve a higher diversity of niches which would result in a higher diversification rate. Under this scenario, mycorrhizal diverse families would have had more chances to take advantage of a new ecological opportunity, than families with most species within a single mycorrhizal state. It is interesting to note that mycorrhizal diverse families have not only higher diversification when compared to low diverse families with the ancestral state, but also higher rates than families that have switched from the ancestral state to one novel mycorrhizal state (NM and EM families).

The mycorrhizal diversity index might not capture well the effects of mycorrhizal shifts on diversification rates if shifts occurred only once within each family. However, we observed that mycorrhizal shifts in mix families occurred multiple times, because more than 97% of mixed families contain genera that have multiple mycorrhizal states, this means that the MS do not form monophyletic sub-clades and shifts occur even below the genus level. This suggests that diversification rates are not the result of a single mycorrhizal shift, but a result of high lability of the mycorrhizal types within the mixed families. These results together suggest that rather than a key innovation scenario, it is the evolutionary variability of mycorrhizal state what promotes diversification rates of plant seed families. Our results also highlight the evolutionary role of specialization at

different organization levels: even if species are mycorrhizal specialized within a mixed family, the possibility to switch to different mycorrhizal states might increase the diversification of the family.

Because biodiversity dynamics could be rather complex, with clades either expanding, at equilibrium and even declining in diversity, simple metrics like the average rate of diversification might not be able to separate them (Quental & Marshall 2010). The use of an average rate as a descriptor of a clade diversification dynamics assumes (or at least equates to) a scenario of expanding diversity (Quental & Marshall 2010), and it might be especially problematic if lineages have a carrying capacity because the average rate might be diluted as time goes by (Rabosky 2009). Moreover, with an average rate is not possible to distinguish between speciation and extinction rates or to test directly the effect of one trait on diversification dynamics. Ideally one would use more complex tests, but that would require a lot more phylogenetic data than what is currently available. Overall, we used a relatively simple and limited macroevolutionary method and our conclusions arose from a limited ecological and phylogenetic data. These conclusions might be revisited in future studies, when more data on mycorrhizal states and more complete phylogenies of plants are available.

Acknowledging the limitations of our study, the results suggest that a higher diversity of mycorrhizal strategies promotes diversification of lineages, possibly related with new ecological opportunities that each mycorrhizal state provides to plants. Our results finally suggest that the associations between soil fungi and plants has been key for plant diversification, not only due to the foundational association that allows plants colonize land (Pirozynski & Malloch 1975) but also for further diversification of seed plant lineages.

Acknowledgements

We thank all members of the Lab MeMe in the Institute of BioScience at the University of São Paulo for discussions and suggestions; and Guillermo Bueno for his contribution to the database. T.B.Q. thanks FAPESP for financial support (grants # 2012/04072-3 and #2018/05462-6), M.F.P thanks CONICYT for financial support (grants #1171369), G. B. thanks FAPESP for financial support (grant # 2018/04821-2) and M.IM. thanks CONICYT for the doctoral scholarship # 21151009.

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Figure captions

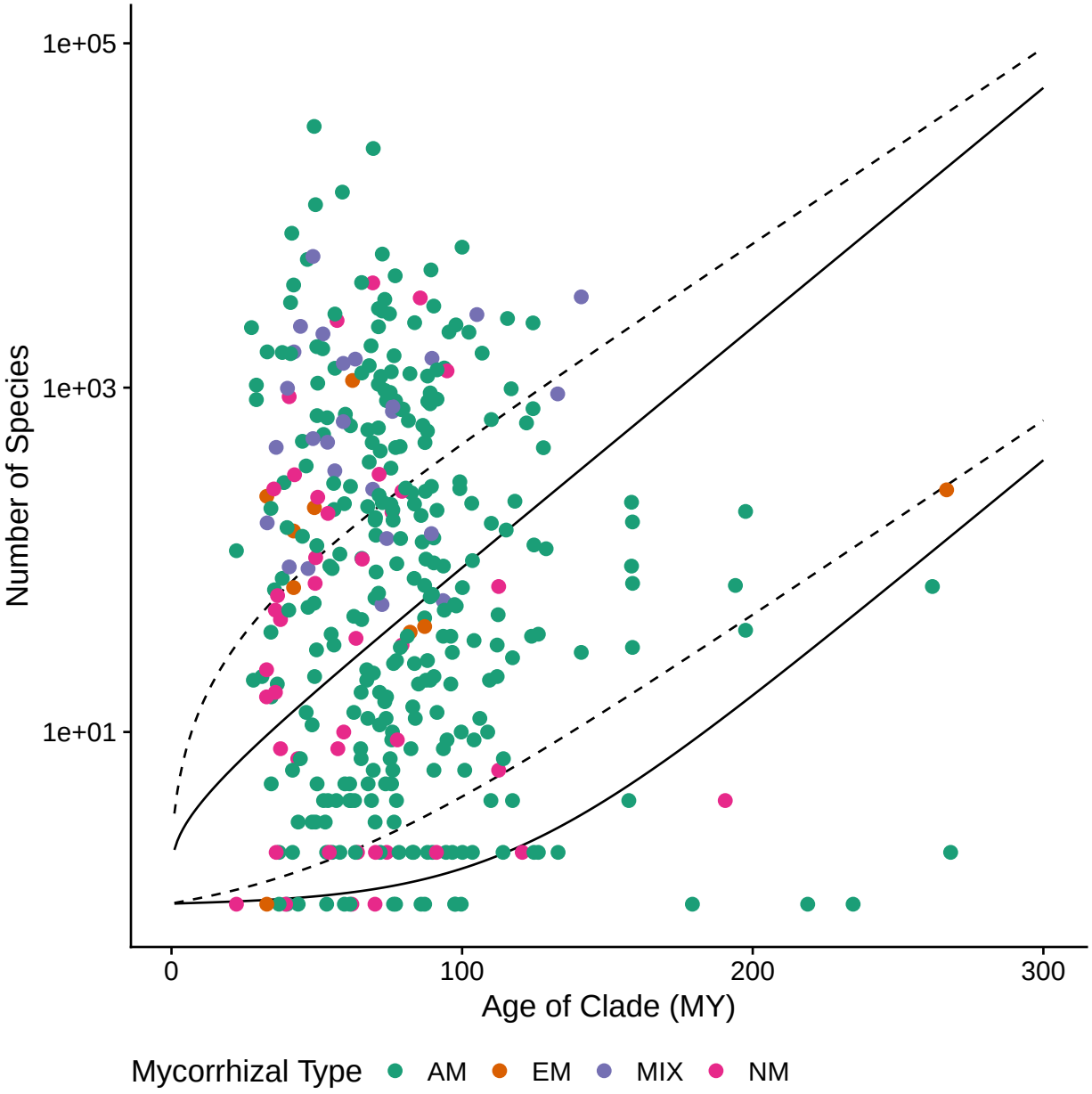
Figure 1: Relationship between number of species and age for each lineage when compared to the confidence intervals based on the global diversification rate of seed plants. The solid and dashed lines represent the expected richness for epsilon = 0 and epsilon = 0.9, respectively. The color of the points represents the mycorrhizal state for the 60% threshold.

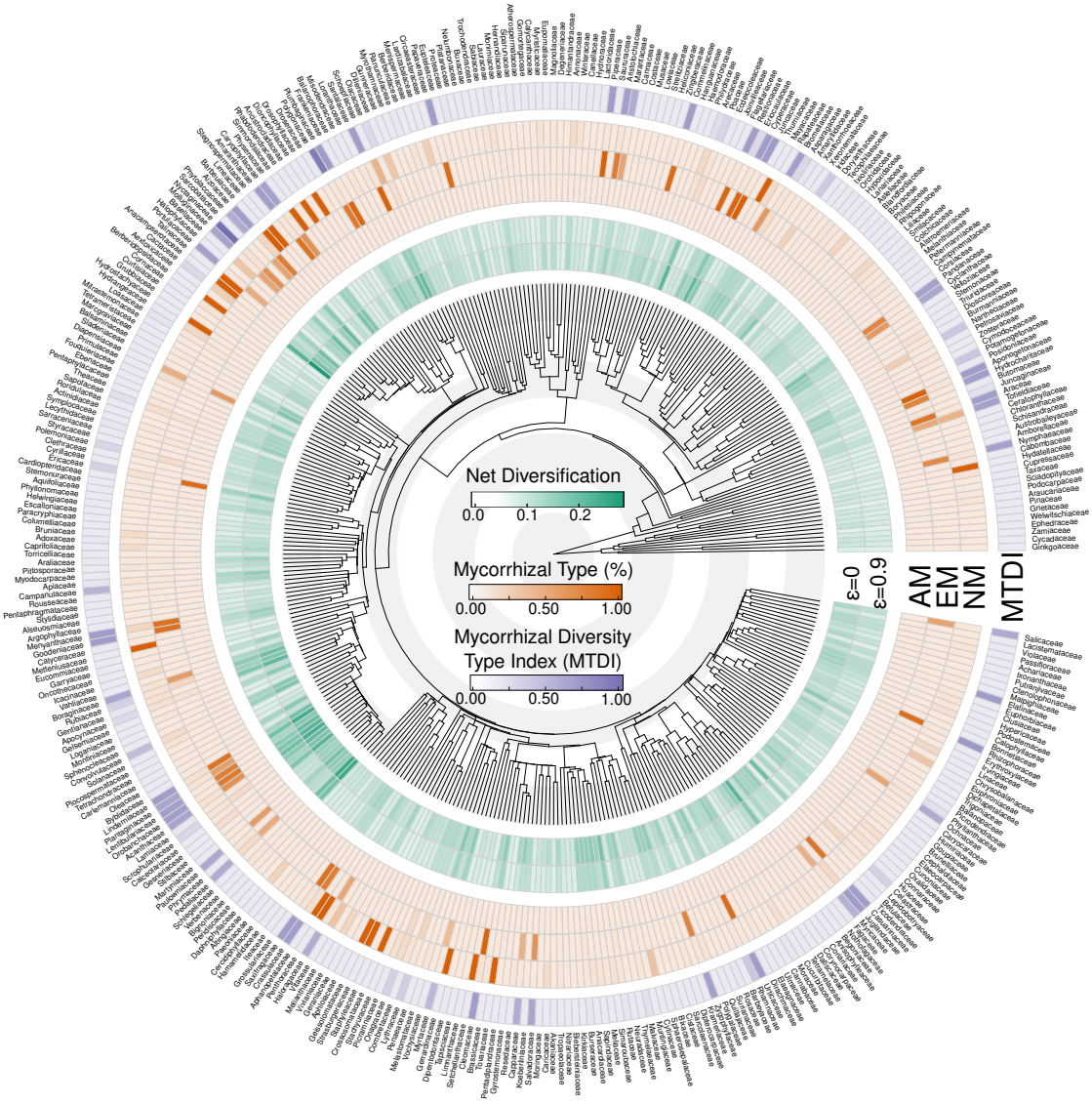
Figure 2: Family-level, time-calibrated phylogeny for the 367 seed plant families included in the analyses. For each family, the proportion of species within each mycorrhizal type is represented in the rose-to-red boxes, AM: Arbuscular mycorrhiza, EM: Ectomycorrhiza and NM: non-mycorrhizal. The mycorrhizal diversity index (MDI) is represented in the purple boxes and the diversification rates (r) are shown in the green boxes. To illustrate the timescale of the phylogeny, the width of concentric white and gray circles represents 50 million years.

Figure 3: Relationship between mycorrhizal type and diversification rates. a) diversification rate estimated with ϵ (relative extinction fraction) = 0 and b) diversification rate estimated with ϵ= 0.9. AM: Arbuscular mycorrhiza, EM: Ectomycorrhiza, NM: non-mycorrhizal and MIX (families with no dominance of any specific mycorrhizal association). The size of the points indicates the Mycorrhizal Type Diversity Index value for each lineage, indicating a predominance of larger indices with higher diversification rates.

Figure 4: Scatterplots showing the relationship between mycorrhizal diversity index and diversification rates (a and c), species richness (b) and age family (d). Diversification rates were estimated with ϵ (relative extinction fraction) = 0 (a) and with ϵ = 0.9 (c). The red and blue lines indicate the results of a linear model and a phylogenetic generalized least squares (PGLS) fit, respectively. Respective p-values and R^2 are shown in each panel.

Figures





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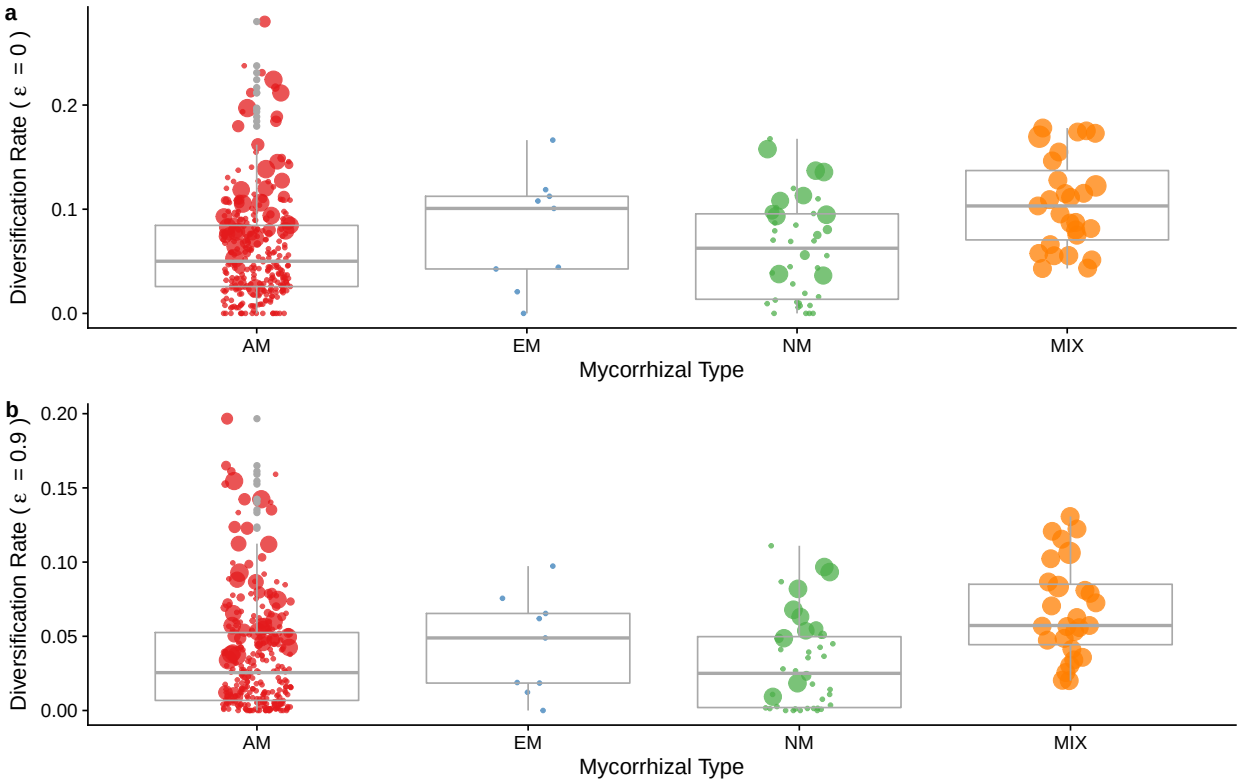

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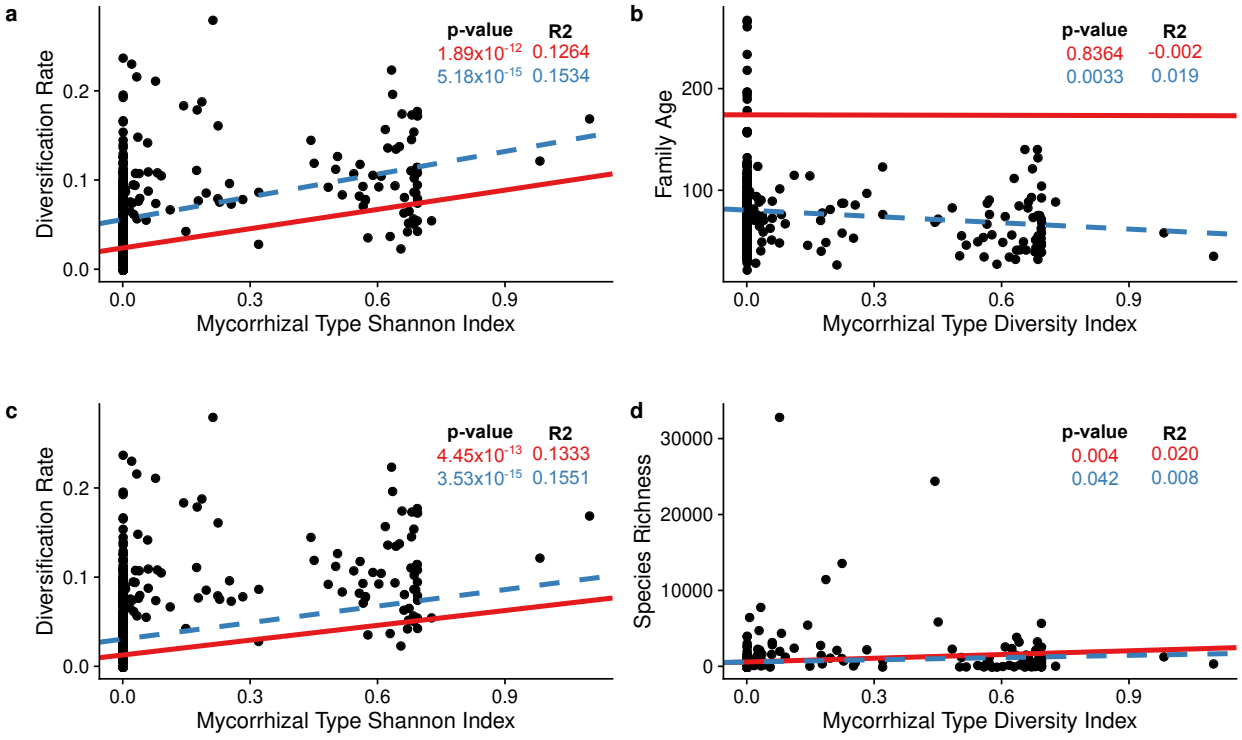

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