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On the evolution of ectomycorrhizal fungi

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Abstract

Ectomycorrhiza is a symbiosis between plants and fungi. It is the type of mycorrhiza that involves the highest number of fungal species and it involves many stand forming tree species. As the ectomycorrhizal trees include the majority of all the worlds tree stems, it has a huge impact on the nutrient and carbon cycles. Here, we review the evolution of ectomycorrhiza within fungi. It has evolved many times in different clades. The rate of evolution has not varied much through time, but has varied between clades. Thus, no time period seems to have been more important than others for the evolution of new ectomycorrhizal lineages. It seems like once becoming ectomycorrhizal the probability of becoming saprotrophic again is very low. Despite the large change in nutritional strategy, it does not seem like becoming ectomycorrhizal is a key innovation that by itself gives a high rate of speciation and/or adaptive radiations. Genomic studies have shown little commonality in the evolution of ectomycorrhiza except for the loss of decay genes, expansions of transposable elements, and expansions of Mycorrhiza-induced Small Secreted Proteins. Although this fits with a biotrophic lifestyle and possibly an elevated rate of genome evolution, it does not hint at any other functional similarity. As ectomycorrhizal lineages have evolved many times during the evolutionary history of fungi, and it is not unlikely that there are ectomycorrhizal lineages that have gone extinct, the available functions provided by ectomycorrhiza to plants may have varied over time.

Keywords – Evolutionary analysis – Evolutionary transition – Genome evolution – Key innovation – Symbiosis

Introduction

Mycorrhiza is a symbiotic relationship between plants and fungi where fungi receive energy-rich carbon compounds from the plants, and the plants receive nutrients, water, and other benefits from the fungi (Smith & Read 2010). The contact takes place in the plants' roots, hence termed mycor- (fungus) rhiza (root). Mycorrhiza was most likely key to the successful colonization of land by plants (Pirozynski & Malloch 1975, Humphreys et al. 2010, Wang et al. 2010), one of the most important transitions in the history of life as plants are the major terrestrial primary producers and largely influence the terrain. Mycorrhiza is arguably the most diverse biotrophic nutritional mode within fungi as seen by the combination of the number of disparate clades, and the number of species involved (Rinaldi et al. 2008, Tedersoo et al. 2009).

Mycorrhiza has been divided into many different classes based on the structures of the interface between the plant and fungi. Ectomycorrhiza (ECM; Fig. 1) is characterized by three

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structures, 1) The Hartig net where the fungal hypha encloses the outer cortical cells of the fine roots of the plant, 2) the mantel layer where the fungi form a dense tissue around the surface of the fine root, and 3) the extraradical mycelium where the fungal hypha extends out in the soil. The first of these can be seen as the defining character of ECM, while the two latter may be more or less well developed. Another characteristic (or lack of) is that the fungi do not cross the cell wall of the plant. There are other classes of mycorrhiza that share the characteristics of a Hartig net, a mantel layer and an extraradical mycelium, but for which the fungi penetrate the plant cell wall. Some of these ECM-like classes are specific for certain plant taxa (e.g. Arbutoid and Monotropoid mycorrhiza), while others involve particular combinations of plants and fungi (Ectoendomycorrhiza; Smith & Read 2010).

Ectomycorrhiza is the most diverse type of mycorrhiza among fungi, with more than 20,000 species (Rinaldi et al. 2008) in more than 80 separate clades (Tedersoo et al. 2009). Even if most plants form mycorrhiza it is only about 2% of the species that form ECM (Brundrett 2017). However, these 2% include the most dominant tree species in forest ecosystems covering a large proportion of the globe and have been estimated to associate with 60% of all the tree stems (Steidinger et al. 2019). This symbiosis thus has a huge global impact on nutrient and carbon cycling, and their importance for many forest ecosystems has led to many studies focusing on different aspects of ECM. One aspect that has received relatively little attention is the evolution of ECM. Here, we review what has been learned on some of the big questions about the evolution of ECM fungi, draw some general conclusions from these findings, and also discuss how analytical shortcomings may give misleading results.

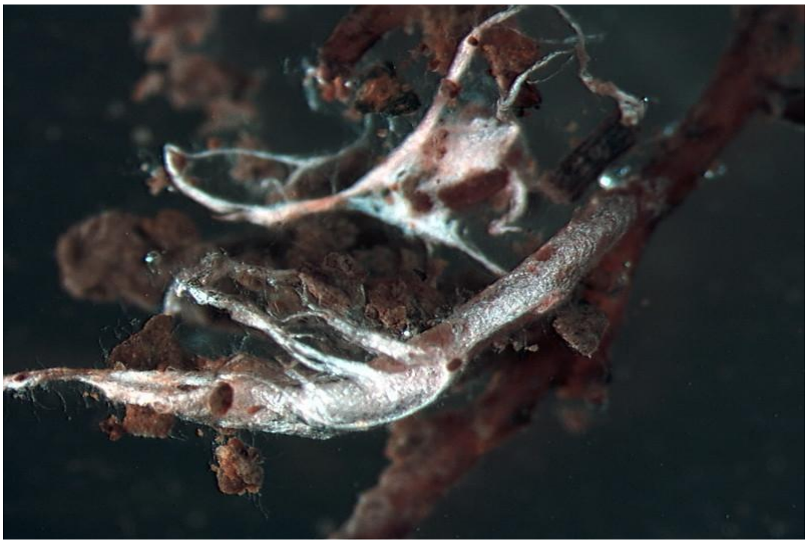

Figure 1 – Ectomycorrhizal root tip. This picture is copyright of Martin Ryberg.

Parametric vs non-parametric methods

There are two major ways to statistically compare hypotheses: 1) parametric, where you have a model with different parameters giving the outcome of the process; and 2) non-parametric where you analyse the process without being dependent on or considering any parameters. When analysing trait changes from an evolutionary perspective (phylogenetic), parsimony is the most frequently used non-parametric method. It simply selects the history that infers the least number of changes, or less commonly the history that gives the lowest score according to a scoring matrix (e.g. in DIVA; Ronquist 1997). The parametric methods, for analysing trait changes, usually use a probabilistic model with parameters giving the probability of going from one character-state to another or changing along one or more continuous axes. The model parameters are then estimated in a maximum likelihood (ML) or Bayesian statistical framework.

In parsimony, the score will entirely depend on your scoring scheme, so a comparison between different scoring schemes can only say how much you need to change in the scoring to get another reconstruction of the evolutionary history, but there is no intrinsic statistic to say if this is significant or not. It is also difficult to judge what difference in score is significant even if using the same scoring scheme, and therefore to say if one result is significantly better than another. When looking at specific traits bootstrapping does not work, as you need many characters to bootstrap. It is however possible to make randomisation tests to check if the trait depends on the topology at all, or is just randomly distributed among the taxa (Archie 1989). If given only one result, it is easy to get overconfident in that result, so parsimony works best for traits with few changes and a clear pattern. As parsimony does not estimate parameters it is also unsuitable to test hypotheses about parameters.

Many questions about the evolution of ECM fungi are best addressed as hypotheses formulated in the form of models and model parameters, for example, differences in rates to and from the ECM habit, or differences in the probability of becoming ECM during different time periods, as other ways of formulating them often require a near complete sample of the diversity of ECM fungi and relevant related taxa. However, no model is true but may just be a better or worse representation of reality. It is therefore important to evaluate the results of a model-based analysis and consider alternatives. Using explicitly parameterized probabilistic models it is possible to compare different hypotheses of how evolution has occurred. This can be done by including different parameters and/or restricting parameter values, such that they correspond to the different hypotheses. It can then be tested on how well they fit with the data. It is also possible to generate random datasets using the model and then see if some characteristics of the observed data correspond to the data generated by the model, and therefore if the data is likely to have been generated by the hypothesised process (Goldman 1993, Boettiger et al. 2012).

How many times has ECM evolved, and has any ECM lineage evolved into a saprotroph?

As ECM is present in many disparate clades an important question has been how many times it has evolved, and if each clade represents a separate origin of the ECM lifestyle or if there have been reversals to a non-ECM lifestyle. The discussion of this question was boosted by the publication of Hibbett et al. (2000), which found that there have been reversals and that several orders were likely to have an origin as ECM fungi. This paper has however been largely questioned (Bruns & Shefferson 2004), and Matheny et al. (2006) did not find the order *Agaricales* to be ancestrally ECM, as inferred by Hibbett et al. (2000), based on parsimony analysis. Tedersoo et al. (2009) inferred that each clade of ECM fungi has evolved separately, however, the basis of their analysis was unclear, but parsimony analysis was suggested. In addition, their topology was partially based on unpublished data. Hibbett & Matheny (2009) showed that many of the clades inferred to be ancestrally ECM in Hibbett et al. (2000) are older than the oldest ECM plants, and therefore unlikely to have been ECM.

It has now been widely accepted that each ECM lineage has a separate origin (Ryberg & Matheny 2011). However, a recent publication by Sánchez-García et al. (2020) indicated that even if most orders have a non-ECM origin, a reversal could have occurred in *Thelephorales*. Sheikh et al. (2022) showed that if a constant rate of evolution through the fungal tree is assumed, reversals are very likely while parsimony inferred only one reversal in *Thelephorales*. However, when the rate is allowed to vary along the tree, then a higher rate of evolution in certain clades, including *Thelephorales*, can explain the pattern and reversals are not needed, and such a model fits the overall pattern better than models with constant rate through the tree. To complicate the story, there are also models with different rates during different time periods that also infer a high probability of going from ECM to non-ECM, and these models cannot be rejected as fitting the pattern of ECM among taxa worse than models without transitions from ECM to non-ECM. However, the models with different rates during different times also infer that the most recent common ancestor of *Mucoromycota* and *Dikarya* was likely ECM. However, this seems questionable since this ancestor very likely predates the vascular plants as members of both *Mucoromycota* and *Dikarya* can be found in the early (although not in the earliest) fossil record of vascular plants (Taylor et al. 2009, 2014). The pattern of ECM among the tips in the fungal tree of life may not be enough to decisively determine this question, the process that fits best with this pattern, the evolutionary history of plants, and patterns in the evolution of genome characteristics (Miyauchi et al. 2020) suggest that reversals from ECM are very unlikely (but perhaps not impossible). It should be pointed out that many ECM fungi have enzymes that are involved in the decomposition of complex carbon compounds, and such genes may even proliferate within ECM lineages (Bödeker et al. 2009). There are even species that can form ECM but have also been inferred to be able to live saprotrophically (Vaario et al. 2012, Wu et al. 2021). These seem to be the rare exceptions and there is probably a cost to maintaining that nutritional flexibility. It is also unclear to what extent this flexibility extends to natural conditions. Even if it is not inconceivable that reversals may happen and that there is probably not enough power in the present-day patterns to exclude a very low rate of transition from ECM to saproptrophy, it is unlikely that any major and ancient clade of saprotrophic fungi stems from an ECM ancestor. However, to infer that there have been no reversals to saprotrophy we would need data for every possible case, which given our present state of knowledge about fungal diversity seems to be far off. There are examples of ECM lineages that were suggested to have evolved into biotrophic parasitic lifestyles, such as *Entoloma clypeatum*, *Pseudoboletus parasiticus*, *Chroogomphus* and *Gomphidius* (Sánchez-García et al. 2020), indicating that becoming ECM is not a dead-end evolutionary trajectory.

When and where did ECM evolve?

Several hypotheses have been proposed on when ECM evolved. Bruns et al. (1998) suggested that the expansion of temperate forests after the mid-Eocene climate maximum was important for the diversification of ECM. Others have suggested that ECM fungal lineages evolved together with the major ECM plant lineages (e.g. Halling 2001) or in the early Jurassic - or even Triassic or Late Permian - with Pinaceae, and Late Cretaceous with the rise of ECM angiosperm lineages (Taylor et al. 2009). It does seem like the oldest lineages correspond reasonably well to the age of Pinaceae (Jeandroz et al. 2008, Sheikh et al. 2022), but there is a great uncertainty in these estimates (Berbee & Taylor 2010). Separate fungal ECM lineages have been found to have evolved during the Late Cretaceous and after the mid-Eocene climate, but Sheikh et al. (2022) did not find any consistent evidence that any combinations of the suggested three periods have had an increased rate of evolution in ECM lineages. It rather seems like ECM has evolved at a fairly constant rate through time, but some clades have a high rate of evolution, while others have a lower rate. The fact that many ECM lineages seem to have evolved fairly recently (Late Cretaceous or more recently; Ryberg & Matheny 2011, Looney et al. 2016, Varga et al. 2019, Sheikh et al. 2022) may thus just be a statistical effect that there are more recent than ancient lineages if only present-day taxa are included in a phylogeny. A simple count of the ages of ECM clades through time is thus not a good way to address a hypothesis that a particular period has been more important than others for the evolution of ECM. It is not unlikely that distinct ECM lineages have gone extinct, given estimates of the extinction proportions of Agaricomycetes (Sánchez-García et al. 2020). There has thus probably been a turnover in ECM communities through evolutionary times, and the first ECM lineage may not be around anymore.

It is unlikely that the geographic region where the first ECM lineage evolved can be reconstructed from present-day patterns because it is still uncertain which ECM lineage was the first, most of ECM lineages seem to have a wide distribution (Tedersoo et al. 2009), dispersals between continents are not expected to be rare on a relevant time scale (Houdanon et al. 2022), and given current limitations in biogeographic models (Landis et al. 2013). However, assuming an early evolution together with Pinaceae, the origin could have been on Laurasia, or even the supercontinent Pangaea (Domogatskaya & Herman 2019, Matsunaga et al. 2021).

Individual fungal ECM lineages seem to have very different origins. *Laccaria* for example seems to have a clear southern hemisphere origin (Wilson et al. 2016), while *Inocybaceae* has been inferred to have a Paleotropical origin (Matheny et al. 2009). Given that the earth was largely tropical during the Eocene, it is likely that lineages that are older than that have gone through tropical habitats. In spite of this *Cortinarius* seems to be older than that (Ryberg & Matheny 2011, Varga et al. 2019, Sheikh et al. 2022), and has relatively few tropical species that seem to be nested within otherwise temperate lineages (Peintner et al. 2003, Houdanon et al. 2022), which is more consistent with a temperate origin.

Is ECM a key innovation that has led to adaptive radiations?

Many people have suggested that ECM is a key innovation and that transitions to an ECM habit should lead to adaptive radiations. However, most studies indicate that this is not the case. Ryberg & Matheny (2011) found no evidence of any rapid radiation early in the history of nine separate clades of ECM fungi, a key pattern that is expected for adaptive radiations. Patterns of early radiations may however be obscured by later radiations within the group. It seems like many other things may affect species diversification within ECM lineages, for example, Wilson et al. (2016) indicated that the transition of *Laccaria* from the southern to the northern hemisphere led to a radiation of species, and Kennedy et al. (2012) found an increased rate of diversification after a shift to temperate regions in *Clavulina*. This means that even if the expansion of the temperate forests after the mid-Eocene climate may not have had a great impact on the number of distinct ECM lineages, it may have had a significant effect on the number of ECM species, but that remains to be tested for a large number of cases. There may also be other processes increasing the rate of species diversification (Sánchez-Ramírez et al. 2015) that may be even more important. So even if we cannot exclude that transitions to ECM may lead to radiation of species, adaptive radiation does not seem to be the major process explaining the species diversity we see today. When looking for adaptive radiations in ECM lineages it may be better to study younger lineages, as these will have had less time to be affected by other processes. Adaptive radiation is also expected to affect the rate of evolution of adaptive traits, but this has not been studied in detail in ECM lineages. Perhaps because it is difficult to identify such traits and code them for a large number of species. In Arbuscular Mycorrhizal (AM) fungi there are indications that such early radiations into major partitions of niche space have happened (Maherali & Klironomos 2007), but as the evolutionary history between AM and ECM fungi is so different it is difficult to say if this has any bearing on ECM evolution.

If ECM is a key innovation in fungi, we would also expect to see an increase in species diversification rate in ECM lineages. It has been found that ECM clades sometimes are larger than their sister clades, for example, the ECM *Amanita* (Wolfe et al. 2012), and ECM *Laccaria* (Wilson et al. 2016). However, Sánchez-García & Matheny (2016) and Sánchez-García et al. (2020) found no evidence of a general increase in diversification rates in ECM lineages, but some ECM lineages (not including *Amanita* and *Laccaria*) do have increased rate of diversification when compared to their non-ECM sister clades. It seems like species diversification rates depend more on other traits, such as sporocarp morphology than nutritional mode (Sánchez-García et al. 2020).

One reason to expect ECM to be a key innovation is that switching to an ECM habit would open an entirely new niche space to radiate into, but as ECM have evolved asynchronously, the niche space was probably not entirely open for most lineages when becoming ECM. This could, in theory, lead to older lineages having more opportunities to radiate as they in turn would already occupy part of the niche space when younger lineages became ECM. However, this prediction was tested and rejected in *Boletales* by Sato & Toju (2019), and many of the most diverse lineages seem to have an Eocene or Cretaceous origin, and not to be among the oldest lineages (Sheikh et al. 2022, Varga et al. 2019). The lack of a positive correlation between the age of a clade and the species diversification rates could be due to that part of the niche space that is available to the lineage depends on the traits that it brings with it from before it became ECM. The available niche space will then be highly lineage-specific and may, or may not, be larger than for older ECM lineages, saprotrophic sister groups, or offering room for adaptive radiation. Younger ECM lineages may also intrude on the niche space of older lineages, reducing their diversity, and obscuring any patterns of previously high diversification rate. Alternatively, the niche space may not be the limiting factor for speciation or even co-existence, and other factors may matter more (Hubbell 2001).

What has been learned from whole-genome comparisons, and what unites ECM fungi?

Different ECM lineages have derived independently from functionally diverse saprotrophs with different decay capacities e.g brown rots, white rots, soil and litter decayers etc (Kohler et al. 2015, Martin et al. 2016, Miyauchi et al. 2020). The convergent losses of much of the ancestral saprotrophic decay apparatus is a hallmark of most (but not all) of the ECM genomes studied so far (Kohler et al. 2015, Peter et al. 2016, Hess et al 2018, Murat et al. 2018, Chang et al. 2019, Miyauchi et al. 2020, Wu et al. 2021, Looney et al. 2022). The contraction of gene families coding for CAZymes (Carbohydrate Active Enzymes), particularly Plant Cell Wall Degrading Enzymes (PCWDEs) act on lignocellulose (Kohler et al. 2015, Peter et al. 2016, Miyauchi et al. 2020) suggests a reduced capacity of ECM fungi to decompose plant cell walls in comparison to their saprotrophic ancestors, as well as an increased dependence on the plant hosts for the acquisition of carbon compounds. Nevertheless, different ECM species have retained different unique sets of PCWDEs, showing their potential to degrade substrates in soil organic matter (Kohler et al. 2015, Miyauchi et al. 2020, Wu et al. 2021).

Another characteristic of ECM genomes is the expansion of effector-like Small Secreted Proteins (SSPs). These symbiosis-related genes facilitate plant-fungal interactions by suppressing hosts' immune system and help in the establishment of mycorrhiza (Plett et al. 2011, 2014). Among symbiotic effectors, Mycorrhiza induced Small Secreted Proteins (MiSSPs), are found in most of the ECM genomes (Martin et al. 2008, Kohler et al. 2015, Miyauchi et al. 2020) and several sub-categories of MiSSPs are involved directly in the early development of symbiotic structures like Hartig net (Pellegrin et al. 2019). A major chunk of the symbiosis genes is lineage-specific orphan genes, which include SSPs, genes of unknown function and genes involved in different signalling pathways. Though frequently reported by different comparative genomics studies, the elevated gene copies of SSPs are not shared by all ECM genomes e.g. species in different ECM lineages like *Amanitaceae*, *Russulaceae* and *Endogonaceae* families have moderate to low content of SSPs as compared to their closest saprotrophic relatives, suggesting that different ECM lineages may have adopted different ways to interact with their hosts, have a lower need for other functions of the SSPs, or are just able to duplicate functions for the same SSP genes (Hess et al. 2018, Murat et al. 2018, Looney et al. 2022).

Most of the ECM genomes have also been marked by the overload of repeat content and large genome sizes (Peter et al. 2016, Murat et al. 2018, Chang et al. 2019, Miyauchi et al. 2020, Looney et al. 2022). A strong correlation found between the genome size and content of Transposable Elements (TEs) in ECM genomes is suggestive of the role of TEs proliferation in genome size expansions (Murat et al. 2018, Miyauchi et al. 2020, Looney et al. 2022). Transposable elements appear to invade ECM genomes independently as different TE categories were found in different ECM lineages (Chang et al. 2019, Miyauchi et al. 2020). Furthermore, TEs are located in close proximity to SSP genes in ECM *Russulaceae* and *Boletaceae* lineages indicating their potential involvement in gene innovation (e.g. duplications) and regulation (Wu et al. 2021, Looney et al. 2022).

Most symbiosis-related genes in ECM lineages have orthologues in their non-symbiotic relatives and these genes are suggested to be co-opted from already existing ancestral genes during the divergence process from saprotrophy (Hess et al. 2018, Miyauchi et al. 2020, Wu et al. 2021, Looney et al. 2022). Different sets of ancestral metabolic and signalling genes have been used by different independent lineages of ECM to serve the new functions required in symbiosis (Miyauchi et al. 2020). After the origin of mycorrhiza, novel genes unique to each ECM lineage have also evolved and diversified. Examples of these lineage-specific ECM-related changes include upregulation of aquaporins in *Cenoccocum geophyllum* genome, reduced NRPS (Nonribosomal Peptide Synthase)-like secondary metabolite clusters in *Russulaceae*, upregulation of fruiting body genes and genes involved in volatile compounds metabolism in *Tuberaceae*, and host-specific secondary metabolites evolution in *Suillineae* lineage genomes (Peter et al. 2016, Hess et al. 2018, Murat et al. 2018, Miyauchi et al. 2020, Lofgren et al. 2021, Looney et al. 2022).

The rate of evolution of changes in the genome has been suggested to vary through the evolution of ECM lineages. Hess et al. (2018) showed that most of the contractions of families coding for CAZymes happened during the transition to ECM, while the proliferation of TE content happened before that. Wu et al. (2021) suggested that there has been an increase in gene duplication rates since the Oligocene within *Boletales*. This would be consistent with an increased rate of evolution at the same time as the temperate forests expanded, and may suggest a diversification consistent with the hypothesis of Bruns et al. (1998). However, the demonstrated pattern is based on counts of duplications reconstructed by a parsimony fit of the gene trees into the species tree (i.e. branching in the gene tree that does not correspond to speciation events). It is thus not a parametric estimation of the rate of duplications, but a secondary estimation not considering the loss of genes. Losses will effectively delete branching points from the tree but not in a regular fashion and will create an apparent increase in duplications towards the present (Cotton & Page 2005). It is therefore unclear if their pattern represents an increase in duplication rates or if it is just consistent with a process of constant rates of duplications and losses through time (Fig. 2). A pattern of increased rate towards the present may also be due to an increased rate of evolution in separate clades, i.e. the pattern is not so much about time as it is about the evolution of individual lineages. Wu et al. (2021) indicated that, if there is any change, this may indeed be driven by changes in rates in *Boletaceae* and *Suillineae* rather than a general increase in duplication rates after the Oligocene.

It is not always straightforward to draw conclusions from parametric methods, and it should be remembered what hypothesis is tested. Looney et al. (2022) inferred an increase in the rate at the terminal branches in *Russulaceae* using a parametric method. However, the maximum likelihood estimate may very well infer different rates between branches even if the rate has been constant (Fig. 3). It is therefore important to test if the inferred process indeed fits the data significantly better than a simpler process, for example with a constant rate through time, which is not clear from the analysis of Looney et al. (2022).

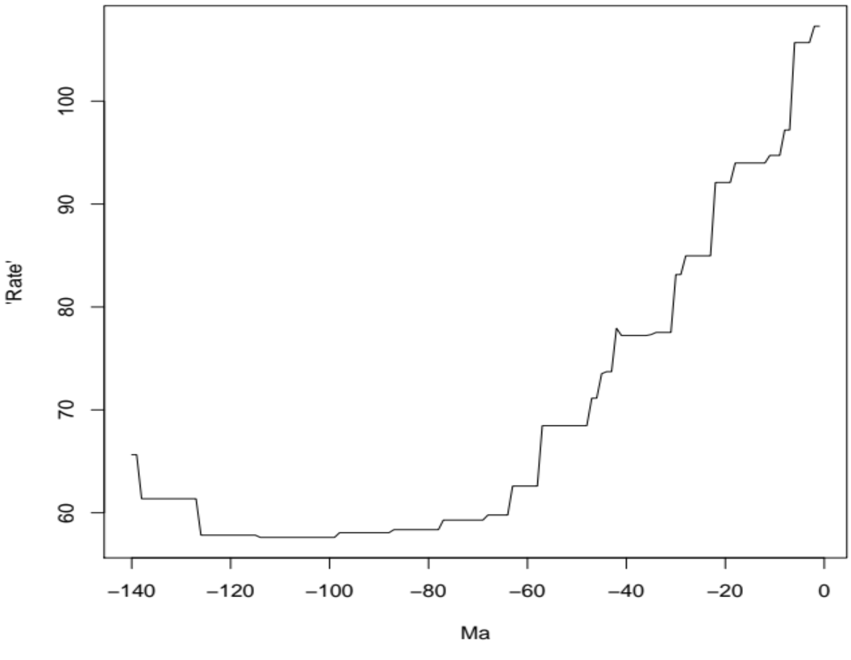

Figure 2 – Apparent mean duplication rate among branches calculated every 1 Myr, based on 10,000 simulated gene trees with constant rates of 0.2 duplications and 0.2 losses per Myr throughout the tree. Phylogeny was based on Wu et al. (2021). Simulations were made with PrIME-GenPhyloData (Sjöstrand et al. 2013).

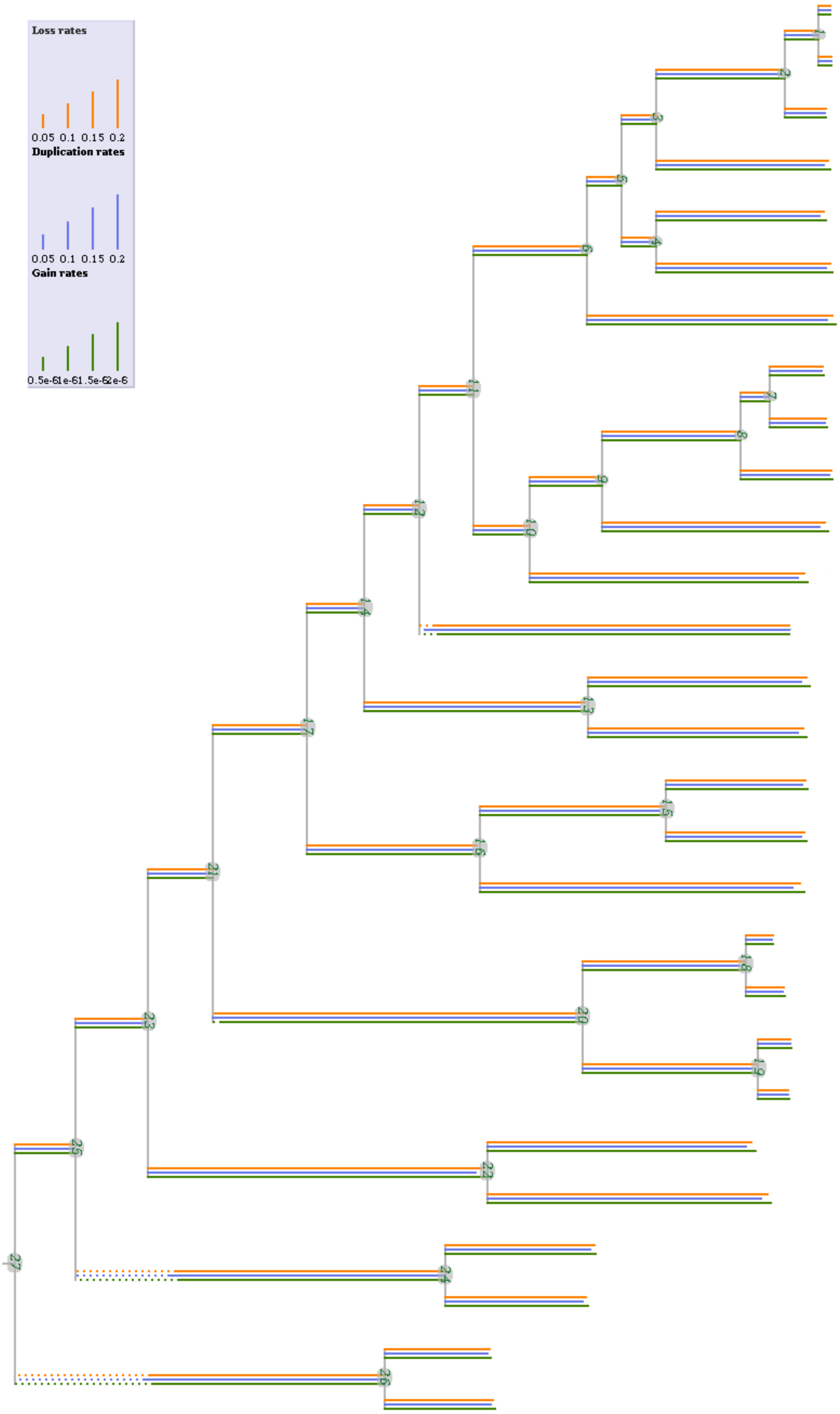

Figure 3 – Estimations of gene duplication, loss, and gain rate using Count (Csűös 2010). Allowing for different rates in each branch, based on the same simulations with constant rate through the tree as in Fig. 1.

To get a better understanding of the evolution of gene families in ECM lineages, and if recent rate increases are common, we need to have a thorough understanding of gene family evolution and make explicit tests of different hypotheses. The methods in use today have different strengths and weaknesses, which should be considered when making inferences about gene family evolution. The most commonly used parametric methods only consider gene counts of the different families (Csűös 2010, Mendes et al. 2020), while the parsimony methods can take into account phylogenetic information, but then do not give direct estimates of duplication and extinction rates. Which one is best will depend on the question at hand.

Studies of ECM morphology reveal both differences and similarities between lineages. Agerer (2001) described five main types of exploration types, with one further subdivided into three categories. Each of these categories include members from separate clades of ECM fungi, and some clades have members of different types. These exploration types have been suggested to have great functional importance (Agerer 2001, Koide et al. 2013), however, it is not known if these exploration types correlate with any patterns in the genome.

Conclusions

ECM lineages have evolved at different times, and in different places. There seem to be few general patterns in the evolution of ECM in fungi beyond contractions of gene families coding for CAZymes, expansion of TEs, and SSPs. ECM does not seem to be any key innovation in fungi leading to rapid speciation. However, transitions to new habitats such as temperate forests seem to have increased diversification rates in at least some lineages. Given that ECM lineages have originated independently they may have fairly different functions depending on what traits they bring when becoming ECM. The diversity of functions is also indicated by the differences in exploration types and lack of clear similarity in genome evolution. As the global pool of fungal ECM lineages has not been stable over time, new lineages have been recruited and it is not unlikely that ECM lineages have gone extinct, many functions provided by the fungal ECM communities have probably changed over time too.

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