Evolutionary ecology of pine-mycorrhizal interactions in the Caledonian pinewoods of Scotland

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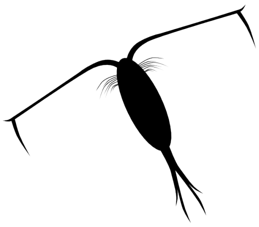
To Frank Evans, who showed me that a career in biology was possible.



Lay Summary

Most species of plants form symbiotic associations with a group of organisms known as mycorrhizal fungi. These fungi grow on a host plant's roots and trade nutrients they collect from the soil to their host plants in exchange for sugar produced by the plant during photosynthesis. This normally results in benefits to growth and survival for both the plant and fungus, resulting in a symbiotic interaction. However, not all fungal species provide equal benefits to plants, and some even appear to act parasitically. If avoiding associations with these parasites or poor-quality partners is important for survival or growth, then plant populations may evolve to prefer associations with the highest-quality fungi or to avoid bad partners. Different species of these fungi are found in different places, depending both on the plant species present and properties of the soil. Because of this, different populations of plants may be evolving to account for the unique combination of fungal species in their local area, resulting in adaptation to local fungi.

In this thesis, I looked to see if there was any evidence for this kind of evolution in populations of Scots pine (*Pinus sylvestris*) from the Caledonian pinewoods of Scotland. Firstly, I determined whether seedlings from different populations were bigger when grown with fungi from their home site, and the degree to which this increase in growth was determined by the genetic origin of the seedling. I found no evidence that seedlings grew bigger with their local fungi, but I did find that increase in biomass in response to the fungi had a strong genetic origin.

Secondly, I conducted an experiment where seedlings from different populations were planted in the wild in each of the originating populations. After three months, I identified how much of each seedling was colonised by each fungal species. I then assessed the importance of a seedling's location versus its genetic origin in determining what fungi colonised it. I also explored whether compounds produced by pine trees to defend against fungal pathogens might also affect what fungi colonised each seedling. I found that the location of a seedling was the only factor that explained any variation in colonisation, and that there was no effect of a seedling's genetic origin.

Finally, using data from the same experiment, I looked to see if different species of fungi were competing with each other to colonise seedlings. The two most common fungal species appeared to be good competitors for roots, and often colonised a seedling's root system to the exclusion of other fungal species. Thus, the interactions between fungi colonising the seedlings may be a lot more important in determining what species colonise seedling than its genetic origin.

Overall, I found little evidence to suggest that Scots pine trees in Scotland are evolving because of interactions with mycorrhizal fungi. Because these trees live a long time, if the fungi present at a site change within the lifespan of an individual tree, then even if evolution had occurred then the effects may have been disrupted. Instead, this kind of evolution may be more important for species of plants with shorter lifespans, such as grasses or herbs. Further research is required

in order to determine whether factors such as plant lifespan determine the evolutionary response to these fungi.

Abstract

Mycorrhizal fungi are important mutualistic symbionts of most plant species, exchanging soil-bound nutrients with their host plants in exchange for photosynthetically derived sugars. Although generally beneficial, not all mycorrhizal fungi provide the same amount of benefit to their hosts, and in some contexts they can also have negative effects on their hosts. Because of this variation in potential benefit, and because different fungal species are found in different places, host populations may be under selection to modify which fungi they interact with, or how they interact with fungi, in order to increase the amount of benefit they receive from the interaction. If selection occurrs differently at different host populations, this would then also lead to local adaptation. In this thesis, I explore whether evolution in response to ectomycorrhizal (EM) fungi has occurred in the Caledonian pinewoods of Scotland, the remnant fragments of *Pinus sylvestris* woodland that established in Scotland 10,000 years ago.

Firstly, I set up a reciprocal inoculation experiment to estimate the amount of genetic variation present amongst families of Scots pine for response to mycorrhizal inoculation, and to look for potential local adaptation of populations to local fungi. We used seedlings and soil from four populations in the Caledonian pinewoods, measuring seedling total biomass after four months. While I found that ectomycorrhizal responsiveness was highly heritable, there was no evidence that pine populations were locally adapted to fungal communities. Instead, I found a complex suite of interactions between pine population and soil inoculum. These results suggest that while Scots pine has the potential to evolve in response to mycorrhizal fungi, evolution in Scotland has not resulted in local adaptation. Long generation times and potential for rapid shifts in fungal communities in response to environmental change may preclude the opportunity for such adaptation in this species, and selection for other factors such as resistance to fungal pathogens may explain the pattern of interactions found.

To explore whether there was genetic variation in the association of pine seedlings with different species of EM fungi, or whether pine defensive compounds (monoterpenes) affected the colonisation of EM fungi, we set up a reciprocal transplant experiment. Seedlings from six populations of Scots pine were grown reciprocally in grids at each of the originating populations. EM communities on each seedling were characterised using a combination of microscopy and molecular barcoding, and we used gas chromatography to characterise the monoterpene chemotype for a subset of seedlings. While there was strong evidence that mycorrhizal communities varied between sites, and among grids within sites, I found no evidence that either maternal family or monoterpene chemotype explained any variation in EM community composition. These results again suggest the importance of life history: if a seedling is too selective, it may impose penalties to competitiveness at an important life stage. Instead, a lack of selectivity may allow a seedling to gain the competitive advantage needed to outcompete conspecifics.

Finally, I explored the spatial ecology of EM communities from the dataset collected previously. Although I previously found that EM community composition varied between sites and grids, approximately 30% of the variation remained unexplained. I conducted spatial correlogram analysis on EM communities at the 0 - 70 cm scale, and performed an analysis of co-occurrence of species pairs to find evidence of positive and negative interactions between fungal species. I found that most grids showed no spatial autocorrelation in EM root tips at this scale, suggesting relatively homogeneous EM communities. However, some grids were autocorrelated up to 20 cm in distance, suggesting patchy distributions of some EM fungi. I found evidence for negative interactions between species, with the two most abundant species being involved in over half of negative interactions detected. I also found evidence of positive interactions between species pairs, with most involving the genus *Suillus*, a group of fungi that have been previously found to host N-fixing bacteria. The presence of positive interactions with these species may suggest a facilitative effect of *Suillus* on other EM species.

Overall, I found that while mycorrhizal traits in Scots pine had a genetic component, in natural conditions ectomycorrhizal interactions may be determined much more strongly by aspects of the ecology of individual mycorrhizal species than by host genetics. More generally, the outcomes of these interactions may depend on the life history strategy of the host, and selection pressures may be stronger in species with shorter lifespans or which occur in competition-heavy environments. Further work investigating these effects of life history should also aim to incorporate more natural conditions, as ecological interactions between fungi may mitigate genetic effects.

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Contents

List of Tables

List of Figures

1

Introduction

1.1. Local adaptation in trees

Spatial and environmental variation in the patterns of natural selection is well understood to result in the local adaptation of populations, where the genotypes of a population are more suited to local environmental conditions, such as climate or soil, than other environments. This adaptation has long been recognised as ecologically important by foresters, who for hundreds of years have noted that tree stock transferred long distances performs poorly compared to local stock (Boshier et al. 2015). Local adaptation of trees to abiotic factors has been investigated in many tree species, and adaptations have been found to a wide variety of environmental factors such as latitude, growing season length, and frost (Savolainen et al. 2007).

However, abiotic factors are just one set of selective pressures imposed on trees. Interactions with other organisms, such as pathogens, predators, competitors, and symbionts can also impose selective pressures, resulting in evolution in the traits involved in these interactions (Thompson 2005; Guimarães Jr et al. 2011). Changes in these traits can in turn present selective pressures on these interacting organisms, resulting in reciprocal selection. Under certain conditions, reciprocal selection can then lead to local coadaptation, where the specifics of reciprocal selection differ between locations (Thompson 2005, 2016). The outcomes of such coevolutionary adaptation thus depend on the abiotic environment, the community of species present in a locale, genetic variation within those species, and gene flow between populations. Because all of these factors vary in space, coevolution is an inherently spatially structured process (Thompson 2005).

This spatial structuring of the coevolutionary process has been termed the “geographic mosaic theory of coevolution” (GMTC) (Thompson 2005). In particular, the GMTC hypothesises that coevolution has three inherently spatial characteristics: geographic hot- and cold-spots, selection mosaics, and trait remixing (Gomulkiewicz et al. 2000). Selection hotspots occur where reciprocal selection occurs between two species (a genotype x genotype interaction for fitness); comparatively, a selection cold spot occurs where the fitness of at least one of the species is unaffected by the other (for example, due to the absence of one of the species or the presence of an additional species). Selection mosaics thus result when the character of this reciprocal selection varies between environments, that is, there is a G x G x E interaction. Finally, trait remixing encompasses processes that shift genetic material potentially undergoing coevolutionary selection around, including gene flow, genetic drift, and extinction and recolonisation (Gomulkiewicz et al. 2000, 2007; Thompson 2005; Hoeksema 2010).

1.2. Pines and ectomycorrhizal fungi

Among the organisms that might impose strong selective pressures on trees are symbionts such as ectomycorrhizal (EM) fungi. These root-associated fungal symbionts are highly important for plant nutrition, exchanging otherwise plant-inaccessible nutrients for access to plant-derived photosynthate (Smith and Read 2008). They have also been suggested to increase access to soil water resources (Duddridge et al. 1980; Brownlee et al. 1983) and aid in disease resistance by sheathing vulnerable root tips (Marx 1972; Bennett et al. 2017). Such processes are mediated through the formation of an organ on the root tips of their host plants known as mycorrhizas, which allows nutrient exchange between the host and fungus (Brundrett 2004). Unlike arbuscular mycorrhizal (AM) fungi, which form intracellular structures within host root cells, ectomycorrhizal fungi, as the name suggests, form extracellular associations on the root tips of host plants. These mycorrhizas consist of a fungal sheath or “mantle” surrounding the outside of a root tip or hair, as well as the “hartig net”, a network of fungal hyphae that that grow in the cell walls and extra-cellular matrix of the host root cells, through which exhange occurs (Smith and Read 2008). Ectomycorrhizal fungi are typically hosted by woody shrubs and trees, paticularly conifers in the Pinaceae, as well as members of the Fagaceae, Betulaceae, Dipterocarpaceae, and others (Koele et al. 2012).

Mycorrhizal fungi as a whole are an ancient group, which likely evolved concomitantly with the origin of land plants (Pirozynski and Malloch 1975; Humphreys et al. 2010), with the earliest fossils dating back 400 MYa to the Devonian (Remy et al. 1994). However, the ectomycorrhizal symbiosis is likely a lot younger. Although the oldest EM fossils known date from around 50

MYa (LePage et al. 1997; Beimforde et al. 2011), it is likely that EM fungi evolved between approximately 270 and 174 MYa, around the time of the split of the Pinaceae from the Gnetophytes (Strullu-Derrien et al. 2018). Pines appear to be the original hosts for these fungi, and the association of EM fungi with angiosperms appears to have occurred later, with a shift to Rosids occurring approximately 100 MYa (Strullu-Derrien et al. 2018). The ectomycorrhizal habit has evolved independently among fungi between 82 - 86 times, usually from saprophytic and brown- and white-rot fungi (Tedersoo and Smith 2017), and many of these fungi have retained some level of ability to break down organic matter (Kohler et al. 2015).

As a result of this long evolutionary history, and multiple evolutionary origins, EM fungi are highly diverse, with estimates predicting a global diversity of 20,000 to 25,000 species (Rinaldi et al. 2008; Suz et al. 2018), and local levels often between 20 and 50 species, even within monospecific stands (Horton and Bruns 2001). EM fungi are also functionally diverse in terms of their morphology and biochemistry. The fungal mantle of EM fungi can be smooth, exploring the soil a short distance from a root with few hyphae, or “hairy” explorers with many hyphae; additionally, some species form rhizomorphs aiding water transport over long distances. Because EM fungi place phylogenetically within clades of saprotrophic fungi, many species thus express enzymes for the degradation of organic matter such as lignin (Agerer 2001). This variation across phylogeny, morphology, and function may thus create a wide suite of traits upon which selection can act.

As noted, the Pinaceae were likely among the first hosts of these fungi, evolving approximately concurrently to the suspected origin of EM fungi in the mid to late Jurassic (Lin et al. 2010; Rothwell et al. 2012). Members of the Pinaceae are highly prevalent in the boreal forests of the northern hemisphere, where cool temperatures mean that decomposition is slower and biomass accumulation is greater (LePage 2003). The spread of Pinaceae and subsequent dominance of these ecosystems may have been facilitated by their association with these novel symbionts. By priming symbionts with host-derived photosynthate for the purposes of nutrient acquisition from recalcitrant substrates, the pines may have received a competitive advantage over other groups (LePage 2003). Furthermore, EM fungi are known to have stronger weathering capabilities of calcium-magnesium silicates than AM fungi, drawing CO_2 from the atmosphere (Thorley et al. 2015). This reduction in CO_2 following the origin of the EM symbiosis may have helped drive the climate towards cooler temperatures, reinforcing the burgeoning dominance of pines in these regions with their novel symbiosis (Quirk et al. 2014; Strullu-Derrien et al. 2018).

1.3. Community ecology of ectomycorrhizal fungi

Given the diversity, both phylogenetic, morphological, and functional, among EM fungi, it should be of no surprise that the community composition of these fungi varies at large and small scales. EM fungal community composition is known to vary between environments (Suz et al. 2014). Climate variables such as annual precipitation, temperature and nitrogen deposition have been found to exhibit strong effects on fungal community composition (Cox et al. 2010; Jarvis et al. 2013; Suz et al. 2014; van der Linde et al. 2018). For example, at sites featuring heavy N deposition, there was a strong change in EM community composition, which may have represented a community shift toward EM species which have low C requirements (Cox et al. 2010; Suz et al. 2014). Given that some fungal species provide more benefit to plants under certain environmental conditions, such as high soil moisture (for example Boucher and Malajczuk 1990), these shifts in composition may thus help maintain overall benefits to plants and fungi.

EM fungi also exhibit varying host preferences, and the host makeup of a site has often been shown to affect the community of fungi present (Bruns et al. 2002; van der Linde et al. 2018). Some EM fungi show specificity, or at least a marked host preference, to particular host genera or even species. The most well-understood specialists are the Suilloid group, a clade of fungi including the genera *Suillus* and *Rhizopogon*. Species in this clade all associate only with hosts in the Pinaceae, with many species associating only with a single genus or even sub-genus (Bruns et al. 2002; Liao et al. 2016). Similarly, members of the genus *Leccinum* are also usually highly specific, with individual species restricted to restricted phylogenetic clades of hosts (Den Bakker et al. 2004). Even species which are considered generalist may still show preference for particular host clades (van der Linde et al. 2018). Thus, host identity can also strongly affect the makeup of individual EM fungal communities.

Variation in EM fungal distributions is also present at small scales, from the sub-metre scale to tens of metres. At such scales, variation in the abundance of particular fungal species is likely determined by the distribution of fungal “individuals”, or genets (Pickles and Anderson 2016). These genets are often distributed through the soil space in distinct patches, with the sizes of these patches varying both within and between fungal species (Sawyer et al. 1999; Anderson et al. 2001; Guidot et al. 2001; Beiler et al. 2010). This patchiness is likely driven my a number of factors. Small-scale environmental variation in the soil can drive changes in the presence or absence of fungi due to niche partitioning on specific soil and host resources (Bahram et al. 2015), and biotic interactions, such as competition and facilitation interactions, may also restrict or enhance patch sizes (Hortal et al. 2008; Kennedy 2010; Pickles and Anderson 2016). Finally, life history strategies, such as dispersal mechanism (for example vegetatively, wind-dispersed, animal-dispersed) can affect distributions at both large and small scales. For example, ruderal species characterised by strong dispersal abilities may be more likely to be found in disturbed or novel environments with lower competition, whereas more competitive fungi may persist longer and be found more commonly in old-growth forests (Taylor and Bruns 1999; Redecker et al. 2001; Twieg et al. 2007).

Finally, it is worth considering how the age of hosts may affect the communities of their associated EM fungi. Older trees have large, extensive root systems which, by virtue of sampling a larger area, are likely to host a greater diversity of fungal species than seedlings or saplings with small rooting areas. Furthermore, mature trees are likely to be able to provide an increased amount of photosynthate to their symbiotic fungi (Kranabetter et al. 2005; Twieg et al. 2007; Teste et al. 2009), and thus may have more choice in their ability to allocated it to different fungal species depending on their nutritional requirements (Bever 2015). Such effects have been noted in previous studies: Aučina et al. (2011) found that while seedlings associated with the same species as neighbouring mature trees, they associated with these fungi at different frequencies, with a lower Shannon diversity index on seedlings compared to mature trees. Similar results were obtained by Twieg et al. (2007) in Douglas-fir growing in chronosequences, but were not found to occur in paper birch. Another study found no difference in colonisation of seedlings compared to mature trees (Obase et al. 2009), but it was limited by poor sample sizes in young seedlings. Overall, these results suggest that the EM fungal communities of seedlings are not always fully commensurate with those of nearby mature trees, and such effects need to be considered when inferring effects from one age-class to another.

1.4. Coevolution

It is now well established that the outcome of these symbiotic associations can range from parasitism to mutualism (Karst et al. 2008; Hoeksema et al. 2010). Mutualistic outcomes occur when both the fungus and the plant gain a benefit from the interaction, for example through the beneficial exchange of nutrients and C resulting in the growth of both partners. Mutualism appears to be typical of mycorrhizal interactions (Frederickson 2017), but parasitism is a noted outcome in a subset of studies. Parasitism on the part of either the plant or the fungus occurs when one party to the interaction draws a benefit to the overall cost of the other partner, either not providing enough benefit to their partner or forgoing the exchange entirely ("cheating") (Jones et al. 2015). The distinction between these two forms of parasitism is important, as the former implies a poor-quality partner attempting to engage in an exchange, while the latter suggests an evolutionary strategy to profit at the expense of the partner through the loss of traits related to exchange (Jones et al. 2015). The outcomes of these interactions, then, depend on numerous factors including the genotypes and phenotypes of host and partners, as well as the environmental context in which the interaction occurs (Hoeksema et al. 2010). One key environmental parameter which may affect these outcomes is the host-accessible nutrient availability of the soil. High levels of host-accessible nutrients should reduce the host's dependence on such nutritional mutualisms, as they are able to access their required nutrition without engaging in the costly maintenance of another organism (Thrall et al. 2007; Hoeksema et al. 2010). Because of this variation in context, both genetic and environmental, there is potential for varying selection on the species traits involved in these interactions (Hoeksema 2010), depending on the local environment and local population factors such as genetic variation in these traits.

Selection is likely to act on traits at different levels of organisation (Hoeksema 2010). At the lowest level, selection is likely to act on traits relating to signalling and recognition between host trees and their fungal partners. These signalling pathways are complex and there is much that is yet to be understood (Garcia et al. 2015); however, to the degree with which these pathways allow specificity in association with fungi, they are likely under selection to avoid associations with poor symbionts or parasitic fungi. In turn, these non-beneficial fungi are likely under selection to avoid detection by the plant (Hoeksema 2010). It has been suggested that host mechanisms such as killing off root tips colonised by poor symbionts (Hoeksema and Kummel 2003) or preferential allocation of C to beneficial fungi (Kiers and Heijden 2006; Bever 2015) are important in maintaining this kind of selection, but evidence is sparse for EM fungi. However, evidence from AM interactions suggest plants are able to preferentially allocate carbon to good symbionts (Bever et al. 2009).

At higher levels, traits such as the degree of colonisation of host roots by a fungus are likely also under coevolutionary selection (Tagu et al. 2005; Hoeksema 2010). These traits integrate lower-level molecular traits as well as traits such as the degree of allocation of resources to roots and root length. Selection on these traits is likely mediated both by the interaction strength of the fungal association (e.g. Hoeksema et al. 2009), as well as adaptations to edaphic factors such as water or nutrient levels (Johnson et al. 2010). Similarly, traits such as fungal allocation to extraradical hyphae or enzyme production affect (Courty et al. 2011) the amount of nutrition the fungus is able to provide to the plant, and thus these traits may also experience coevolutionary selection (Hoeksema 2010).

Integrating all these traits, as well as ecological and coevolutionary interactions between mycorrhizal fungal species, are community phenotypes (Whitham et al. 2006), where the community of EM fungi on a plant is related to host genotype. These traits are among the most commonly investigated in EM studies, as they are relatively easy to measure and do not require the isolation of particular fungal strains. In common garden experiments, host genotype has been found to be an important factor structuring EM community composition on roots in both conifers (Korkama et al. 2006; Leski et al. 2010; Velmala et al. 2012) and in angiosperms (Courty et al. 2011; Lamit et al. 2016), and these differences have been shown to correlate with host traits such as growth rate, insect resistance, and tannin content. A significant problem with common garden experiments, however, is that they often concentrate genetic variation from wide spatial scales into a small space, and hence can exaggerate the effects of host genotype (Tack et al. 2012). Examinations of host genotype within wild populations, in comparison, have found varying effect strengths. While Sthultz et al. (2009) found an effect of host genotype, in this case of a locus pertaining to insect resistance, fine-scale investigations by Bubner et al. (2013), Lang et al. (2013), and Saari et al. (2005) found host genotype to be unimportant in structuring EM communities, with roots from different trees in the same soil core having a similar community structure regardless of host genotype. This underscores the importance of considering ecological as well as genetic context in understanding these interactions.

In addition to host genotype, fungal genotype has also been shown to be important in controlling mycorrhizal interactions. For example, an experiment by Burgess et al. (1994) between 20 isolates of EM fungus *Pisolithus tinctorius* from across the globe and a half-sib family of *Eucalyptus grandis* found wide variation in the ability and rate of isolates to develop ectomycorrhizae, ranging from no mycorrhiza formation at all to fully developed mycorrhizae. This variation was usually, but not always, correlated with plant growth, with fully developed mycorrhizae providing the greatest benefit to the plants. Similarly, a study investigating the effects of two fungal genotypes of *Rhizopogon occidentalis* from within a single *Pinus muricata* forest found that host and fungal genotype interacted differently in different environmental conditions, producing variation in both host relative growth rate and % root colonisation by the fungus (Piculell et al. 2008). By comparison, a follow-up study utilising genotypes from a *P. contorta* var. *contorta* stand found that only fungal genotype was important for plant growth, with one genotype inducing significantly negative growth in the pine seedlings (Hoeksema et al. 2009).

The organisation of this genetic variation in hosts and fungi is unlikely to be random. Recent work investigating local adaptation in mycorrhizal interactions has shown that the benefit of mycorrhizal inoculation is dependent on the geographic origin of the fungi relative to the plant (Rúa et al. 2016a). Most of the evidence for this trend comes from work with arbuscular mycorrhizas: for example, an experiment by Johnson et al. (2010) on prairie grasses found that sympatric combinations of plants, soils, and arbuscular mycorrhizal (AM) fungi resulted in the greatest performance of both plants and fungi. More generally, local adaptation has been demonstrated in a meta-analysis of 1170 studies on AM plants, which showed that sympatric combinations of plants, fungi or soil resulted in higher plant growth than allopatric combinations (Rúa et al. 2016a). However, they also found that in situations where the fungi and soil were sympatric, but allopatric to the plant, plant growth response was no better than in all-allopatric situations.

Local adaptation in EM systems is harder to determine, as EM-associated host plants are often long-lived, making common measurements of fitness such as survival rates or reproductive output time consuming to measure (Pickles et al. 2015). However, local adaptation has been recorded among various tree species for a variety of abiotic factors (e.g. Salmela et al. 2013; Donnelly et al. 2016; Boshier et al. 2015), and the life history strategies of many tree species are likely to result in rapid local adaptation (Cavers and Cottrell 2015). High degrees of gene flow in many tree species (Wachowiak et al. 2011, 2013; Cavers and Cottrell 2015) and the production of many thousands of offspring, combined with heavy density-dependent mortality at young ages, present a strong selective pressure that can act to produce local adaptation (Petit and Hampe 2006). Thus, if EM fungi do present a strong selective pressure to seedlings, it is highly possible this will be visible through experimentation.

Because of the difficulty in conducting evolutionary experiments on mature trees, studies investigating local adaptation in EM systems have examined traits such as seedling growth rate, root:shoot allocation, % root colonisation, and community phenotype factors such as the EM community on the plant in question (e.g. Hoeksema et al. 2012). The fitness value of such traits is harder to ascertain, as such trait values do not have a simple 1:1 correlation with survival or reproduction, and will vary by environment (for example, slower growth rates may be favoured in low-nutrient soils) (Hoeksema 2010; Hoeksema et al. 2012). This makes determining whether sympatric-allopatric combinations are adaptive or maladaptive more difficult. Furthermore, there have been few studies directly assessing the potential for local adaptation in EM host plants; however, a meta-analysis based on a small number of studies found no evidence for local adaptation (Rúa et al. 2018).

The most well-studied system for coadaptation in EM fungi are the native pines of western North America, including the Monterey pine (*Pinus radiata* D. Don), Bishop pine (*Pinus muricata* D. Don), and shore pine (*Pinus contorta* var. *contorta* Dougl. ex Loud.). In one study (Hoeksema and Thompson 2007), two half-sib families each from 9 coastal pine populations (3 shore, 4 Monterey, 2 Bishop) ranging from Canada to Mexico were grown reciprocally with one of four *Rhizopogon occidentalis* genotypes (2 from Bishop pine hosts, 1 from shore pine, and 1 from Monterey pine). They found no evidence of host specificity between fungal genotypes, and no increase in plant growth was attributable to fungal genotype. However, they found that there was a decline in average fungal colonisation of host roots with increasing distance between the host and fungal source populations, suggesting a clinal pattern of local adaptation to pine populations in the fungi. Another study looking at within-population genetic variation among both trees and fungi found that the outcome depended on the environmental contect (Piculell et al. 2008), as well as fungal genotype: one fungal genotype colonised seedlings less intensively than another, and had a negative effect on plant growth (Hoeksema et al. 2009). Scaling up to a study at the community level, an investigation of the “resistant propagule community” of EM fungi in Monterey pine found that host population and EM community interacted to produce differences in mycorrhizal colonisation of the pine roots as well as differences in plant growth (Hoeksema et al. 2012). In particular, the Mexican populations associated more with the fungal OTU Wilcoxina1 compared to the more northern populations. They also found interactions between plant genotype and soil, which produced differences in plant growth rate and root length. They also found that plant growth rate was lowest in sympatric combinations of plants and soil, suggesting potential local adaptation or maladaptation to soil characteristics; however, they could not determine from the experimental design whether this was an effect of soil chemistry, EM fungi, or pathogenic fungi. Further work has now identified putative SNPs that underlie variation in association frequency with different EM fungi (Piculell et al. 2019).

Another study investigated local adaptation of interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) to local soils, both in the biotic and abiotic components (Pickles et al. 2015). They found that matching seed source to climatically and geographically matching soil sources improved seedling survival, but that survival was also mediated by soil fungi. Growth was significantly higher in soils not treated with fungicide, but as the transfer distance increased, plants in untreated soils also had a lower survival rate. Mismatch of plants and fungi causing decreased survival may have been the result of maladaptation between the plants and EM fungi or increased susceptibility to non-local soil-borne pathogenic fungi, underscoring the importance of biotic interactions in adaptation to edaphic conditions.

Recent evidence also suggests that host traits involved in defence against pathogens and predators may also have knock-on effects on mycorrhizal associations. A study in the pinyon pine (*Pinus edulis*) forests of New Mexico found that trees with an allele conferring resistance to a stem-boring moth maintained a consistently different EM community than those without, particularly in terms of the proportion of Ascomycete to Basidiomycete fungi colonising the seedlings. These differences were mediated by innate genetic factors rather than the effects of herbivory on the host plant: experimental removal of insects over a 20 year period did not significantly change community composition (Sthultz et al. 2009). These EM communities maintained separate trajectories over a 10 year period of environmental warming, with insect-susceptible trees maintaining stable community composition whilst insect-resistant trees changed over time (Gehring et al. 2014). These differences, particularly those relating to the proportion of Ascomycete to Basidiomycete fungi, have additionally been shown to be replicable in a common garden experiment (Patterson et al. 2018). In another study, Lamit et al. (2016) found that senescent leaf chemistry, particularly condensed tannin concentration, correlated with EM community composition in poplar, and Tagu et al. (2005) found that a quantitative trait locus (QTL) relating to EM formation was in a linkage group containing several QTLs involved in interactions with a pathogenic leaf rust fungus. Finally, Piculell et al. (2018) found evidence for a G x E interaction between pines resistant to the pine decline fungus, with resistant pines associating with more of a particular fungal OTU in one soil treatment. Overall, these results suggest that elements of plant defence pathways may play a role in the colonisation of plant roots by mycorrhizal fungi.

Although there have been few studies to date, the evidence thus far suggests that many of the ingredients for ongoing evolution in mycorrhizal interactions may be present in ectomycorrhizal host trees. In particular, there appears to be evidence for genetic variation within and between populations in terms of both the response to mycorrhizal inoculation and the association frequencies with different fungal species, as well as variation in the composition of mycorrhizal fungi at both large and small scales. However, the lack of basic research means that attempts to detect any trends across datasets have lacked statistical power (Rúa et al. 2018). Much of the work to date has also been conducted on seedlings grown in pots in glasshouses inoculated with spores or soil collected from natural environments. While such experiments are invaluable disentangling the relative contributions of different factors, particularly those which cannot be controlled in the field, they do not represent natural conditions for either the host or fungi. There is consequently a need for further basic research, particularly to replicate such effects in natural conditions where evolution actually occurs.

1.5. Methodological considerations for studies involving EM fungi

There are a few methodological considerations that need to be made when conducting studies on EM fungi. Early studies of EM fungi were limited, with little ability to identify the fungi colonising the roots of plants. Prior to the ready availability of genetic technologies, such as Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and molecular barcoding, identification of fungi was based around the morphological description of the fungal mantle, at both the macro- and micro-scopic level (Agerer 1997; Smith and Read 2008). While such techniques are sufficient to describe a wide variety of species, due to the many distinct morphological categories for example 1.1, many of these morphologically described fungi lacked true scientific names as the causal fungi was not known (Agerer 1997). However, with the cheap availability of direct sequencing, identification of EM fungi directly from sequences obtained from root tips is now commonplace. In particular, the Internal Transcribed Spacer regions (ITS), two regions of DNA located within the ribosomal array, have become standard targets for the molecular identification of fungi (Schoch et al. 2012). This is aided through the availability of the UNITE fungal database (http://unite.ut.ee, Nilsson et al. 2019), a curated database of fungal ITS sequences with reference sequences available for each characterised fungal species. By combining these morphological and genetic approaches, high-quality community data can be generated for root samples, that characterise the complete number of root tips colonised by each morphological type, with high-certainty IDs from sequencing to classify each type.

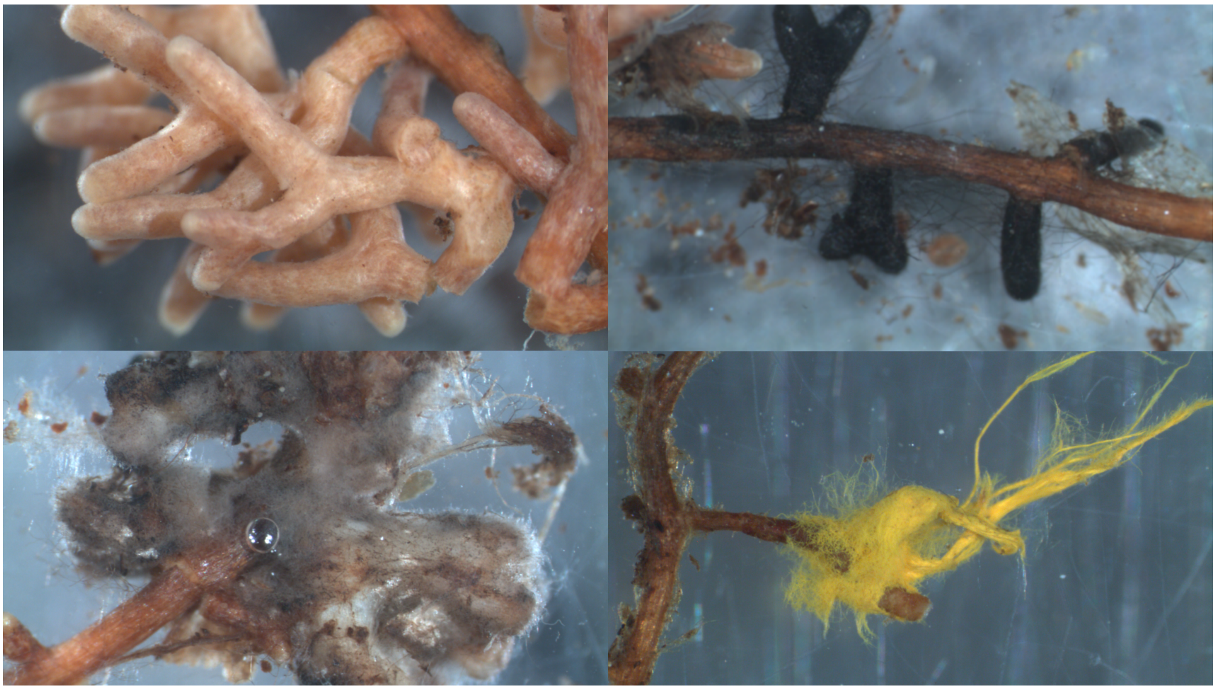

Figure 1.1.: Various ectomycorrhizal root tips encountered while conducting the research for this thesis, showing the wide range in morphology among different species. From top left, left to right and top to bottom: 1. unidentified Cantharellales sp. 2. *Cenococcum geophilum*; 3. *Suillus variegatus*; 4. *Piloderma olivaceum.*

Further to this, sampling of EM fungi in the wild requires consideration of spatial processes that can strongly bias community composition at small scales. EM fungi show strong patchiness at small scales, with patches ranging from tens of cm to tens of metres (Sawyer et al. 1999; Anderson et al. 2001; Guidot et al. 2001; Beiler et al. 2010). This patchiness is driven by a mixture of processes, including specialisation on specific soil resources, the autoecology of fungal species, including dispersal strategies, and biotic interactions between fungal species, such as competition and facilitation (Pickles and Anderson 2016). Where factors such as host genotype are of interest, this patchiness can bias estimates of these effects, because communities are not well-mixed and point estimates may occur within a single patch, which may appear as a host effect in a model while actually being driven by other factors. To counter this, spatially-explicit sampling methods can be a powerful tool to control for effects such as spatial autocorrelation. By sampling at regular intervals, potentially using a nested sampling design that incorporates different distances between samples, spatial autocorrelation can be estimated (Legendre and Legendre 2012), and spatial effects can be controlled for using modelling techniques.

1.6. The Caledonian pinewoods of Scotland

In my thesis, I will explore questions about coevolution between trees and ectomycorrhizal fungi using the Caledonian pinewoods of Scotland as a study system. These Scots pine (*Pinus sylvestris*) populations are the last remnants of the primaeval forest that colonised Scotland following the end of the last ice age, arriving 10,000 years ago (Salmela et al. 2010). Now highly fragmented, there are 84 woodland remnants that are recognised as native (Fig. 1.2). These woodlands occur patchily between latitudes 55°N and 57°N and longitudes 3°W and 1°W, and at altitudes from near sea level to above 600 m. These fragments also vary in size from below 1 ha to above 2,000 ha, with some having tree densities of below 1 tree ha^{-1} (Salmela et al. 2010). Despite evidence of high levels of gene flow between populations (Wachowiak et al. 2013), evidence for adaptive variation between populations has been shown in a variety of traits, much of which has been linked back to the longitudinal climate gradient that forms the main axis of climate variation in Scotland. This strong gradient in precipitation and temperature (annual precipitation ranges from ~3,000mm y^{-1} to ~700mm y^{-1} west to east, and the mean growing season length (the number of days above 5° C) ranges from ~300 to ~100 (Salmela et al. 2010)) has been found to correlate with variation in needle morphology (Donnelly et al. 2016), photochemical capacity (Salmela et al. 2011), spring phenology (Salmela et al. 2011, 2013), waterlogging tolerance (Donnelly et al. 2018), and resistance to needle pathogen *Dothistroma septosporum* (Perry et al. 2016a; b).

Additionally, the EM fungal communities of these pinewoods are already well-characterised. Jarvis (2013) investigated the diversity and community composition in a number of Caledonian

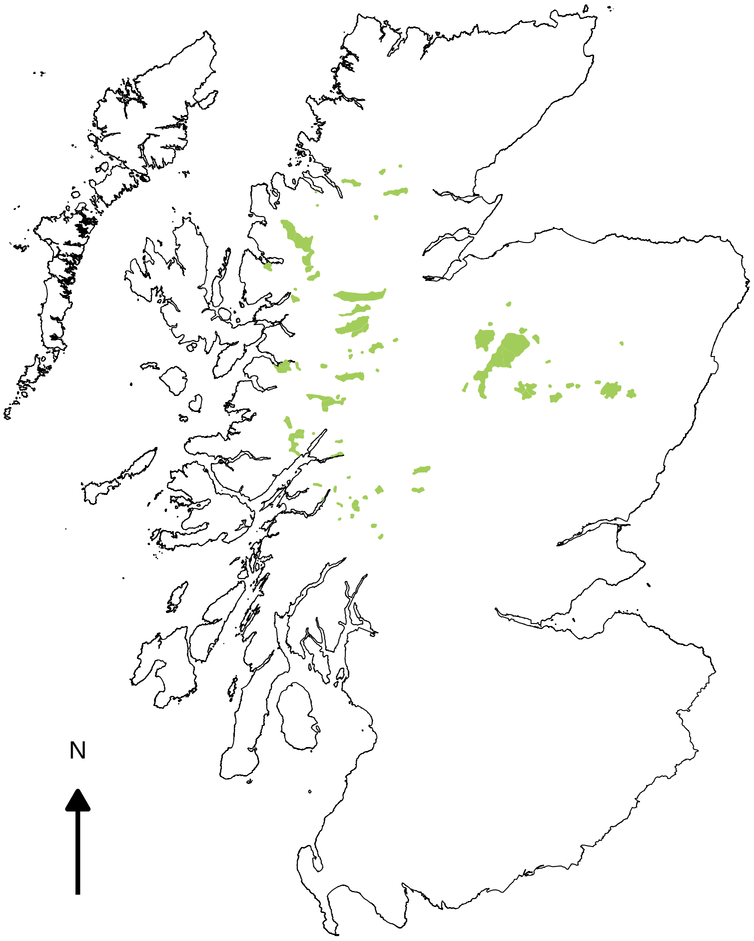

Figure 1.2.: Distribution of Caledonian pinewood remnants in Scotland (green), from the core pinewood areas of the National Pinewood Inventory.

pinewoods, producing species lists and identifying environmental drivers of community composition at large and small scales. At the regional scale, shifts in EM community composition were found to vary as a result of rainfall, soil moisture, nitrogen deposition, as well as the degree of isolation from other pinewoods (Fig. 1.3). At smaller scales, community composition of EM fungi was found to change with altitude, which co-varied with soil moisture and temperature. Soil organic matter and pH were also found to be important in determining community composition (Jarvis et al. 2015). Preliminary investigations into population-level differences in ectomycorrhizal preference have also been carried out in this system. Seedlings from two populations of Scots pine, from the extremes of Scots pine's longitudinal range in Scotland, were grown in an inoculum created from a mixture of soils from both forests, with three different watering treatments were also applied. Host genotype was found to be the most important factor in determining the EM community composition, suggesting that populations may have diverged in their compatibility with different EM communities (Jarvis 2013, chap. 5).

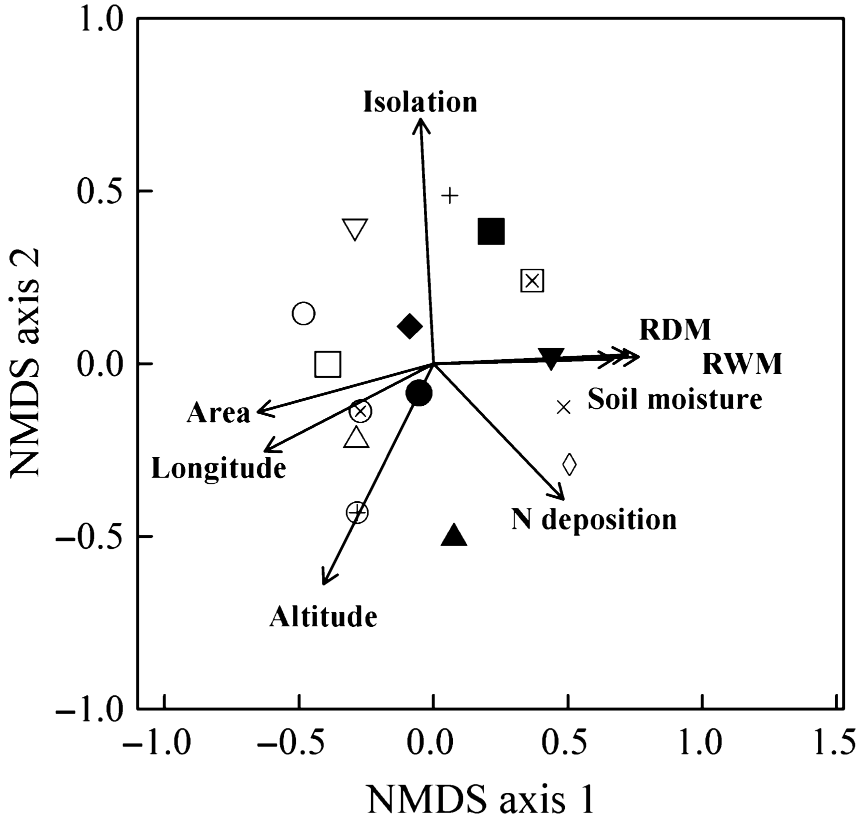

Figure 1.3.: NMDS ordination of EM fungal community composition from 15 Caledonian pinewoods. Arrows show significant environmental predictors at $p < 0.05$. RWM = Rainfall in Wettest Month. RDM = Rainfall in Driest Month. From Jarvis et al. (2013).

The Caledonian pinewoods also show geographic variation in their terpene chemotype: trees from populations in the north-west of Scotland typically lack the monoterpene 3-carene (Forrest 1980; Thoss et al. 2007), which is usually the second-most abundant terpene in Scots pine (O'Reilly-Wapstra et al. 2007). Terpenes have previously been shown to be important in deterring herbivory (Iason et al. 2011), and through leaf litter can change the community composition of the surrounding vegetation (Iason et al. 2005). In addition to this, terpenes have demonstrated anti-fungal properties (Eckhardt et al. 2009; Marei et al. 2012; Zhang et al. 2016), and have been shown to be active against both saprophytic and ectomycorrhizal fungi (Melin and Krupa 1971; Ludley et al. 2008). It is thus plausible that the terpene profile of individual trees, particularly the presence or absence of 3-carene, may affect the ability of particular mycorrhizal species to form successful associations.

The Caledonian pinewoods thus provide an excellent system within which to explore coevolution between pines and EM fungi. The strong climatic gradients and complex topography, contained within a relatively small geographic extent, create a mosaic of environments, which combined with varying EM communities may create the conditions necessary for coevolution to occur. Finally, geographic variation in defensive phenotypes between the east and the west also allows an exploration of a potentially important trait that might be involved in determining mycorrhizal interactions.

1.7. Aims of this thesis

The aim of this thesis is to determine the extent to which genetic differentiation between populations or genotypes of Scots pine affects interactions with mycorrhizal fungi, both in the glasshouse but particularly in the field. More specifically, I aim to answer the following questions:

1. Is there genetic variation between Caledonian Scots pine populations or genotypes in terms of their response to inoculation?
2. If so, is this genetic variation geographically structured (is there local adaptation)?
3. In field conditions, how relatively important are environmental conditions and pine genotype in determining what EM fungi colonise seedlings?
4. Does a pine's defensive (terpene) phenotype explain variation in mycorrhizal associations?

5. At small scales, to what extent does the ecology of mycorrhizal fungi affect mycorrhizal community composition?

In Chapter two, I address questions 1 and 2 using a reciprocal inoculation experiment. Seedlings from four Scots pine populations representing the east-west variation are grown reciprocally in soil from each population in order to determine both the response to mycorrhizal inoculation, and to determine whether there is geographic variation in the response.

In Chapter three, the relative importance of environmental conditions and pine genotype are evaluated in a reciprocal transplant experiment. Seedlings from 12 pine maternal families are planted in the field at their six originating populations, and the community of mycorrhizal fungi colonising each seedling is assessed. A number of the seedlings were assessed for their terpene character, allowing us to model pine genotype in terms of defensive phenotype.

In Chapter four, I explore the potential for competitive or facilitative interactions between mycorrhizal fungi at small scales. I assess the degree to which mycorrhizal fungi in the previous experiment show patchy distributions at small scales, and estimate the extent to which such interactions between pairs of fungal species determine their presence or absence on individual seedlings.

In Chapter five, I discuss the results of the preceding three chapters and consider them in the context of life history evolution, suggesting areas for potential future research. I also consider the importance of incorporating more realistic conditions in studies on evolution in mycorrhizal interactions, as well as whether the results have any implications for the reforestation of the Caledonian pinewoods of Scotland.

2

Heritable genetic variation but no local adaptation in a pine-ectomycorrhizal interaction

(This chapter has been published in the journal **Mycorrhiza** under the title **Heritable genetic variation but no local adaptation in a pine-ectomycorrhizal interaction** (Downie et al. 2020), see Appendix A for the published copy.)

2.1. Introduction

Mycorrhizal fungi are near-ubiquitous across the plant kingdom, forming associations with approximately 80% of plant species (Smith and Read 2008). Despite our understanding of their importance in terrestrial ecosystems in terms of both host performance and soil nutrient cycling, it is only relatively recently that we are beginning to understand the evolutionary landscape that helps shape the outcomes of these interactions. In particular, local adaptation (the differential success of a genotype in its home environment compared to a foreign environment) has been explored as a potentially important process in structuring some of the context-dependency in host benefit seen in many mycorrhizal studies (Hoeksema 2010; Rúa et al. 2016b). Clear evidence of the importance of these adaptive processes has been shown for plants forming arbuscular mycorrhizal (AM) symbioses (Johnson et al. 2010; Rúa et al. 2016a), but there have been comparatively few studies exploring local adaptation in ectomycorrhizal (EM) systems. While there is some evidence suggesting the existence of genotype x genotype interactions in ectomycorrhizal hosts (Hoeksema and Thompson 2007; Piculell et al. 2008; Hoeksema et al. 2012; Pickles et al. 2015), overall, there appears to be little evidence so far for local adaptation in host populations (Rúa et al. 2018).

For local adaptation to occur, populations must possess sufficient heritable variation in fitness-related traits, and experience differential selection pressures in different places (Cheplick 2015).

Theoretical work on coevolution in mycorrhizal interactions has usually considered factors affecting the outcomes of mutualism, such as partner selection, variation in symbiont community composition, and environmental quality as the drivers of selection (Hoeksema 2010), resulting in pockets of local adaptation or maladaptation depending on the outcomes of these processes (the "geographic mosaic of coevolution") (Thompson 2005). While much work has used reciprocal transplants to investigate the potential for genotype x genotype interactions with mycorrhizal fungi (for example Johnson et al. 2010; Hoeksema et al. 2012), there have been comparatively few studies that have explored the heritability of the traits in question.

In particular, the heritability of ectomycorrhizal host traits, including host performance, degree of colonisation, or compatibility traits, which are potentially important for local adaptation, have received only cursory attention. Many tree populations show very high amounts of genetic variation (Petit and Hampe 2006; Cavers and Cottrell 2015), which means that robust estimates of heritability will often require large amounts of replication both at the family and individual level, which can be time consuming to achieve. An even greater degree of replication may be required in situations where cloning of the host is difficult, such as in *Pinus* species. To our knowledge, only one study has estimated the heritability of host performance in response to inoculation, reporting high levels of heritability in *Pinus elliotii* inoculated with a single genotype EM inoculum (Rosado et al. 1994). Other studies have reported heritability in other mycorrhizal traits, such as the number of mycorrhizal root tips and degree of colonisation (Rosado et al. 1994; Tagu et al. 2005; Courty et al. 2011; Velmala et al. 2012), and the heritability of ectomycorrhizal community composition on hosts (Velmala et al. 2012; Lamit et al. 2016).

Whether and how heritable variation in traits arises and is acted on by selection can also depend on factors other than those determining mutualistic outcomes. For example, life history strategies employed by the host or symbiont can change the landscape of adaptation through their effects on the amount and distribution of genetic variation (Leimu and Fischer 2008; Cheplick 2015). Mycorrhizal plants exhibit a wide range of life histories, from short-lived annuals to herbaceous perennials to long-lived trees (Smith and Read 2008). The vast majority of ectomycorrhizal hosts are woody perennials, often trees (Smith and Read 2008). As such life history factors, including long generation times, large numbers of offspring, and long distance gene flow, will have significant effects on the ability of such species to evolve in response to symbiotic partners (Petit and Hampe 2006). It has been suggested that long generation times reduce the potential for adaptation, but phenotypic studies in trees often show strong differentiation between populations for adaptive traits, often with high values of heritability (Cavers and Cottrell 2015). This differentiation likely results from strong selective pressures operating in the early stages of the life cycle when there is heavy mortality (Petit and Hampe 2006; Cavers and Cottrell 2015). For hosts to adapt in response to local EM fungal communities, there must thus be both heritable variation in the outcome of mycorrhizal interactions, and mismatched associations with local EM fungi must be deleterious to the fitness of seedlings. In addition, such deleterious effects should occur on similar scales to other selective pressures such as competition, grazing, and environmental stress.

Even in cases where populations show heritability for mycorrhizal traits, local adaptation still may only occur in situations where the identity and quality of their mycorrhizal associates are stable over the course of generations. If fungal community structure is liable to shift within the lifespan of a host, either through neutral processes or in response to shifting environmental conditions, then this can potentially disrupt any local adaptation that has occurred on the part of the host (Iason et al. 2018). Community turnover in EM fungi occurs not only in response to distance, but also in response to environmental variation, including climatic variables such as annual rainfall and temperature, soil factors including pH, and in response to atmospheric deposition of N and K (Cox et al. 2010; Jarvis et al. 2013; Suz et al. 2014; van der Linde et al. 2018). EM fungi also show functional specialisation on specific soil resources (Agerer 2001). Community turnover is thus likely in response to shifting climatic conditions and increasing pollution (Cox et al. 2010; Suz et al. 2014). Under such changing conditions, local adaptation of host populations is likely impossible, and instead shifts in EM community composition in response to environmental change may help facilitate host survival if they are more able to provide host benefit under new conditions (Batstone et al. 2018; Iason et al. 2018).

To measure the heritability of host performance and test for local adaptation in an ectomycorrhizal host, we set up a reciprocal inoculation experiment using seedlings and soil from populations of Scots pine (*Pinus sylvestris*) from the Caledonian pinewoods of Scotland. Established approximately 10,000 years ago, these forests have experienced gradual historic climatic warming (Salmela et al. 2010) and currently occur in a wide variety of climatic conditions and altitudes across their limited range, with a west to east rainfall gradient of approximately 3,000mm to 500mm per year over little more than 150 km (Donnelly et al. 2016). Local adaptation of these populations has previously been demonstrated along this climatic gradient (Salmela et al. 2011, 2013; Donnelly et al. 2016), and heritable variation has also been found for both resistance to needle pathogen *Dothistroma septosporum* (Perry et al. 2016a; b) and fungal endophyte community composition (Cavers, unpublished). The forests also have a well-documented ectomycorrhizal flora, and EM community composition at these sites has been shown to vary strongly in response to both rainfall and altitude (Jarvis et al. 2013, 2015).

I sought to answer the following questions: 1. Is there heritable variation within Scots pine for response to inoculation by EM fungi? 2. Does this response differ depending on the pine population of origin? 3. If so, do populations perform better when paired with their local EM community compared to others?

2.2. Methods

I set up a reciprocal cross-inoculation experiment to investigate the effects of soil biota on pine performance. The experiment used seedlings and soil from four populations of Scots pine across a longitudinal rainfall gradient in Scotland, resulting in a 4 x 4 factorial cross. To control for variability within populations and allow estimates of heritability, I used seed from six maternal families per population. Treatments consisted of seedlings grown in sterilised compost inoculated with field soil. Each treatment combination was replicated 8 times, with an additional 3 replicates containing sterilised inoculum to act as a control, resulting in a total of 1056 individuals. Plant performance was measured at the end of the experiment as aboveground and belowground biomass, and mycorrhizal colonisation was measured for a subsample of root systems.

Table 2.1.: Coordinates of each population used in the trial and the trial site, as well as climatic variables (Met Office) and soil types (Soil Information For Scottish Soils, James Hutton Institute). MAP = mean annual precipitation.

Forest	Latitude	Longitude	Altitude (m)	MAP (mm)	Soil type
Beinn Eighe	57.63	-5.35	21	2476	Peaty gleyed podzol
Strath Oykel	57.98	-4.61	67	1234	Peaty gleyed podzol
Abernethy	57.21	-3.61	362	1060	Humus-iron podzol
Glen Tanar	57.05	-2.86	177	801	Humus-iron podzol
Common garden	55.86	-3.21	190	-	-

2.2.1. Source material

Cones were collected from four native stands of Scots pine in Scotland: Beinn Eighe and Strath Oykel in the west, and Abernethy and Glen Tanar in the east (Fig. 2.1). These sites sit along a strong west-east rainfall gradient, and additionally vary in both altitude and soil type (Table 2.1). Cones were collected from Beinn Eighe and Strath Oykel in late January 2018, and from Abernethy and Glen Tanar in late February of the same year. At each site, cones were collected from six open-pollinated mother trees. Seeds were cold-stratified at 4°C for three weeks, before being surface sterilised in 2% sodium hypochlorite for ten minutes. They were then rinsed, imbibed in water for 3 hours, and germinated on sterile agar before planting.

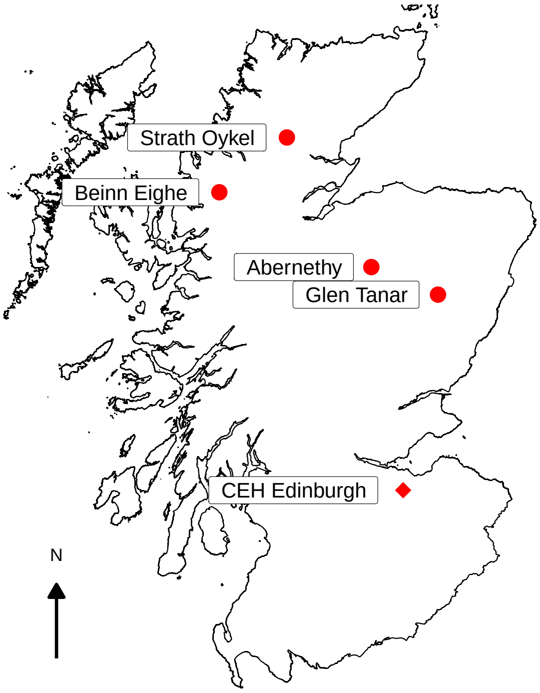

Figure 2.1.: Map of each pine population used in the experiment (circles) and the location of the glasshouses (diamond) where the experiment was conducted.

Field soil for use as inoculum was collected in late April 2018. At each site, soil from 4 small soil pits was collected to a depth of 30 cm from within the drip line of mature pine trees. These trees were chosen haphazardly, with a focus on collecting from beneath trees with low amounts of undergrowth, to allow for easy soil collection with minimal interaction with roots of other species. Collected soil for each site was sieved through a 6 mm mesh to remove stones and roots.

The samples from each site were then homogenised to produce inocula for the experiment.

2.2.2. Glasshouse set-up

Soil from each site was mixed with sterile potting soil (a compost:sand mix, 2:1 Levington stock nursery compost:Royal Horticultural Society Sharp Sand, sterilised by steam autoclave at 121°C for 30 minutes) in a ratio of 7 potting soil:1 inoculum, and placed into 0.46 L square pots (9 x 9 x 9.5 cm). Germinated seedlings were cut out of agar and planted in the centre of the pot on the 31st May 2018. Each set of replicates was arranged randomly in blocks on two greenhouse benches, for a total of 8 blocks, 4 per bench; controls using sterilised inoculum (also autoclaved at 121°C for 30 minutes) were placed into the blocks at each end of one glasshouse bench and at one end for the other. Pots were placed directly onto an unmatted flood bench base to limit root growth outside of the pots. After a week, any seedlings that had not taken hold were replaced with spares. Seedlings were watered in by hand following potting, and subsequently were watered two to three times a day (depending on moisture levels) by an overhead sprinkler system. Seedlings were grown in this way for 4 months.

2.2.3. Seedling analysis

Seedlings were harvested between the 18th of September and 1st of October 2019, in block order to capture any additional growth in the block effect. Seedlings were first cut at the soil level to separate above and belowground biomass. Pots were then emptied and the contents were carefully sieved and then washed to extract the roots with minimal damage. Root systems were inspected by eye for visible presence of mycorrhizal colonisation, evident as thickened, coloured and/or bifurcated root tips, and were scored for presence or absence. Subsequently, 5+ root systems per population:soil treatment were randomly selected from among 4 families per population for detailed analysis of root colonisation. Colonisation was assessed as the number of colonised ectomycorrhizal root tips per cm root length. Root length was measured following the gridline intersect method as described by Tennant (1975) using 5 randomisations. The shoots and roots for all seedlings were dried in an oven at 50°C for 48 hours, before being weighed to obtain a measure of above and belowground biomass.

2.2.4. Statistical analysis

In order to separate out the component of growth that was due to the soil biota, I used mycorrhizal response ratios instead of raw seedling measurements. These were calculated as the ratio of a seedling's biomass or root:shoot ratio to the mean value of the sterile control seedlings for a given soil treatment and maternal family. In addition, I grouped soil treatments into two categories: western soils (Beinn Eighe and Strath Oykel) and eastern soils (Abernethy and Glen Tanar). This allowed us to simplify the interpretations of our model interactions to account for the major axis of variation between our sites. All statistical models were conducted in R (R Core Team 2019) using the *lmerTest* package (Kuznetsova et al. 2017).

2.2.4.1. Heritability and coefficient of genetic variation

I estimated narrow-sense heritability (h^2), the proportion of phenotypic variance (V_P) that can be explained by additive genetic effects, by fitting models for each trait in which maternal family and block were included as random effects (Eq. 2.1), where μ is the mean and ϵ is the residual error. These models were fitted for each trait both within each inoculum treatment and for the full dataset.

$$Trait = \mu + Family + Block + \epsilon \tag{2.1}$$

From these models, h^2 was estimated using Eq. 2.2, where V_A is the additive genetic variance, V_P is the phenotypic variance, V_{fam}, V_{block}, and V_{res} are the among family, among block, and residual variances respectively, and R is the relatedness of individuals. As the relatedness of the seedlings was unknown, I estimated heritability for three values of R: seedlings are all half-siblings (R = 4), seedlings are 50% half-siblings and 50% full siblings (R = 3), and seedlings are all full siblings (R = 2).

$$h^2 = \frac{V_A}{V_P} = \frac{RV_{fam}}{V_{fam} + V_{block} + V_{res}} \tag{2.2}$$

Standard errors were calculated following Visscher (1998) (Eq. 2.3), where s is the mean number of offspring per family, and f is the number of families.

$$SE_{h^2} = R\sqrt{\frac{2(1-\frac{h^2}{4})^2[1+(s-1)\frac{h^2}{4}]^2}{s(s-1)(f-1)}} \tag{2.3}$$

I also calculated the coefficient of genetic variation (CV_A) (Houle 1992) (Eq. 2.4), a measure of genetic variation normalised by the trait mean. Heritability is an estimate only of the proportion of trait variance attributable to genetic components, so by accounting for the total amount of phenotypic variance, CV_A provides a more realistic measure of the ability of a trait to respond to selection.

$$CV_A = \frac{\sqrt{V_A}}{\mu_{Trait}} \times 100 \tag{2.4}$$

2.2.4.2. Genotype x environment interactions

To investigate whether there were interactions between pine genotype and soil treatment, I used linear mixed modelling to test models structured as in Eq. 2.5. I included the specific soil origin as a random effect to control for variation between sites in our east/west soil categorisation. In order to test for local adaptation, I used the same model structure, but replaced the soil term with a term indicating sympatry or allopatry of the pine population and soil inoculum.

$$\begin{aligned} \text{Response} \sim \text{Population} \times \text{ Soil (east/west)} +& \\ \mathit{random}(\text{Family}) + \mathit{random}(\text{Block}) \ + \ &\mathit{random}(\text{Soil}) \end{aligned} \tag{2.5}$$

2.3. Results

In total, 1045 of 1056 seedlings survived through to the end of the experiment. Of these, 93% of inoculated seedlings showed signs of successful EM colonisation, and 89% of sterile control seedlings remained uncolonised at the end of the experiment. Of the 81 seedlings for which I measured the degree of colonisation, I found no effect of the degree of colonisation on host biomass response ($F_{1,73} = 1.2$, $p = 0.28$). However, I did find that the presence of EM fungi on seedlings, regardless mycorrhizal treatment, had a net positive effect on growth (Mycorrhizal presence: $F_{1,1018.9} = 25.9$, $p < 0.001$; Mycorrhizal treatment: $F_{1,1013.5} = 0.004$, $p = 0.95$)

(Fig. 2.2). For this reason, I assumed growth benefits to the seedlings were likely due to the presence of EM fungi rather than the other biotic components of the soil inocula. For all further analysis I removed all seedlings from the data set that did not meet treatment standards. This left 960 seedlings in the dataset in total (709 inoculated seedlings, 251 control seedlings).

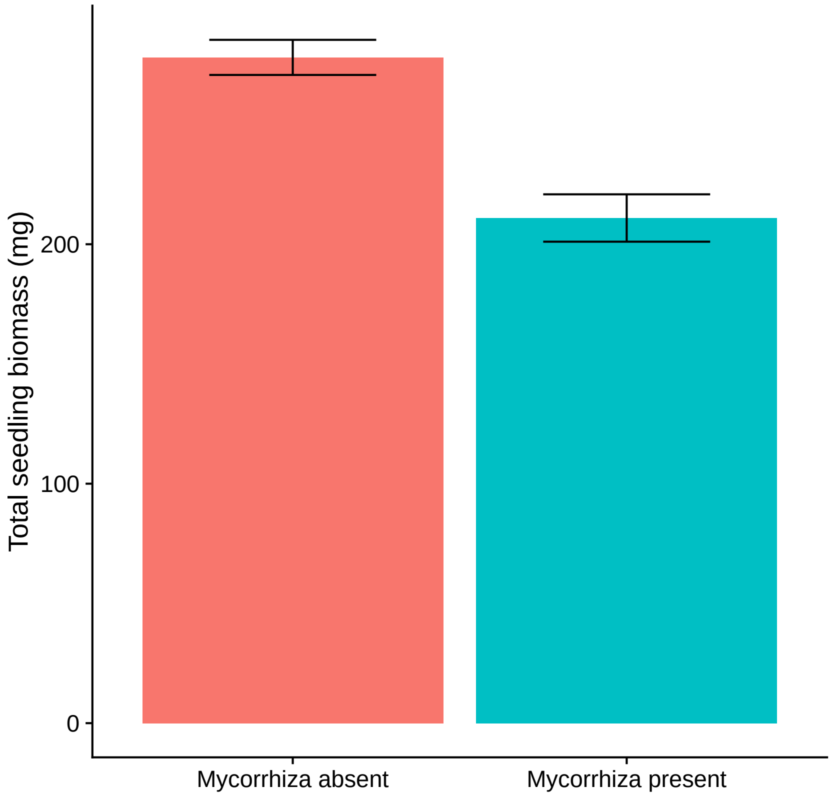

Figure 2.2.: Bar chart showing the response of seedlings to the presence or absence of mycorrhizal fungi, regardless of mycorrhizal treatment. Error bars show 95% confidence intervals.

On average, seedlings inoculated with mycorrhizal fungi grew an additional 62.6 (SE ± 3.9) mg larger than non-inoculated seedlings. However, the range of responses was wide, with 29.3% of seedlings across all treatments showing reduced growth compared to the control. Additionally, inoculated seedlings allocated relatively less biomass to roots than shoots, with root:shoot ratios on average 7.5% (SE &plusmn 0.88%) smaller than those of control seedlings. Again, however, there was a wide range in the response of this trait, with 40.6% of all seedlings increasing their root:shoot ratio in response to inoculation.

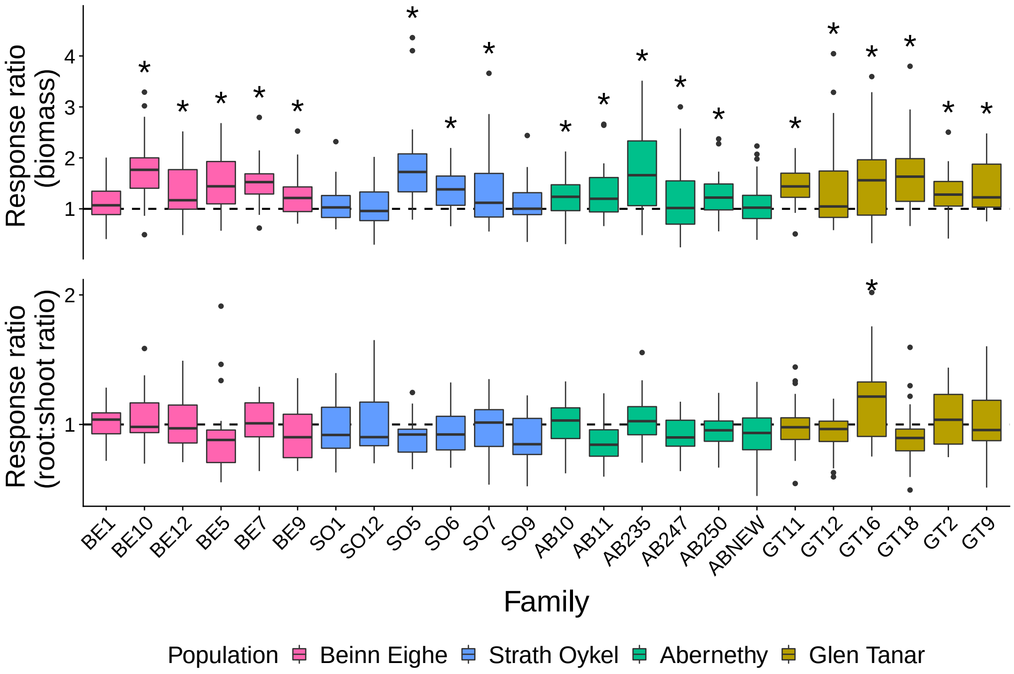

Figure 2.3.: Box plots showing the range of response ratios of seedling families for biomass (top) and root:shoot ratio (bottom). Middle line shows the median, box shows the first and third quantiles, and the whiskers show no more than 1.5x the interquantile range. Colours indicate the population of origin for the seedling. Dashed line indicates no change compared to a non-inoculated seedling, with a * indicating a significant difference compared to non-inoculated controls. Populations Beinn Eighe and Strath Oykel are located in the west, and Abernethy and Glen Tanar in the east.

Table 2.2.: Heritabilities (SE) of mycorrhizal response traits in each of the three soil categories (western soils only, eastern soils only, all soils). Each R column shows heritability estimated under different relatedness assumptions (R = 2: seedlings are full siblings; R = 3: seedlings are 50% full siblings and 50% half-siblings; R = 4: seedlings are all half-siblings). V columns: proportions of variance attributable to family (V_f), and block (V_b). CV_A: Coefficient of genetic variation. I considered heritabilities to be significant when the standard errors did not overlap with zero.

Trait	Soil origin	h^2 (R = 2)	h^2 (R = 3)	h^2 (R = 4)	V_f (%)	V_b (%)	CV_A
Biomass response	West	0.38 (0.09)	0.57 (0.16)	0.76 (0.24)	19.11	2.74	41.50
	East	0.52 (0.1)	0.78 (0.19)	1.05 (0.28)	26.17	2.89	41.36
	Full	0.2 (0.05)	0.3 (0.09)	0.4 (0.14)	10.06	2.51	28.24
Root:shoot response	West	0.32 (0.08)	0.47 (0.14)	0.63 (0.22)	15.79	3.31	17.89
	East	0.13 (0.06)	0.2 (0.1)	0.27 (0.15)	6.73	2.21	10.77
	Full	0.15 (0.04)	0.23 (0.07)	0.3 (0.12)	7.50	2.42	11.89

2.3.1. Heritability

Although the amount of within- and between-family variation was very large (Fig. 2.3), I found evidence for significant heritability of both mycorrhizal response traits under all soil conditions (Table 2.2). Estimates varied from 0.13 to 1.05, although in many cases standard errors were large. Variation in traits due to block effects were low for all estimates; however, in all cases residual variance accounted for the vast proportion of error.

2.3.2. Genotype x environment interactions

I found evidence for a significant G x E interaction between seedling populations and soil treatments for biomass response to inoculation (Population x Soil: $F_{3,673.6} = 12.6$, $p < 0.001$), but no individual effects of population or soil (Fig. 2.4). In particular, I found that eastern inocula had a uniformly beneficial effect on growth for all populations, whereas western inocula had markedly different growth benefits depending on the population of origin for a seedling. Pines from either extreme of the longitudinal range in this study (Beinn Eighe in the west and Glen Tanar in the east) grew better with western inocula than eastern inocula, whereas pines from the middle two populations showed no difference in growth when inoculated with either inoculum.

I also found evidence for a significant G x E interaction between population and soil treatment for a shift in root:shoot ratio following inoculation (Population x Soil: $F_{3,674.2} = 2.7$, $p = 0.042$), but no individual effect of soil or population (Fig. 2.5). More specifically, I found a complex pattern of interactions between soil types and populations: one population (Strath Oykel) allocated more biomass to shoots over roots when grown with western inocula, while the others (Abernethy, Beinn Eighe, and Glen Tanar) showed no differentiation in root:shoot response between inocula.

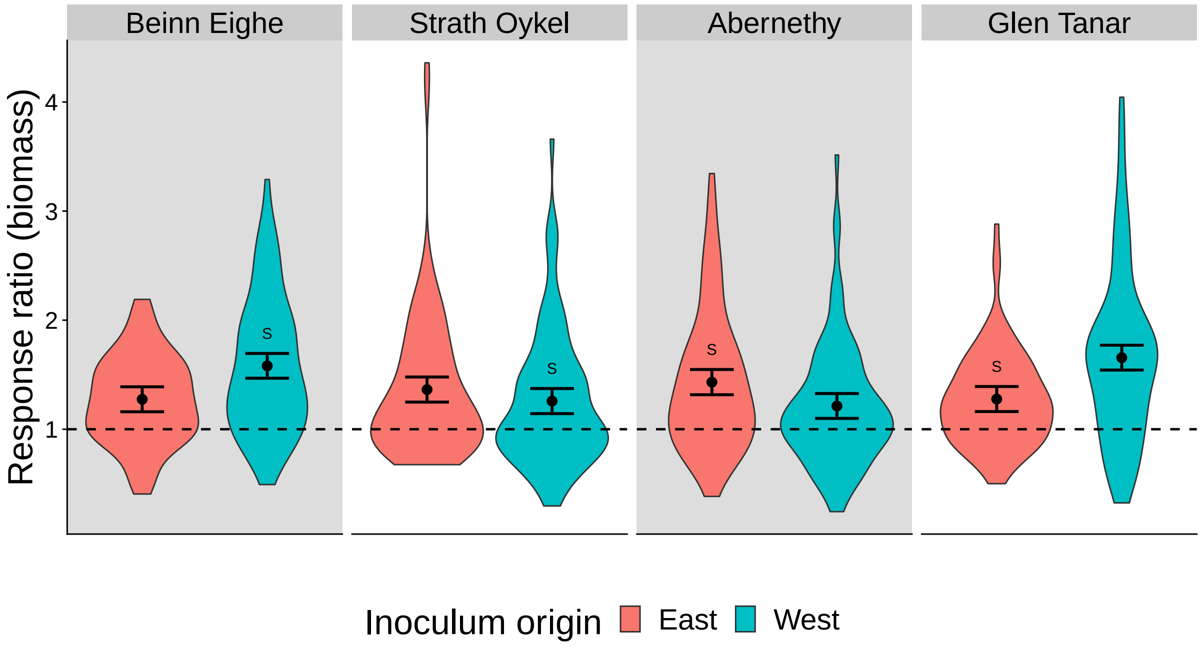

Figure 2.4.: Response ratio of total seedling biomass among treatment classes. Violin plots show the distribution of raw data; error bars show model predictions ± SE. Red violins = eastern soil inoculum (Abernethy, Glen Tanar); Blue violins = western soil inoculum (Beinn Eighe, Strath Oykel). S = sympatric combination. Pine populations are ordered from most western to most eastern. There were no significant differences apart from a significant interaction between Strath Oykel seedlings grown in western soil. Dashed line = no change compared to a non-inoculated seedling. Model predictions and SE were fitted using R package *glmmTMB*.

2.3.3. Local adaptation

Assuming that the locally adapted phenotype would show increased biomass accumulation when grown with its local soil inoculum, I found no evidence for any main effect of growing in home soil vs away soil, either considering soils as four individual treatments (Effect of sympatry on biomass response: $F_{1,674.8} = 0.95$, $p = 0.33$; Effect of sympatry on root:shoot response: $F_{1,675} = 3.07$, $p = 0.08$) or on an east-west basis (Effect of sympatry on biomass response: $F_{1,673.8} = 0.06$, $p = 0.81$; Effect of sympatry on root:shoot response: $F_{1,674.3} = 2.1$, $p = 0.14$).

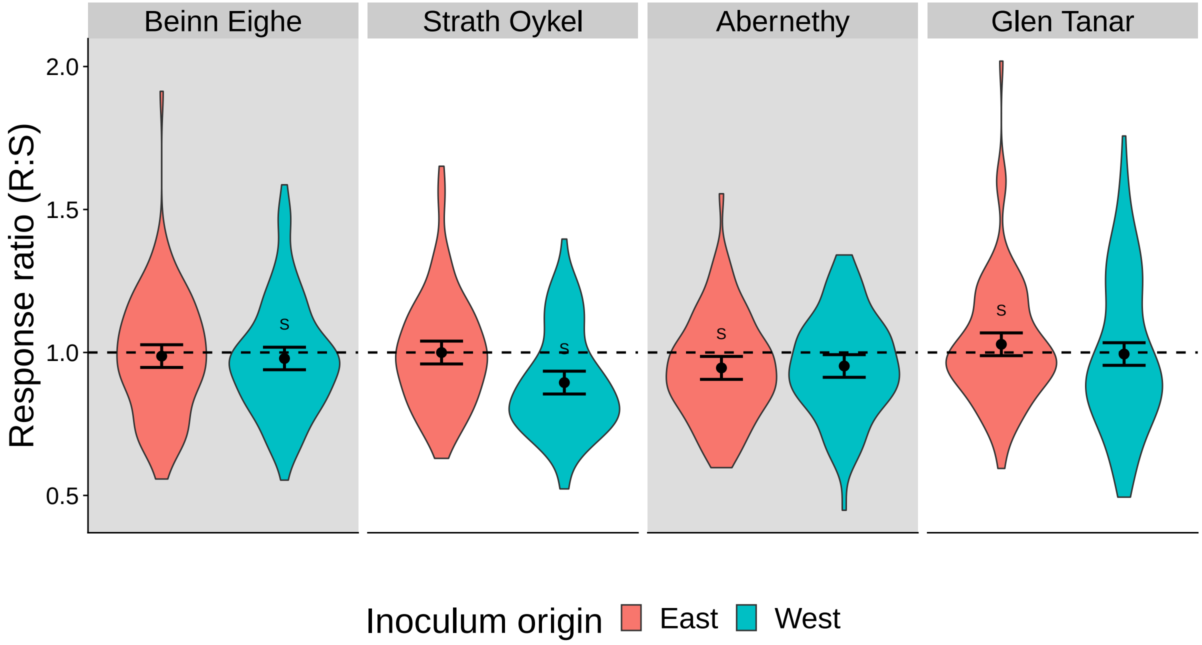

Figure 2.5.: Percentage change in seedling root:shoot ratio following inoculation. Violin plots show the distribution of raw data; error bars show model predictions ± SE. Red violins = eastern soil inoculum (Abernethy, Glen Tanar); Blue violins = western soil inoculum (Beinn Eighe, Strath Oykel). S = sympatric combination. The only significant interaction was a decrease in root:shoot ratio when Strath Oykel populations were grown in western soil. Pine populations are ordered from most western to most eastern. Dashed line = no change compared to a non-inoculated seedling. Model predictions and SE were fitted using R package *glmmTMB*.

2.4. Discussion

Overall, I found evidence for heritability in both host performance and change in root shoot ratio in response to inoculation in all of our soil treatments. In line with previous studies in this system, I expected to find little overall differentiation between populations, but to find variation that fell along the major east-west climatic axis of Scotland. Although I did find evidence for G x E interactions between pine populations and soil inocula, suggesting some degree of genetic differentiation between populations in their response to specific fungi, I was unable to detect any clear pattern of geographic variation, either along the east-west gradient or in terms of local adaptation.

2.4.1. Heritability

For both growth in response to inoculation and shift in root:shoot ratio, I found significant heritability within as well as across all soil treatments. Estimates of heritability were high under the most realistic relatedness scenario (R = 4, all half-sibs), but had wide estimated errors. Previously reported values for plant performance were broadly comparable to those found here for plant growth. Rosado et al. (1994) reported values of heritability for plant growth between 0.55 and 1.2 in inoculated seedlings, higher than in non-inoculated seedlings (0.26 to 1), in *Pinus elliottii* inoculated with *Pisolithus tinctorius*, suggesting a greater degree of variability in these traits in response to EM inoculation than without. However, the authors reported no errors for their estimates. A few other studies have shown heritability in other EM-associated host traits, including the number of EM root tips, root enzyme activity, and species-specific compatibility (Tagu et al. 2005; Courty et al. 2011; Velmala et al. 2012; Lamit et al. 2016), suggesting that host genotype is an important component of EM interactions. Understanding how these lower-level traits integrate to produce higher-level responses in host or fungal growth would be valuable to further our understanding of the process of adaptation in these organisms (Hoeksema 2010).

Values of heritability are hard to accurately estimate, and require large amounts of replication at the family and population level. Estimates can also be influenced by maternal effects: particularly in the case of young seedlings, these effects can inflate measures of variance between families, leading to overestimation of heritability. Differences in environment between populations can also affect seed development, which may lead to additional differences in performance that are conflated with genetic variance (Falconer and Mackay 1996). These latter effects are particularly difficult to control for without multigenerational trials, which are very time consuming when working with trees. I did not control for such non-genetic factors in this study, which may explain why our estimates of heritability were particularly high for our estimates of biomass response. It is also worth noting that the measurements of heritability in both this study and in Rosado et al. (1994) were conducted on seedlings grown in individual pots under glasshouse conditions. Seedlings in natural environments are normally connected to a common mycelial network, such that they are connected to fungal partners that are maintained by nearby mature trees (Teste et al. 2009). It has been suggested that many of the negative outcomes seen in mycorrhizal studies are a result of such laboratory conditions, and that experiments conducted in field conditions should produce more positive results (Frederickson 2017). It would be instructive for future work to test for heritability of mycorrhizal response under field conditions as well.

2.4.2. Local adaptation

In Scotland, mycorrhizal communities have been shown to vary strongly in response to the W-E rainfall gradient (Jarvis et al. 2013). Because of this, I assumed that community composition in the two western and two eastern inocula would be broadly similar. Our results here suggest that populations of Scots pine within Scotland have diverged in their interactions with EM fungi in terms of both growth and biomass allocation. This effect does not appear to be a simple effect of pine population, but instead depends on the specific combination of both population and inoculum in question. However, these G x E interactions show no clear geographic pattern. In particular, I found no effect of either local adaptation or variation along the east-west climate gradient. Instead, the seemingly random pattern of interactions suggests that other unmeasured traits, such as resistance to fungal pathogens, may play a role determining the divergence of these populations.

Previous studies have reported mixed results for evidence of local adaptation in EM interactions. A previous study using 5 pine populations at a wider geographic scale found similar evidence for G x G interactions, but some host traits (relative growth rate and short root length) were smaller in sympatric plant-soil combinations (Hoeksema et al. 2012), while another study on the same system found a clinal gradient of colonisation based on the distance between the host population and fungal population (Hoeksema and Thompson 2007). A study explicitly incorporating climate, altitude and soil effects also found that soil fungi mediated the adaptation of Douglas-fir seedlings in over half of their treatment combinations (Pickles et al. 2015). However, a recent meta-analysis found no overall effect of host-fungal sympatry on host performance in EM systems, though this conclusion was based on a small number of studies (Rúa et al. 2018).

Because I sampled at a small spatial scale, the possibility cannot be excluded that gene flow swamped local selection. However, between the most eastern and most western populations, there is a lag of over two weeks in peak pollen production, which would limit gene flow between the geographic extremes (Whittet et al. 2017). Local adaptation has been demonstrated in these populations along the west-to-east gradient for a number of traits, including needle morphology (Donnelly et al. 2016), response to waterlogging (Donnelly et al. 2018), photochemical capacity (Salmela et al. 2011), and spring phenology (Salmela et al. 2013). Because mycorrhizal fungal communities in this pinewood system have also been shown to vary longitudinally along a rainfall gradient (Jarvis et al. 2013), I believe that the absence of local adaptation found here is due to a lack of selection for mycorrhizal response rather than the homogenising effects of gene flow.

Instead, this overall lack of adaptation may result from life history traits of EM hosts. Over the course of a long lifespan, the importance of mycorrhizal associations for host fitness may vary strongly. For example, trees face varying strengths of intraspecific competition depending on their life stage (Petit and Hampe 2006), and thus for a seedling a boost in growth made by associating with preferred fungi may be much more important for survival than for a mature canopy tree. Alternatively, host preference in seedlings may be disadvantageous, as associations with whatever fungi are available may provide a better chance of survival than being selective. Additionally, if EM community composition is liable to shift within the lifespan of a host, then even if EM fungal associations pose a barrier to survival, then any host adaptation is likely to be quickly disrupted. Instead, the reassortment of EM community composition in response to environmental change may facilitate host survival by providing fungal species or genotypes that are better adapted to current conditions. EM fungi can manipulate host growth, for example by modifying the allocation of biomass to roots or shoots, which may facilitate host survival in a changing environment.

If local adaptation is unlikely in long-lived hosts, host-fungal genotype x genotype interactions may still be maintained through other traits that modify biotic interactions. Recent research is beginning to indicate that host resistance to pests and pathogens can influence mycorrhizal compatibility. For example, *Pinus edulis* individuals resistant to attack by a stem boring moth were found to harbour different EM communities (Sthultz et al. 2009), which provided differing amounts of host benefit under increasing drought (Gehring et al. 2014; Patterson et al. 2018). Similarly, genotypes of *P. taeda* resistant to two fungal pathogens showed both variation in compatibility with specific fungal genera and variation in relative growth rate (Piculell et al. 2018). Evidence of similar effects have also been hinted at in angiosperms, with fungal leaf pathogen communities strongly correlating with EM fungal communities (Lamit et al. 2015). Selection for resistance to these pathogens may thus help maintain the complex G x G interactions found in studies of local adaptation to EM fungi.

It is worth noting that the soil treatments applied here are likely to have included non-EM organisms, such as bacteria and pathogenic fungi. Although I found that the presence of mycorrhizal fungi generally resulted in an increase in plant growth, the G x E interactions found here could be influenced by negative interactions with soil-borne pathogens. If host pathogen resistance and EM response traits are indeed linked, then further work to separate these independent selective effects will be required. In the Caledonian pinewoods, resistance to the foliar needle pathogen *Dothistroma septosporum* has been found both among maternal families (Perry et al. 2016b) and among provenances (Perry et al. 2016a). Additionally, constitutive secondary defensive compounds, particularly monoterpenes, have been shown to influence herbivory by a variety of organisms (Iason et al. 2011), as well as the ground vegetation around a tree (Iason et al. 2005). Variation in the monoterpene $\delta^3 - carene$ shows a geographic bias within Scotland, with high $\delta^3 - carene$ levels uncommon in north-west sites. These or other genetic factors may play a role in determining response to mycorrhizal inoculation, and could explain some of the G x E interactions found in this study. Further work investigating the effects of both $\delta^3 - carene$ and monoterpene chemotype more broadly on Scots pine-EM interactions are underway at present.

2.4.3. Conclusions

The complex set of host-soil interactions found here continues to underscore the complexity of geographic patterns in host-EM interactions. Although I was unable to ascribe a clear, geographic pattern to the host-soil interactions found here, I demonstrated the heritability of mycorrhizal response in Scots pine within a relatively confined geographic area. Given the lack of data on the heritability of ectomycorrhizal responsiveness in the literature, and the importance of heritability to the development of local adaptation, I would recommend that future studies on local adaptation to symbionts explicitly incorporate host genetic structure, such as clones or maternal families, in order generate robust estimates of heritability.

3

Do antifungal monoterpenes affect pine-mycorrhizal interactions?

3.1. Introduction

Mycorrhizal fungi are important components of most terrestrial ecosystems, forming intimate associations with approximately 80% of plant species (Smith and Read 2008). These associations are usually mutualistic, involving an exchange of sugars and soil nutrients, but can vary in outcome, with parasitic or commensal outcomes possible (Karst et al. 2008). Such outcomes are context-dependent, varying between different host genotypes (Hoeksema et al. 2012), fungal genotypes (Burgess et al. 1994), and depending on the environmental context of the interaction (Patterson et al. 2018). Because of this genetic context, it has been suggested that both partners should be subject to ongoing coevolution, in order to maximise the benefit received from the interaction (Hoeksema 2010). Evidence of genetic variation between host genotypes in the amount of benefit received from ectomycorrhizal (EM) fungi has been demonstrated using number of traits, including relative growth rate, height, and final biomass (Hoeksema and Thompson 2007; Hoeksema et al. 2012; Pickles et al. 2015), and this genetic variation can be geographically structured, as a result of local adaptation (Rúa et al. 2016a, 2018).

It is not yet clear which host traits drive this genetic variation in benefit (Hoeksema 2010). One mechanism through which hosts are potentially able to exclude or reduce associations with poor or parasitic partners is preferential allocation, where a host can direct the rewards of a mutualism to more beneficial symbionts (Bever 2015). However, because this mechanism should not depend on symbiont identity, but simply the abundance of quality partners, variation should not produce the complex G x E interactions often seen in studies (Hoeksema et al. 2012; Downie et al. 2020). Instead, variation in the signalling and recognition systems required for successful establishment of a mycorrhiza might underlie such host responses (Hoeksema 2010; Hoeksema et al. 2012). In order for a fungus to successfully colonise a plant root, it must interact with and circumvent the host's defence systems (Garcia et al. 2015). Components of a host's immune system could thus be under selection in order to avoid association with poor or parasitic fungi, which might lead to local adaptation or complex G x E interactions.

Genetic variation in signalling or host defence might be visible as variation in the association frequency of different host genotypes and particular fungal species. Populations of Monterey pine (*Pinus radiata*) have previously been shown to vary in their frequency of association with different ectomycorrhizal species (Hoeksema et al. 2012), and an experiment on Norway spruce (*Picea abies*) found that genotype explained approximately 25% of the variation in associated EM community composition on an individual tree (Velmala et al. 2012). Variation in mycorrhizal associations has also been noted in response to genotypic variation in host defence. Tagu et al. (2005) found that a QTL in *Populus trichocarpa* associated with ectomycorrhiza formation was linked to other QTLs involved in the interaction with a leaf rust fungi, while Piculell et al. (2018) found that susceptibility to two fungal pathogens affected the proportion of root tips colonised by the genus *Thelephora* in multiple soil sources. Patterson et al. (2018) found strong differences in the proportion of basidiomycetes to ascomycetes, as well as a two-fold difference in the Shannon diversity index of fungal communities, between two genotypes of pinyon pine (*Pinus edulis*) which varied in their resistance to a stem-boring moth.

However, all of the studies conducted above were on plants grown in individual pots in glasshouse trials. In natural environments, EM fungi are patchily distributed at small scales, often less than 4 m (Lilleskov et al. 2004; Pickles et al. 2012; Bahram et al. 2016), rather than homogeneously distributed between pots. This small-scale spatial variation may render any effects of host genotype irrelevant if the effect size is small enough. A number of studies conducted on trees from natural environments found that spatial position strongly determined the EM community composition, with no differences in associations between different host genotypes at any position on a transect (Saari et al. 2005; Lang et al. 2013; Bubner et al. 2013). By comparison, a common garden of 15 year old clonally-replicated *Populus angustifolia* trees, host genotype explained 13% of the variation in EM associations (Lamit et al. 2016), and these EM communities have been shown to covary with communities of fungal leaf pathogens (Lamit et al. 2015). Interestingly, the experimental effect seen in the pinyon pine example was also demonstrated in the field, with moth-resistant trees showing much lower percentages of ascomycete colonisation (Gehring et al. 2014). Together, these results suggest that variation in host defence may affect associations with

EM fungi in the field, particularly if it is large, but that genetic variation in other traits may not.

One important defence system in trees, particularly conifers, involves secondary metabolites such as terpenes and phenolics. These diverse families of volatile compounds are produced in shoots, needles and roots (Hiltunen 1976) and have been shown to deter grazing by insects (O'Reilly-Wapstra et al. 2007; Iason et al. 2011), as well as having anti-fungal effects *in vitro* (Eckhardt et al. 2009; Marei et al. 2012; Zhang et al. 2016). These anti-fungal properties have been shown to be active against both saprotrophic and ectomycorrhizal fungi (Melin and Krupa 1971; Ludley et al. 2008), via exposure both to pure vapours and leaf litter. Curiously, EM fungi were found to be more strongly growth inhibited than saprotrophic fungi (Ludley et al. 2008), and reports of the accumulation of phenolic compounds in root tips, particularly in known incompatible host-fungi pairings (Malajczuk et al. 1982; Molina and Trappe 1982; Voigt et al. 2000), suggests a role of secondary metabolites in mediating successful colonisation. However, it is not yet known whether constitutive variation in terpene chemotype can affect the colonisation of hosts by different groups of fungi.

The native Scots pinewoods (*Pinus sylvestris*) of Scotland provide a good system within which to test the effects of genotype and terpene character. Seedlings from different populations in this system have previously been shown to be genetically differentiated in their response to EM inoculation (Downie et al. 2020), and additionally have a well-documented ectomycorrhizal flora, which varies in response to the dominant rainfall gradient in Scotland (Jarvis et al. 2013, 2015). These populations also show a geographic bias in their terpene chemotype; trees from north-western populations in Scotland typically lack the monoterpene 3-carene (Forrest 1980; Thoss et al. 2007), which is usually the second-most abundant terpene in Scots pine (O'Reilly-Wapstra et al. 2007).

To determine whether there is genetic variation in the association of host plants with different ectomycorrhizal fungi in the field, a reciprocal field transplant was set up to investigate whether pine genotype or terpene character affects the selection of mycorrhizal partners by pine seedlings growing in the field. Seedlings from six populations were grown reciprocally in grids in each of the six originating forests in a fully factorial cross. Mycorrhizal communities were assessed through morphotyping and ITS barcoding, and terpene character through gas chromatography.

I sought to answer the following questions:

1. Does the genotype and/or terpene character of a Scots pine seedling affect the diversity of its associated community?
2. Does the genotype and/or terpene character of a Scots pine seedling affect the frequency of association with different species of EM fungi?

3.2. Methods

Cones were collected from six stands of Caledonian pinewoods in Scotland: in the west, Beinn Eighe, Loch Clair and Shieldaig, and in the east, Abernethy, Rothiemurchus, and Glen Derry (Fig. 3.1, Table 3.1). Cones were selected from two mother trees at each site, with one maternal tree having a known high 3-carene chemotype and one with a low chemotype, for a total of 12 maternal families across all populations. Seeds were sterilised and sown on sterile compost in seed trays to germinate. Following germination but before transplanting, the root systems were checked to ensure there were no visible signs of EM colonisation. At each site, three grids were set up at haphazardly chosen locations (between 100m to 2 km apart). Each grid was set up in a patch of moss, outwith the vicinity of any EM host plants, to try and reduce spatial heterogeneity. Grids were 50 x 50 cm, with 5 cm intervals, and 3 to 5 seedlings per maternal family were transplanted in each grid at randomised positions. This grid size was chosen as it was found in previous work (unpublished) to reduce spatial heterogeneity between seedlings while remaining far apart enough that the root systems would not overlap during the growing period. Seedlings were planted into the FH horizon, in order to capture the majority of EM fungal diversity. After three months of growth in the field, seedlings were harvested, and root systems were separated from the shoots. Both were stored in bags at -20°C for later analysis.

3.2.1. Community characterisation

I assessed ectomycorrhizal communities selected by each pine seedling by morphotyping. On each seedling, I viewed root tips under a stereo microscope, and assigned them to morphotypes based on their appearance, with identification where possible (Agerer 2001). I counted the number of root tips for each morphotype on each seedling, with a tip being considered as each individual end where a single colonisation point branched (for example, a bifurcating root tip

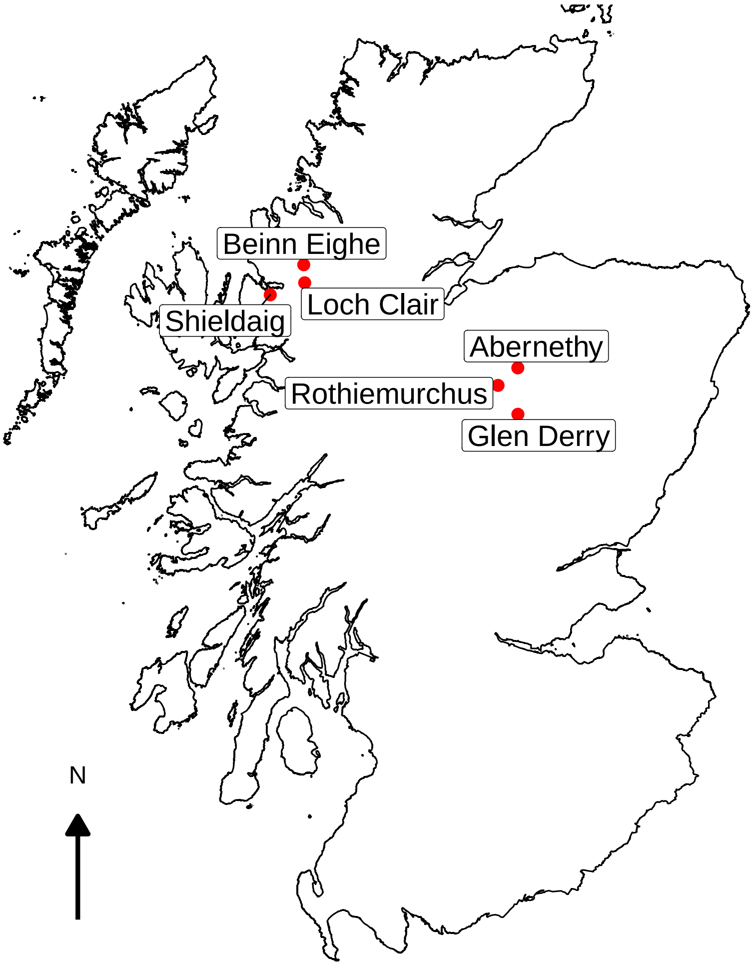

Figure 3.1.: Geographic position of the six populations used in the study.

Table 3.1.: Coordinates of each population used in the trial, as well as climatic variables (Met Office) and soil types (Soil Information For Scottish Soils, James Hutton Institute). MAP = mean annual precipitation, MJT = mean July temperature

Population	Code	Latitude	Longitude	Altitude (m)	MAP (mm)	MJT (°C)	Soil Type
Abernethy	AB	57.21	-3.61	362	1060.7	13.1	Humus-iron podzol
Beinn Eighe	BE	57.63	-5.35	21	2476.1	14.5	Peaty gleyed podzol
Glen Derry	GD	57.03	-3.58	443	1080.4	11.7	Peaty gleyed podzol
Loch Clair	LC	57.56	-5.36	112	2888.2	14.0	Peaty gleyed podzol
Rothiemurchus	RM	57.15	-3.77	307	1060.0	13.5	Humus-iron podzol
Shieldaig	SD	57.50	-5.64	68	2449.7	13.1	Brown earth

was considered as two tips). Tuberculate mycorrhizas were counted as one tip. I also counted the number of non-ectomycorrhizal root tips. For each morphotype on a seedling, 1 to 5 root tips were stored in Eppendorf tubes and frozen at -20°C for identification using molecular barcoding of the ITS region.

For each morphotype from each grid, I selected a number of root tips for sequencing, with a minimum number of 5 tips from different seedlings where possible. For all morphotypes from the three eastern sites, I extracted DNA from root tips using the Qiagen DNEasy Plant Mini Kit, with the following modification to the protocol: at the final elution stage, DNA was eluted from the spin column in 2 x 25 µl steps. I performed PCR for each extract using the fungal specific primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3-) and ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3'). These primers are capable of amplifying DNA from both Ascomycetes and Basidiomycetes, although each primer shows a small amplification bias towards one or the other (Bellemain et al. 2010). PCR was performed in a 25 µl reaction mix with the following makeup: 10.5 µl H_2O, 5 µl 10X PCR buffer, 1 µl each of ITS4 and ITS1-F at 20 µM concentration, 4 µl $MgCl_2$ at 50 micro;M, 1 µl dNTPs at 20 µM, 1 µl BSA at 20 mg ml^{-1}, 0.5 µl BioTaq Polymerase, and 1 µl template DNA. The following settings were used on the thermocycler: 95°C for 5 minutes, then 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for a minute, before at 72°C for 10 minutes and holding at 10°C. Samples which yielded a single band following PCR were then sent for Sanger sequencing at Eurofins GATC using primer ITS1-F. Cleanup of the PCR reactions was performed at this facility.

For morphotypes from the three western sites, I used a different protocol for extraction and PCR, as the previous protocol did not reliably produce amplifiable DNA. I extracted DNA using the Sigma Aldrich REDExtract-n-Amp Plant kit, with the following protocol modifications:

individual root tips were placed into wells in a 96-well plate with 10 µl of the extraction solution, and incubated in a thermocycler at 65 °C for 10 minutes, then 95 °C for 10 minutes. After this, 30 µl of dilution solution was added to each extract. PCR was performed using the accompanying REDExtract PCR mix, in 20 µl reaction mix with the following makeup: 10 µl REDE mix, 8.6 µl H_2O, 0.4 µl each of ITS1-F and ITS4 at 20 µM, and 1 µl of template DNA. The following programme was used on the thermocycler: 94°C for 1 minute, followed by 30 cycles of 94°C for 1 minute, 51°C for 1 minute, and 72°C for 1 minute. Following these cycles, samples were held at 72°C for 10 minutes before being held indefinitely at 10°C. PCR reactions were then cleaned up using Sigma Aldrich illustra ExoProStar 1-step (ref), which was diluted 1 in 4 with PCR-grade H_2O. I then quantified DNA concentrations of each sample on a gel, diluted the samples to recommended concentrations, and sent samples which yielded a single band for Sanger sequencing at Edinburgh Genomics using primer ITS1-F.

Following sequencing, chromatograms from both sequencing sets were basecalled using the ABI KB Basecaller (version 1.4.1; Hyman et al. (2010)), and sequences were trimmed at each end to remove low-quality regions with Phred scores lower than 20. These sequences were used for all downstream analyses. For taxonomic identification of morphotypes, I first filtered all sequences to retain only those over 50 bp in length, as this length was found in preliminary analyses to allow for the identification of a small number of morphotypes which would otherwise have lacked identification. I then searched these filtered sequences against a local copy of the UNITE fungal taxonomy database (Nilsson et al. 2019) using BLAST+ (Camacho et al. 2009), accepting the most biologically plausible result. I collected information on fungal division, genus and species for each sequence, and filtered the results to keep only those sequences of known ectomycorrhizal origin, following Tedersoo et al. (2010). For each morphotype, I manually assigned an identity based on the identities of the tips for which sequence data was available. In cases where I had already putatively identified morphotypes to genus or species, this information was additionally considered when assigning identity.

To ensure consistency of morphological categorisation, where morphotypes had sequences suggesting multiple species, taxonomic IDs were then re-checked against microscope images of each morphotype to ensure that they had been correctly grouped, and split where appropriate according to morphological features. To check the consistency of taxonomic identification, sequences for each genus were aligned in mafft (Katoh and Standley 2013). These alignments were then used to construct rapid bootstrap maximum-likelihood trees in *RAxML* (Stamatakis (2014); trees in Appendix sec. B)), using the GTRGAMMA model and 1,000 bootstrap iterations. These trees were used to determine whether each genus should be considered as a single taxon, or whether multiple taxa were present within each genus. For all further analysis, I removed any OTU that had less than 50 root tips across all samples. This process removed 8 species, leaving 21 species total.

3.2.2. Gas chromatography and terpene data

The presence or absence of 3-carene within Scots pine is strongly genetically determined by a single locus, with homozygotes with the 3-carene allele having high 3-carene proportions, heterozygotes showing intermediate levels, and seedlings without any copy of the allele producing no 3-carene (Hanover 1966; Kinloch et al. 1986; Pohjola et al. 1989). Because of this, it can not be assumed that the 3-carene chemotypes of seedlings would match that of their maternal tree, as the paternal tree was not identified. To account for this, terpenes were extracted from needle tissue to assess the terpene character of each seedling. I assumed that needles would have a similar chemotype to roots, as terpenes in these tissues have previously been shown to be correlated in mature trees (Hiltunen 1976).

Seedlings were collected into small zip-lock bags onto ice and then stored at -20 degrees C until analysis. Needles from seedlings for four sites (Beinn Eighe, Loch Clair, Shieldaig, and Rothiemurchus) were sampled for terpene characterisation. Terpenes were extracted from finely chopped, small needle samples (mean = 36 mg) in duplicate in 500 µl of n-hexane containing 54.5 ug/ml of isobutylbenzene as an internal standard. GC-FID was carried out using a 30m RT BetaDEXsm chiral column with an internal diameter of 0.25 mm and a film thickness of 0.25 µm (Thames, Restek, UK), operated with Helium as a carrier gas at a flow rate of 1 ml/min. One microlitre of extract was injected into a split/splitless inlet operating at 180 degrees C with a split ratio of 40 and an initial oven temperature of 60 degrees C. The initial temperature was held for 2min before increasing at a rate of 3.5 °C/min to 130 deg C and then at 10 deg C/min to 210 deg C. The FID was operated at 250 degC. Mass spectrometer readings were transformed to measurements of µg terpene/g needle dry mass.

In total, there were 16 terpenes (in order of decreasing proportional abundance: alpha-pinene, 3-carene, terpinolene, beta-pinene, beta-caryophyllene, myrcene, limonene, unknown terpene, germacrene-D-4-ol, camphene, alpha-humulene, cis-beta-ocimene, gamma-terpinene, germacrene-D, biocyclogermacrene, tricyclene). For each seedling, each pair of measurements for each terpene was averaged, and then the proportional concentration of each terpene for each seedling was calculated, considering pairs of enantiomers as single terpenes. Using these proportional concentrations, I calculated the Shannon diversity index ($-\sum c_i log(c_i)$), where c_i the is proportional concentration of terpene i), to characterise the chemical diversity of each seedling. I also conducted a DCA ordination using the *decorana* function in *vegan* (Oksanen et al. 2019) in order to investigate how varied seedlings were in their terpene characters. To determine what factors affected terpene character, I used linear mixed models to determine whether there were effects of parental chemotype, population of origin, or location of growth on a seedling's chemical diversity.

3.2.3. Statistical analysis

All statistics were carried out in R (R Core Team 2019). At the end of the experiment, it was found that 127 of the 673 surviving seedlings remained uncolonised. Of the seedlings for which terpene data was gathered, 283 of the 343 seedlings were colonised. These seedlings were kept in the data sets for all analyse except where specified, as these seedlings may have failed to be colonised due to their genotype or terpene character. However, they were removed from data sets for the diversity and richness analyses.

To investigate how EM community composition varied across all samples, I conducted an ordination using NMDS with the metaMDS function in the *vegan* package, with the uncolonised seedlings removed. The number of axes (k) were selected by comparing the stress of ordinations with $k = 1$ to 10, and selecting k such that the stress was < 0.1 and not decreasing by large amounts. Following this method, k was chosen to be 4 (Fig. D.2). For each of the 12 maternal families at each site, I estimated the mean and standard error on each ordination axis.

For all models involving terpenes, I used the smaller dataset of 343 seedlings for which I had terpene character data. Terpene character was included in models as the chemical diversity of terpenes within a seedling. This value was found to correlate strongly with other potential measures of terpene character, including the proportional concentration of 3-carene ($r^2 = 0.84$) and the first DCA axis ($r^2 = 0.86$).

3.2.3.1. Richness and diversity analyses

To determine if the species richness or true diversity (Jost 2006) of the fungal community on a seedling varied between sites, families or with terpene character, I used GLMMs fitted in *glmmTMB* in R (Magnusson et al. 2019). Because true diversity is measured in species richness, I assumed that seedlings with 0 species richness also had zero true diversity. Models investigating terpene character were fitted using the smaller terpene dataset, with the formula in Eq. 3.2, but models investigating family effects used the full dataset, with the formula in Eq. 3.1. For species richness data, I modelled the response using a poisson error structure. The total number of root tips were included in the models to account for root system size, as larger root systems are likely to be colonised by more species. Effects of site, maternal family and terpene character were examined using likelihood ratio tests between models with these effects removed.

$$\begin{aligned}\text{Response} \sim \text{Total tips } + \text{ Site} +\\ \textit{random}(\text{Grid}) \ + \ \textit{random}(\text{Family})\end{aligned} \tag{3.1}$$

$$\begin{aligned}\text{Response} \sim \text{Total tips } + \text{ Terpene diversity} +\\ \text{Site} \ + \ \textit{random}(\text{Grid})\end{aligned} \tag{3.2}$$

3.2.3.2. Turnover analyses

To determine if community composition varied among genotypes or sites, I modified a method utilised by Shutt et al. (2020) to model turnover in community composition of OTUs (β-diversity) for any combination of explanatory variables of interest. This method uses a Bayesian GLMM framework with the abundance of root tips of a fungal OTU on an individual seedling as the response variable, with the log total number of root tips on a seedling used as an offset to control for the number of opportunities for colonisation. Using this framework, explanatory variables included as random or fixed effects interacting with OTU identity provide a test for turnover in community composition. I constructed these models in *MCMCglmm* in R (Hadfield 2019), using parameter-expanded priors for all random effects. Models were run for 2,000,000 iterations each, with the first 40,000 discarded as burn-in and thinning conducted every 200 iterations. Traces of the posteriors were checked visually to ensure good mixing and convergence, and effective sample sizes of the posteriors were checked to ensure they were >~200.

$$\begin{aligned}\text{Number of root tips} \sim\ & \mathit{log}(\text{Total root tips})\ +\ \mathit{random}(\text{OTU})\ + \\ & \mathit{random}(\text{Site} : \text{OTU})\ + \mathit{random}(\text{Grid} : \text{OTU})\ + \\ & \mathit{random}(\text{Family} : \text{OTU})\end{aligned} \tag{3.3}$$

In particular, I conducted two sets of models, one using maternal family and the full dataset, and one using terpene character with the reduced dataset, to estimate the effect of genotype on the proportional abundance of EM OTUs. In the first case, models were fitted with only an intercept as a fixed effect, with OTU fitted as a random effect, as well as interactions between OTU and site, family, and their interaction (Eq. 3.3). For the terpene character models, I fitted carene proportion as a fixed covariate, and fit a random uncorrelated intercept and slope for each OTU in response to carene proportion. I also included an interaction between site and OTU identity as a random effect to control for background variation in OTU abundance between sites (Eq. 3.4).

$$\begin{aligned}\text{Number of root tips} \sim\ & \mathit{log}(\text{Total root tips})\ +\ \text{Terpene diversity}\ + \\ & \mathit{random}(1\ +\ \text{Terpene diversity}) : \text{OTU}\ +\ \mathit{random}(\text{Site} : \text{OTU})\ + \\ & \mathit{random}(\text{Grid} : \text{OTU})\end{aligned} \tag{3.4}$$

3.3. Results

In total, 673 seedlings survived to the end of the experiment, of which, 546 were colonised by EM fungi during the growing period of three months. Uncolonised seedlings typically had small root systems with little to no branching, suggesting poor overall seedling success. I counted approximately 44,400 root tips in total, assigning them to 76 morphotypes. I assigned these morphotypes to 29 EM taxa, with a total of 18 genera and 29 species, but after filtering for low root tip counts, there were 21 total EM taxa in 14 genera (Fig. 3.2). On average, there were 8.8 EM species per site, but any individual seedling only associated on average with 2 ± 0.88 (SD) EM species. Data on terpene character were collected for 349 seedlings from four of the six sites, for which 282 were colonised.

Following the analysis of stress for different dimensions, the number of dimensions selected for the NMDS was four, with a stress of 0.09 Fig. C.2. The NMDS ordination showed that the community composition of EM fungi varied between sites, with communities from Loch Clare and Shieldaig being particularly distinct when viewed on axes 1 and 2 (Fig. 3.3 A). The other sites were broadly similar in community composition, particularly Abernethy and Rothiemurchus, which are geographically very close. Broadly speaking, western sites (Beinn Eighe, Shieldaig and Loch Clare) were more distinct from one another, while all three eastern sites (Abernethy, Rothiemurchus and Glen Tanar) showed higher degrees of overlap. Within each site, maternal families did not appear to differ strongly from one another, with error bars commonly overlapping for most pairs of maternal families. Plots of all other axes are available in Appendix D.

On average, seedlings had a mean chemical diversity of of 1.1 ± 0.33 (SD). However, chemical diversity was strongly bimodally distributed (Fig. 3.4 A), with the bimodal distribution being driven strongly by the proportional increase of α-pinene in seedlings lacking 3-carene (Fig. 3.4 B).

I found that terpene chemical diversity was strongly genetically determined, with seedlings from western seed sources or from maternal trees with a low 3-carene chemotype having lower chemical diversity (Western seedlings: -0.2, SE = 0.03, z = -5.6, $p < 0.001$; Low 3-carene mother tree: -0.3, SE = 0.034, z = -8.6, $p < 0.001$). By comparison, I found no effect of environment on terpene chemical diversity, considered as an effect of seedling location (Site: Chisq = 6.9, df = 3, p = 0.07).

3.3.1. Richness and diversity analyses

On average, seedlings associated with 1.7 fungal species (SD = 1.1). I found no effect of terpene chemodiversity or family on the species richness of a seedling's EM community (Terpene chemodiversity: Chisq = 3.1, df = 1, p = 0.08; Maternal family: Chisq = 0.4, df = 1, p = 0.53), but did find that richness varied between sites in the full data set, but not in the smaller terpene

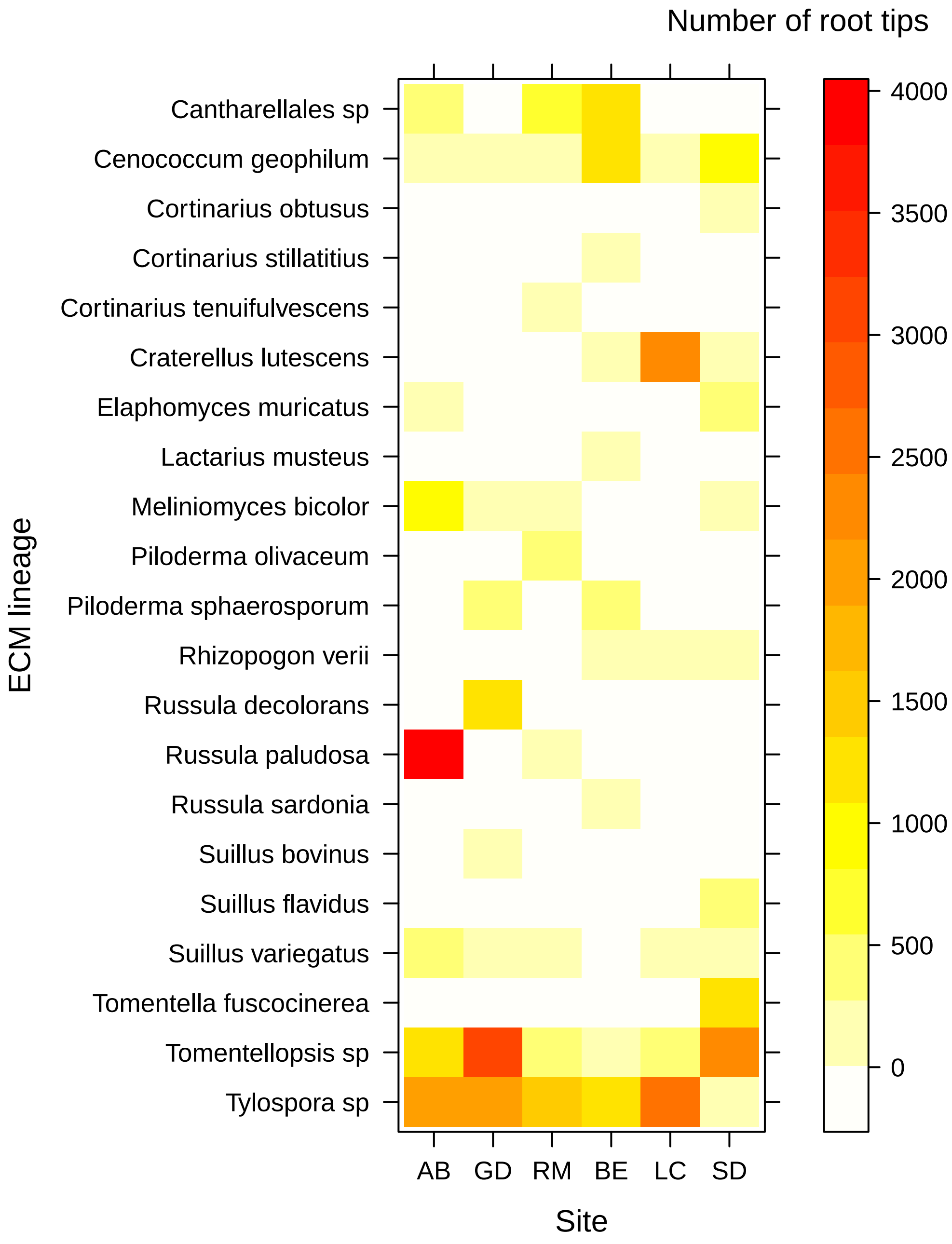

Figure 3.2.: The total number of root tips of each OTU found at each of the six sites.

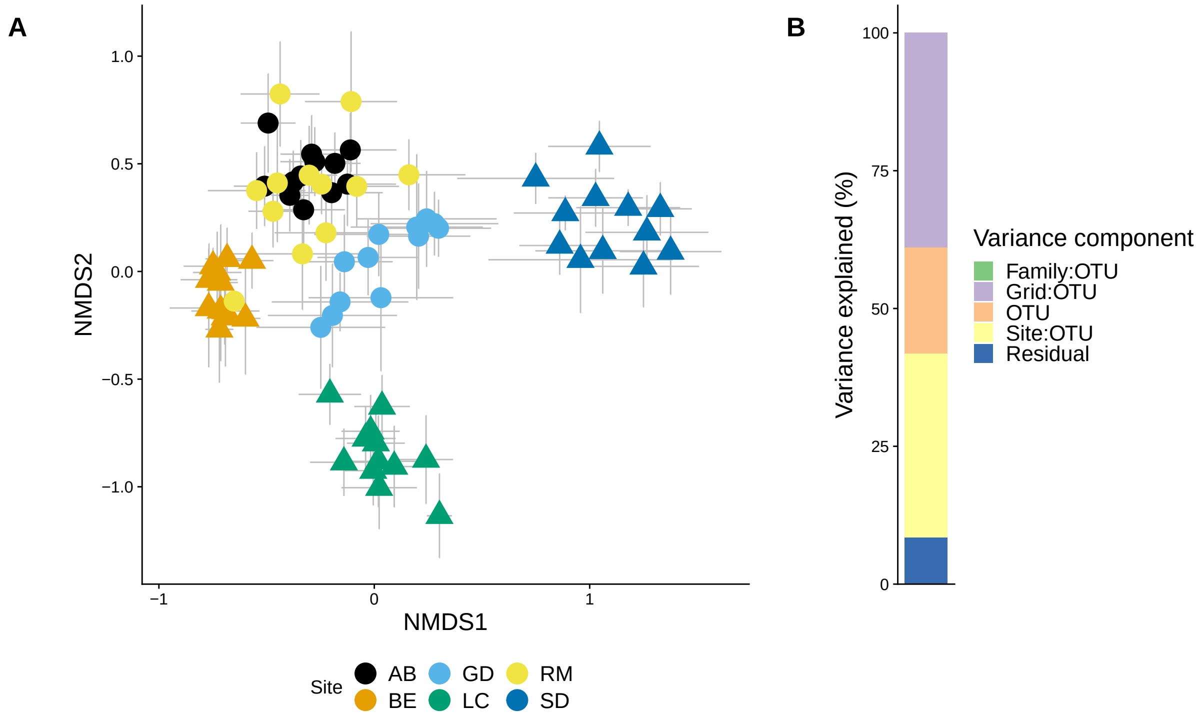

Figure 3.3.: A: NMDS ordination of EM species. Each point represents the mean NMDS score ± SE for one of the 12 maternal families at a particular site. Circles = eastern sites, Triangles = western sites. B: Percentage of variance explained by each component in the turnover model, calculated from the estimates of the posterior modes.

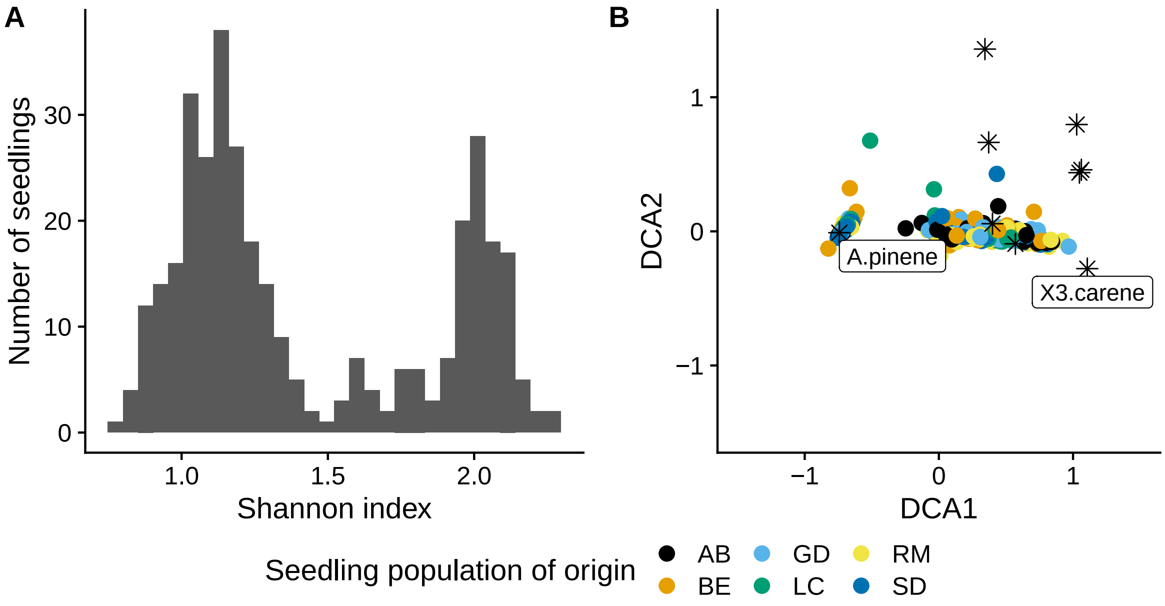

Figure 3.4.: A: Distribution of chemical diversity amongst seedlings. B: DCA ordination of terpene chemotype. Circles = individual seedlings, with colours showing the population of origin. Stars and labels = terpene scores.

data set (full data set: Chisq = 14.0, df = 5, p = 0.02; Terpene data set: Chisq = 7.8, df = 3, p = 0.051).

The mean diversity of a seedling's EM community was lower than the species richness (1.45 effective species, SD = 0.8). I found a difference between sites in diversity of seedling's fungal community (full data set: Chisq = 18.7, df = 5, p = 0.002; terpene data set: Chisq = 8.6, df = 3, p = 0.04), but no effect of terpene chemodiversity (Chisq = 3.4, df = 1, p = 0.07) or maternal family on diversity, as in the richness model [Chisq = 1.1, df = 1, p = 0.3].

3.3.2. Turnover analyses

I found no evidence that seedling genotype, considered as maternal families, had an effect on which EM OTUs associated with a given seedling (σ^2 attributable to maternal family = 0.09, 95% CI: ~0 - 0.24). I found strong evidence for significant variation in OTU abundance between each of the six sites (σ^2 attributable to site = 26.5, 95% CI: 9.8 - 46.1), and strong evidence for variation within sites at the grid level (σ^2 attributable to Grid = 29.0, 95% CI: 17.8 - 41.2). There was also evidence for variation in the overall abundance of different OTUs (σ^2 attributable to OTU = 21.0, 95% CI: 4.5 - 43.5). In total, the residual variance was 5.6 (95% CI: 5.1 - 6.3). This means that turnover due to spatial variation (site and grid) accounted for 72% of the variance in the model (Fig. 3.3 B), with the rest of the variance unexplained.

I found no evidence that terpene character affected EM community composition (Slope of 3-carene proportion: -0.44, 95% CI: -1.0 - 0.1; σ^2 attributable to 3-carene:OTU slope = 0.4, 95% CI: ~0 - 1.4). I also found evidence for variation between sites (σ^2 attributable to site: 47.9, 95% CI: 13.4 - 89.0), as well as variation between between grids (σ^2 attributable to Grid = 19.2, 95% CI: 9.7 - 30.9). In total, the residual variance was 5.0 (95% CI: 5.1 - 6.8).

3.4. Discussion

Overall, I found strong evidence for turnover in community composition between sites, but found no evidence that maternal families associated differently with fungal OTUs. I also found no evidence that maternal families varied in the richness or diversity of their associated mycorrhizal communities. Similarly, I found no evidence that terpene chemodiversity affected the probability of association with different fungal OTUs, nor the richness or diversity of a seedling's EM community.

Despite finding no effect of terpene chemodiversity on the richness or diversity of associated fungal communities, these effects were close to the standard significance threshold ($p = 0.08$ and 0.07, respectively), despite controlling for other factors including root system size, site, and grid. It is possible that these effects are real, and could be revealed with a larger data set. However, as I found no evidence that specific OTUs were affected by terpene chemical diversity, there is also no mechanistic evidence that might explain these changes, such as a particular species being strongly affected by 3-carene. The lack of an effect of maternal family on diversity despite the high heritability of chemodiversity additionally suggests that this effect may be an artefact. Terpenes also exist in the environment outside of host seedlings, released into the air as volatiles and in the soil through needle litter (Ludley et al. 2008). As there is a geographic bias in the prevalence of 3-carene between the west and east of Scotland (Forrest 1980), there may be a filtering effect wherein the high prevalence of 3-carene at eastern sites has already removed or reduced in abundance species that are negatively affected by high quantities of 3-carene, which would render any effect of internal 3-carene concentrations hard to detect. Further work with a larger data set would be warranted to determine whether these effects are true, or simply artefacts.

Instead, these results suggest that it is the location of a seedling, rather than its genotypic identity, that is important in determining what EM fungi it associates with (Fig. 3.3 B). Ectomycorrhizal fungi are very patchily distributed at small scales, with patch sizes for some fungal species smaller than 4 m (Lilleskov et al. 2004; Pickles et al. 2012; Bahram et al. 2016), and 1 m patches being reported in some cases (Pickles et al. 2010). The size of each grid in this experiment was 50 x 50 cm, much smaller than reported patch sizes, and the soil space occupied by an individual seedling is smaller still. Even if a seedling's genotype does alter its ability to associate with a particular fungal OTU, this would not be apparent if the OTU was not present due to small-scale variation in distribution, confounding any ability to detect an effect of genotype.

A number of previous field studies on the effect of host genotype on EM community composition have found similar results highlighting the importance of space over genotype. In a study along a transect in a stand of *Fagus sylvatica*, EM community composition was found to vary spatially, but different host genotypes at each sample point were not found to differ in their EM associations (Bubner et al. 2013). Similar results were found in an experiment by Lang et al. (2013) exploring the EM communities at transect points between 3 individual *F. sylvatica* trees. A study on 5 individual *Pinus sylvestris* trees in a native stand in Scotland also found no differences in EM associations between host genotypes (Saari et al. 2005). However, these studies were not conducted on trees in which genotype was selected, *a priori*, to test for an effect. In a study mapping mycorrhizal traits onto SNPs, Piculell et al. (2019) found that EM traits were largely governed by a few genes of large effect, with no individual gene affecting associations with more than one species. Studies like these, conducted on closely-related trees growing in proximity to one another, may thus not provide much insight into whether genotypes vary in their EM associations to an extent that might be detectable as local adaptation.

Instead, studies that incorporate genotypes known to differ should provide more insight into traits that may underlie interactions with EM fungi. In one such study in a single stand of *P. edulis*, two genotypes of tree varied in their canopy architecture as well as in their resistance to a stem-boring moth. These genotypes were shown to vary in their proportion of ascomycete to basidiomycete EM fungi, as well as the associated diversity of their EM communities (Sthultz et al. 2009; Gehring et al. 2014; Patterson et al. 2018). In the experiment I report here, the two genotypes from each population came from a high 3-carene mother and a low 3-carene mother, which I hypothesised might affect the EM associations of a seedling. The lack of evidence found for an effect of co-occurring host defensive genotypes from multiple populations thus allows us to robustly assess the effects of this particular genotype on community associations.

The lack of genetic signature in EM associations here also highlights the potential importance of life history stage in shaping the evolutionary response to EM fungi (Batstone et al. 2018; Downie et al. 2020). Particularly at such young ages, excessive selectivity on the part of a seedling may impose significant competitive penalties, reducing the potential benefit available to a seedling to aid in competition for light and space. If the predictability of partner quality for particular EM species can vary, for example due to temporal or spatial heterogeneity in soil resources, generalism in interactions on the part of seedlings may be important for survival, precluding adaptation. In agreement with this, a previous study in this system found no evidence of local adaptation of Scots pine seedlings to EM fungi (Downie et al. 2020), and overall evidence for local adaptation in EM interactions is lacking (Rúa et al. 2018).

3.4.1. Conclusions

These results suggest that both large and small-scale variation in the environment and in EM fungal distributions can be much more important than host genetic effects in determining EM associations. In addition, the lack of genetic effects on EM associations found here underscores the importance of conducting experiments on host genotype in more realistic field conditions as well as in the glasshouse. Glasshouse experiments are powerful tools that can disentangle effects that cannot be manipulated in the field, and are useful in understanding traits underlying specific effects. However, they typically remove all factors other than those of interest, with the potential to exaggerate those effects. Thus, it is important that more future experiments looking for genetic variation in host traits incorporate more realistic conditions, particularly if there is an interest determining whether there is potential for selection to act on these traits in natural environments. Combining mechanistic insight from glasshouse trials with practical implications from these field experiments will provide deeper insights into the potential for evolution in these systems.

3.4.2. Contributions

The initial experimental work (experimental design, planting, harvesting, and some morphotyping) was carried out by Andy Taylor and Glenn Iason at the James Hutton Institute. Terpene chemotyping was carried out by Ben Moore (JHI, now at the University of Western Sydney). I finished the morphotyping of all samples, conducted the molecular analyses, and carried out all of the analyses on the data set.

4

Negative interactions structure mycorrhizal root tip communities at small scales

4.1. Introduction

Ectomycorrhizal (EM) fungi are important symbionts of many woody tree species, exchanging soil-bound nutrients for photosynthetically derived sugars from their hosts. The fungi that form these associations are diverse, with upwards of 20,000 to 25,000 species from multiple phyla forming mycorrhizas (Suz et al. 2018; Rinaldi et al. 2008). As these fungal species are not all distributed evenly, there has been a lot of work investigating what factors determine the community composition of EM fungi at both large and small scales. At large scales, at the country or continental level, EM fungal communities vary in response to climate, such as variation in rainfall and temperature; pollution, particularly N deposition; host identity (Cox et al. 2010; Jarvis et al. 2013; Suz et al. 2014; van der Linde et al. 2018), as well as stochastic processes such as dispersal limitation (Talbot et al. 2014). At more regional scales, altitude and soil variables such as pH and organic matter content also drive variation in EM communities (Jarvis et al. 2015). However, factors affecting EM communities at small scales, from less than one metre to tens of metres, are much less well understood.

Spatial patterning of micro-organisms in soil at these small scales was historically regarded merely as stochastic variation, but it is now well-understood that distributions of organisms at these scales follow predictable patterns in scale-dependent ways (Ettema and Wardle 2002). Variation in the spatial distribution of abiotic factors or topography, as well as intrinsic population processes such as dispersal, reproduction and competition, can produce aggregated spatial distributions with distinct patches, as well as spatial autocorrelation, where sample points close together are more similar than would be expected by chance (Ettema and Wardle 2002). With spatially explicit sampling, at a given sampling interval (the distance between samples) and extent (the total area studied), these effects can be described through the application of geostatistical methods (Legendre and Legendre 2012). Using these techniques, the sizes of patches and the degree of autocorrelation can be generated, which can elucidate the processes generating these spatial distributions.

For EM fungal distributions at these scales, the community of fungi in a given sample is likely to be determined by the ecology of fungal genetic individuals (genets). The distribution of genets at such small scales can vary depending on the fungal tissue type studied (for example, EM root tips or sporocarp distributions) (Pickles and Anderson 2016). For fungal root tips, fungal genets appear to be patchily distributed in the soil, with patches varying in size from tens of cm to tens of metres, with variation in patch sizes both within and between fungal species (Sawyer et al. 1999; Anderson et al. 2001; Guidot et al. 2001; Beiler et al. 2010). This patchiness is likely to be driven by a number of factors. For example, whether they colonise primarily vegetatively or through spores, the life history of the fungal species in question is likely to be important in determining patch sizes (for example Taylor and Bruns 1999). Further, segregation in the soil environment due to niche partitioning on soil conditions can also alter the distribution of fungal species at small scales, and specialisation on differing soil resources can also lead to vertical segregation in the soil profile (Rosling et al. 2003; Genney et al. 2006; Bahram et al. 2015). However, studies investigating the effects of environment often leave much variation unexplained (Bahram et al. 2015), suggesting the role of other processes in determining community composition. Interspecific interactions, particularly competition between fungal species, have been explored as a potential factor explaining fine-scale spatial distributions (Pickles and Anderson 2016).

In particular, competition for host root tips has received much attention as a potential determinant of spatial structure (Hortal et al. 2008; Kennedy 2010). In interactions between plants and EM fungi, root tips represent the core organ through which nutrient exchange occurs, and as such represent opportunities for fungi to gain photosynthate from their hosts. Competition for root tips is expected to take two forms: firstly, exploitation competition, in which one fungal species pre-emptively exploits a resource, to the exclusion of other species but without a direct interaction (Kennedy et al. 2009). Secondly, fungal species are likely to participate in interference competition, where fungal species directly interact through antagonistic contact, for example through the excretion of antifungal compounds (Kennedy 2010). Although not every fungal species receives the same amount of host carbon per root tip (Bidartondo et al. 2001), and thus the value of root tips to the fungus will vary from species to species, the colonisation of patches of root tips and the associated soil space may still remain important in preventing displacement by competitor species.

Studies in the lab and the field have provided evidence of the importance and prevalence of competition between fungi. Experimental studies manipulating the order of addition of competitor species have demonstrated the ability of one species to competitively exclude others (Kennedy 2010), and the outcomes of competitive interactions can depend on environmental conditions (Mamoun and Olivier 1993). Direct competition between fungal species has also been demonstrated, where one species produces compounds that retard the growth of competitors (Krywolap 1964), or through taking over the root tips colonised by other species (Wu et al. 1999). However, while lab studies are able to directly demonstrate the presence and outcomes of competitive processes, they are typically restricted to easily-culturable mycorrhizal species that are not necessarily dominant members of wild EM communities. Furthermore, they are typically conducted in unrealistic environments with limited numbers of species, which may make inferences about patterns of competition difficult to transpose to natural communities.

While it is difficult to demonstrate the mechanisms of competition in field conditions, the outcome of competitive processes may be evident in the spatial distribution of fungal tissues such as root tips or mycelium in the field. Studies sampling root tips and mycelium have demonstrated competitive interactions between pairs of EM species within samples (Koide et al. 2005). In a pair of studies, sampling of the same grid in two years found some negative interactions, particularly between pairs of *Cortinarius* species, were the same over both years. However, others disappeared from one year to the next, suggesting that founder effects in the colonisation of root tips could lead to exclusion without interference competition (Pickles et al. 2010, 2012). Similarly, a study by Agerer et al. (2002), extracting root tips from soil cores while keeping the roots in their original positions, found that EM fungi excluded one another at the cm-scale. These studies all suggest that competitive interactions may result in spatial segregation in the soil.

However, all of these studies explored the spatial organisation of root tips from mature trees. During natural regeneration, tree seedlings can reach extremely high densities (e.g. Hallikainen et al. 2019), resulting in the roots of many individuals co-existing in a small space. Regenerating seedlings face strong density-dependent mortality (Petit and Hampe 2006), and are supported in their survival through benefits gained through connections to common mycelial networks maintained by mature trees (Teste et al. 2009). Although seedlings typically associate with the same fungal species as their mature neighbours, some studies have suggested they associate with these fungi at different frequencies (Obase et al. 2009; Aučina et al. 2011), which may represent different the nutritional requirements of seedlings compared to mature trees. Because such regenerating seedlings can occur in high densities in small areas, they present an opportunity to examine the potential for interactions between EM fungi in a potentially more homogeneous environment, with an opportunity to characterise the communities of entire root systems.

However, to my knowledge there are to date no studies exploring the spatial ecology of EM fungi colonising seedlings at these small scales. In order to test for competitive interactions between EM fungi at these scales, I used data from a reciprocal transplant experiment (Chapter 3). Scots pine (*Pinus sylvestris*) seedlings from populations in Scotland were planted in multiple small grids at each of the originating sites, with grid points where seedlings were planted being regularly spaced at 5 cm. Host genotype was previously established to have no effect on the EM community composition of individual three month old seedlings. I explored the spatial turnover of species at large and small scales, and analysed patterns of co-occurrence to explore whether there were positive and negative interactions between pairs of species. In particular, I sought to answer the following questions:

1. Is there turnover in community composition between sites? If so, is this correlated with environmental variables?
2. Is there turnover in community composition at the small (within-grid) scale?
3. Are there patterns of significant positive and negative cooccurrence between pairs of EM species?

4.2. Methods

This chapter uses data collected in Chapter 3, where full details of the experimental protocol, particularly for species identification, can be found. A brief summary is given below.

The experiment consisted of a reciprocal transplant experiment. Seeds from 6 populations of Scots pine, three from the east and three from the west, (Table 4.1) were collected from two trees per population and germinated in sterile soil. At each of the 6 sites, three 50 x 50 cm grids, with 5 cm intervals, were laid out, with grids at a site being between 100 and 2000 m apart. Three to five seedlings per maternal family per population were planted at random positions in each grid. Seedlings were grown for four months before being harvested. The EM fungal community on the root system of each seedling was characterised through morphotyping and subsequent molecular barcoding. In total, 546 seedlings were colonised by EM fungi at the end of the experiment, with an average of 30.3 seedlings (± 6.8 SD) per grid. I filtered out taxa which had less than 50 root tips across all sites in the study. This resulted in a total of 21 fungal taxa in the final dataset, split across a total of ~44,400 root tips.

Table 4.1.: Coordinates of each population used in the trial, as well as climatic variables (Met Office). ALT: altitude, MAP: mean annual precipitation, MFT: mean February temperature, MJT: mean July temperature, ETR: Extreme temperature range, GFD: mean ground frost days.

Population	Code	Latitude	Longitude	ALT (m)	MAP (mm)	MFT (°C)	MJT (°C)	ETR (°C)	GFD
Shieldaig	SD	57.50	-5.64	68	2449.7	3.42	13.1	30.3	115.9
Loch Clair	LC	57.56	-5.36	112	2888.2	3.66	14.0	33.9	128.3
Beinn Eighe	BE	57.63	-5.35	21	2476.1	3.87	14.5	35.4	127.4
Rothiemurchus	RM	57.15	-3.77	307	1060.0	1.70	13.5	37.4	153.1
Abernethy	AB	57.21	-3.61	362	1060.7	1.47	13.1	36.1	167.2
Glen Derry	GD	57.03	-3.58	443	1080.4	-0.17	11.7	36.0	167.2

All statistical analyses were carried out in R (version 3.6, R Core Team 2019).

4.2.1. Variation in community composition between sites

To explore the variables associated with the change in community composition between sites, I conducted an NMDS ordination on communities aggregated at the grid level using the *metaMDS* function from the R package *vegan* (Oksanen et al. 2019), with communities characterised as the proportion of the total number of root tips (including non-mycorrhizal tips) of each species. The number of axes (k) were selected by comparing the stress of ordinations with k = 1 to 10, and selecting k such that the stress was < 0.1 and not decreasing by large amounts. Following this method, k was chosen to be 4 (Fig. D.1). To examine whether specific environmental factors were associated with changes in community composition, I fitted environmental variables to the ordination using the *envfit* function. Environmental variables comprised grid-level measurements of altitude, as well as site-level measurements of the mean July and February temperatures, mean annual precipitation, the extreme temperature range at a site, and the number of ground frost days. The variables were extracted from MET office data collated by Whittet (2017), and were selected as they have previously been shown to be important determinants of mature tree EM fungal community composition in this system (Jarvis 2013).

4.2.2. Spatial turnover

To explore patterns in the change of community composition over space (β-diversity), I calculated Mantel correlograms using the *mantel.correlog* function in the R package *vegan* (Oksanen et al. 2019) for each experimental grid. Community similarity was calculated as the Bray-Curtis dissimilarity index using the *vegdist* function in *vegan*, with communities characterised as the proportional abundance of each species on each seedling, out of the total number of root tips including non-mycorrhizal tips. The distance between grid points was calculated as the euclidean distance between each seedling based on their X and Y positions within each grid.

Mantel correlograms partition the geographic distance matrix into discrete distance classes, and the *mantel.correlog* function allows for custom distance classes to be defined. Distances were partitioned with steps of 10 cm from 0 cm to 80 cm, the maximum possible distance between seedlings in any grid. For each distance class, a Mantel test was performed using 1,000 permutations, and p values were corrected for multiple comparisons using the Holm correction (Holm 1979). Significantly positive values of Mantel r indicate positive spatial autocorrelation, while significantly negative values indicate negative spatial autocorrelation.

To explore autocorrelation of individual fungal taxa within each grid, I also calclulated Moran's I correlograms using the proportional abundance of each fungal taxon as a response variable. These correlograms were calculated using the *correlog* function in the R package *ncf*, and used the same distance classes as in the Mantel correlograms (Bjornstad 2020).

4.2.3. Co-occurrence analysis

To explore patterns in co-occurrence between pairs of species, I used the *cooccur* package (Griffith et al. 2016, developmental version), on a presence-absence transformed community matrix. Common tests of co-occurrence use a data randomisation process, where the community matrix is permuted a given number of times in order to produce a null distribution for a test statistic estimating the degree of co-occurrence, and then testing the observed co-occurrence score against this null distribution to estimate significance. However, such models involving permutation are prone to Type I errors due to improper randomisation (Veech 2013). The method implemented in the *cooccur* package is by comparison entirely probabilistic, using a combinatorics approach to calculate the probabilities that the observed number of co-occurrences of a species ($\mathrm{Q_{obs}}$) is more or less than expected if the pair was randomly distributed. This method has been shown to have a much lower rate of Type I error, with maximum error rates of approximately 0.05 (Veech 2013).

The expected number of co-occurrences is calculated simply, as the product of the probability of each species occurrence multiplied by the total number of sites each species can occur at: $E(N_1, N_2) = P(1) \times P(2) \times N$, where the probability of occurrence P(i) is the number of samples species i occurs at divided by the total number of samples N. To calculate the pair of probabilities that the observed number of co-occurrence is smaller or larger than this expected value, a series of probabilities, $\mathrm{p_j}$, are calculated for each species pair, where $\mathrm{p_j}$ is the probability of co-occurrence of two species at exactly j sites (Eq. 4.1), where N is the total number of sites where both species could occur, and $\mathrm{N_1}$ and $\mathrm{N_2}$ are the number of sites where species 1 and 2 are found, respectively (Griffith et al. 2016). $\mathrm{p_j}$ is calculated for j to $\mathrm{N_1}$ samples; the probability that two species occur at a frequency less than expected if their distributions were random ($\mathrm{p_{lt}}$) is $p_{lt} = \sum_{j=0}^{Q_{obs}} p_j$, and the probability that two species occur at a frequency greater than expected if their distributions were random ($\mathrm{p_{gt}}$) is $p_{gt} = \sum_{j=Q_{obs}}^{N_1} p_j$ (Veech 2013).

$$p_j = \frac{\binom{N_1}{j} \times \binom{N-N_1}{N_2-j}}{\binom{N}{N_2}} \tag{4.1}$$

However, by default the calculations assume that all species can occur in all samples (N = total number of samples, in this study 546), and would lead to erroneous conclusions if one is interested in patterns of co-occurrence within grids rather than between sites. To account for this, I used the *site_mask* function in the *cooccur* package, which takes a separate matrix that defines which samples each species could occur at. This has the function of changing the value of N for each species pair. I allowed each fungal taxon to be present at each seedling within a grid if that taxon had occurred on at least one seedling within a grid. Because each grid was exhaustively sampled, with every root tip assigned to an identified taxon, I was confident that absences within grids were due to the non-occurrence of a given taxon rather than poor sampling. The value of N for each species pair is provided in Fig. 4.1.

	Tylospora sp	Tomentellopsis sp	Tomentella fuscocinerea	Suillus variegatus	Suillus flavidus	Suillus bovinus	Russula sardonia	Russula paludosa	Russula decolorans	Rhizopogon verii	Piloderma sphaerosporum	Piloderma olivaceum	Meliniomyces bicolor	Lactarius musteus	Elaphomyces muricatus	Craterellus lutescens	Cortinarius tenuifulvescens	Cortinarius stillatitius	Cortinarius obtusus	Cenococcum geophilum
Tomentellopsis sp	258																			
Tomentella fuscocinerea	0	70																		
Suillus variegatus	286	213	0																	
Suillus flavidus	36	76	40	0																
Suillus bovinus	31	31	0	0	0															
Russula sardonia	36	36	0	0	36	0														
Russula paludosa	83	51	0	83	0	0	0													
Russula decolorans	27	49	0	49	0	0	0	0												
Rhizopogon verii	172	70	0	137	0	0	0	0	0											
Piloderma sphaerosporum	66	31	0	0	0	31	0	0	0	35										
Piloderma olivaceum	26	26	0	26	0	0	0	0	0	0	0									
Meliniomyces bicolor	157	128	0	126	0	31	0	37	0	34	31	26								
Lactarius musteus	33	0	0	0	0	0	0	0	0	33	0	0	0							
Elaphomyces muricatus	32	30	30	32	0	0	0	32	0	0	0	0	0	0						
Craterellus lutescens	105	106	70	103	40	0	0	0	0	138	35	0	0	0	30					
Cortinarius tenuifulvescens	31	31	0	31	0	0	0	14	0	0	0	0	0	0	0	0				
Cortinarius stillatitius	71	36	0	0	36	0	36	0	0	35	35	0	0	0	0	35	0			
Cortinarius obtusus	0	30	30	0	0	0	0	0	0	0	0	0	0	0	30	30	0	0		
Cenococcum geophilum	339	205	0	204	36	31	36	83	0	172	66	0	102	33	32	105	31	71	0	
Cantharellales sp	159	62	0	55	36	0	36	0	0	68	35	26	55	33	0	35	0	71	0	104

Figure 4.1.: Matrix showing the number of seedlings each fungal pair could potentially co-occur at (N in Eq. 4.1). Zero = species pair does not co-occur within any grid.

4.3. Results

On average, there were 8.8 fungal taxa per site, but each individual seedling only associated with 2 ± 0.88 (SD) taxa. The average fungal diversity in effective species count (exponential of the Shannon index, see Jost (2006)) was lower, at 1.66 effective species (SD = 0.64), suggesting that fungal species were not equally abundant on each seedling. Following the analysis of stress for different dimensions, the number of dimensions selected for the NMDS ordination was three, with a stress of 0.1 Fig. C.2. The ordination showed that community composition differed within and between sites, with weak differentiation between eastern and western sites on axis 2 (Fig. 4.2 A). This east-west difference was found to be associated with differences between the east and west in rainfall and temperature, with increasing rainfall and winter temperature increasing towards western sites (Fig. 4.2, Table 4.2).

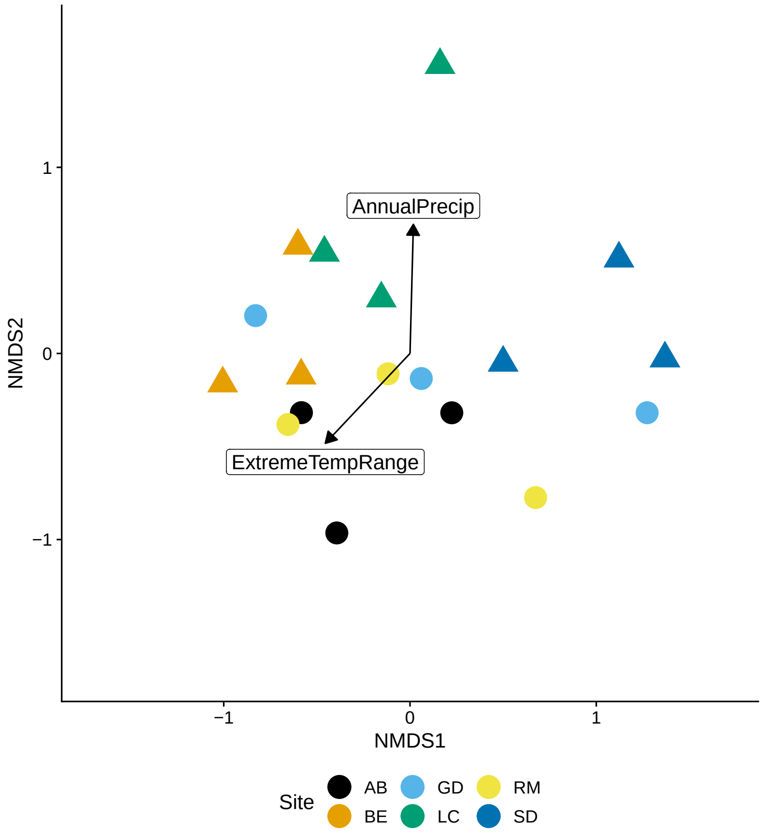

Figure 4.2.: Results of NMDS ordination of fungal communities at the grid level, with significant environmental vectors from the envfit analysis plotted. Each point represents a grid, with colours indicating site. Western sites = triangles, eastern sites = circles. Biplot arrows indicate increasing values of a given environmental variable.

Table 4.2.: Environmental variables fitted to the NMDS ordination in Fig. 4.2. Values in bold indicate p values below 0.05.

Environmental Variable	R^2	p
Mean July Temperature	0.18	0.20
Mean February Temperature	0.22	0.16
Mean Annual Precipitation	0.54	**0.001**
Extreme Temperature Range	0.43	**0.017**
Ground Frost Days	0.46	0.06
Altitude	0.14	0.30

4.3.1. Spatial turnover

At small scales, there was some evidence for spatial autocorrelation within grids (Fig. 4.3), with approximately 40% of grids showing evidence of spatial autocorrelation within the first two distance classes (either 0 - 10 cm or 10 - 20 cm). The rest of the grids showed either no significant autocorrelation at any distance class, or autocorrelation either in the middle or at the end of the correlogram.

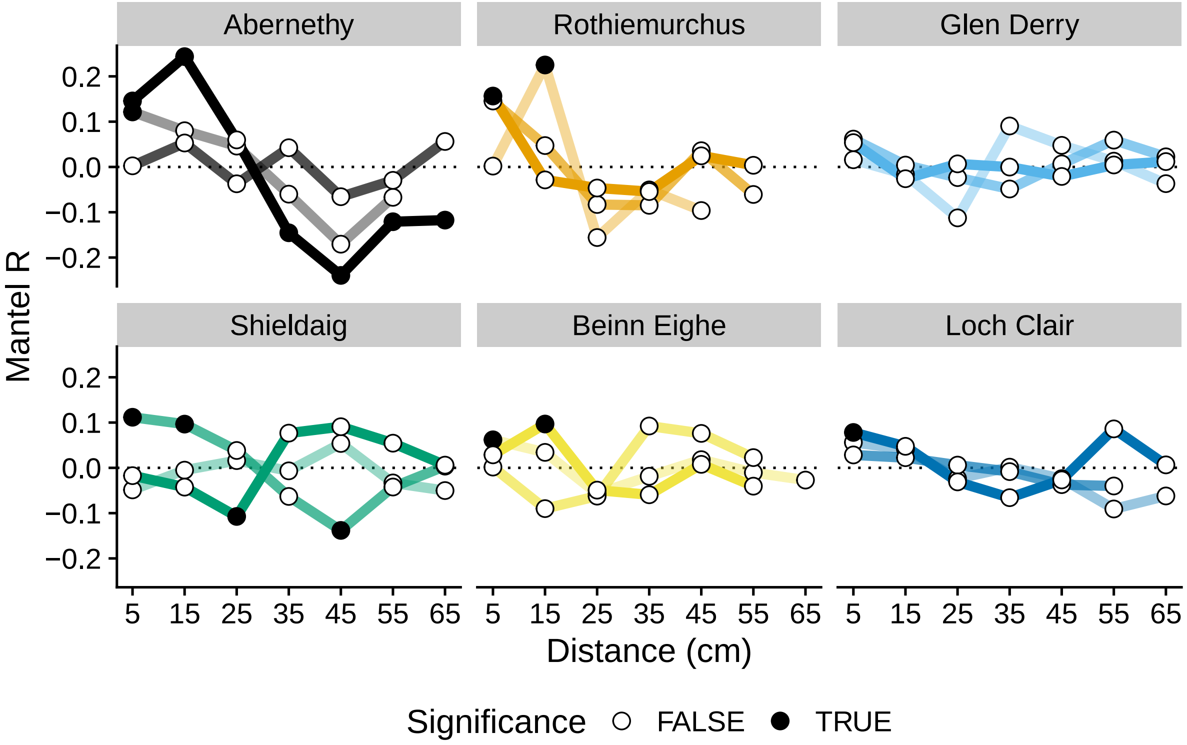

Figure 4.3.: Mantel correlograms of spatial autocorrelation in seedling EM fungal community composition, calculated for each grid. Colours indicate different sites, and shading indicates different grids within sites. Error bars on coloured lines indicate 95% confidence intervals. Top row = eastern sites; Bottom row = western sites. Filled circles indicate significant estimates, hollow circles not significant.

4.3.2. Co-occurrence analysis

In total, I was able to estimate co-occurrence probabilities for 70 of 210 total possible species pairs. Of these 70 species pairs, I found evidence for random associations for 56 species pairs, negative co-occurrence for 10 species pairs, and positive co-occurrence in 4 species pairs (Fig. 4.4). Of the species pairs for which tests were conducted, no pairs were defined as unclassifiable due

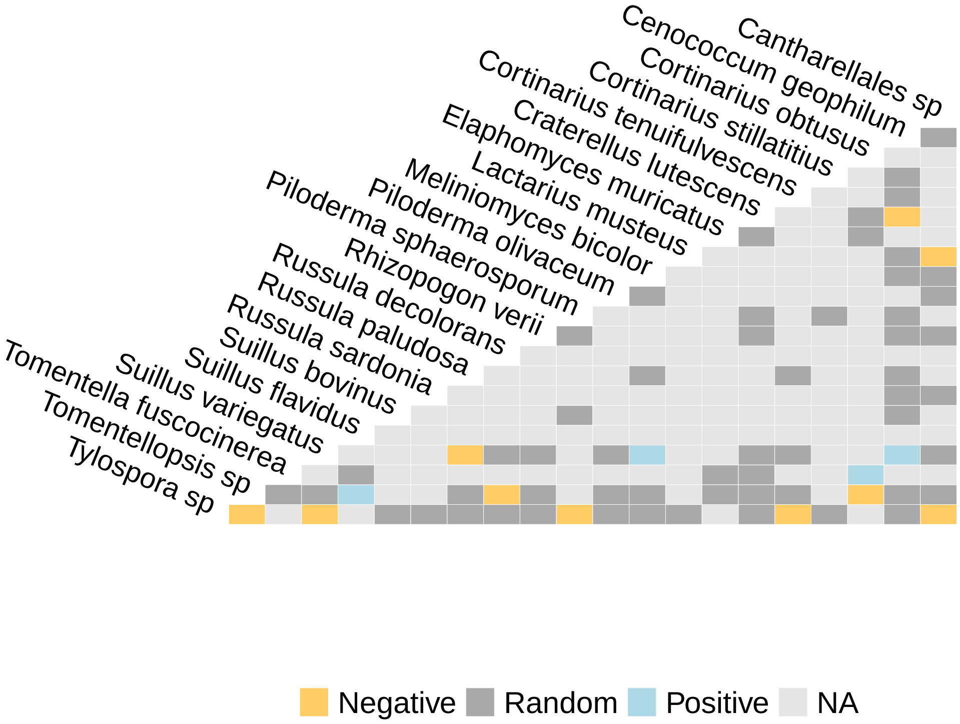

Figure 4.4.: Results of the co-occurrence analysis. Yellow = species pair occurs less frequently than would be expected by chance. Blue = species pair occurs more frequently than would be expected by chance. Dark grey = species pair associations are random. Light grey = species do not co-occur within any grids (no effect calculated).

to low statistical power. Of the species involved in negative interactions, 7 of the 10 species pairs involved either *Tylospora sp* (with *Tomentellopsis sp*, *Suillus variegatus*, *Piloderma sphaerosporum*, *Cortinarius tenuifulvescens*, and an unidentified Cantharellales species) or *Tomentellopsis sp* (with *Tylospora sp*, *Russula decolorans*, and *Cortinarius obtusus*), which were the two most abundant species across all of the sites by a high margin. Additionally, there were negative interactions between *S. variegatus* and *R. paludosa*, *Craterellus lutescens* and *Cenococcum geophilum*, and *Lactarius musteus* and an unidentified Cantharellales species. Of the species involved in the 4 positive interactions, three out of the four species pairs involved a member of the genus *Suillus*, with *S. variegatus* having two positive interactions (with *Meliniomyces bicolor* and *Cenococcum geophilum*), and *S. flavidus* having one positive interaction, with *Tomentellopsis sp*. There was an additional positive interaction between *Tomentella fuscocinerea* and *Cortinarius obtusus*. Three species had no negative interactions (*Suillus flavidus*, *Tomentella fuscocinerea*, *Meliniomyces bicolor*) (Fig. 4.5), but it is worth noting that these species were typically rare where they occurred.

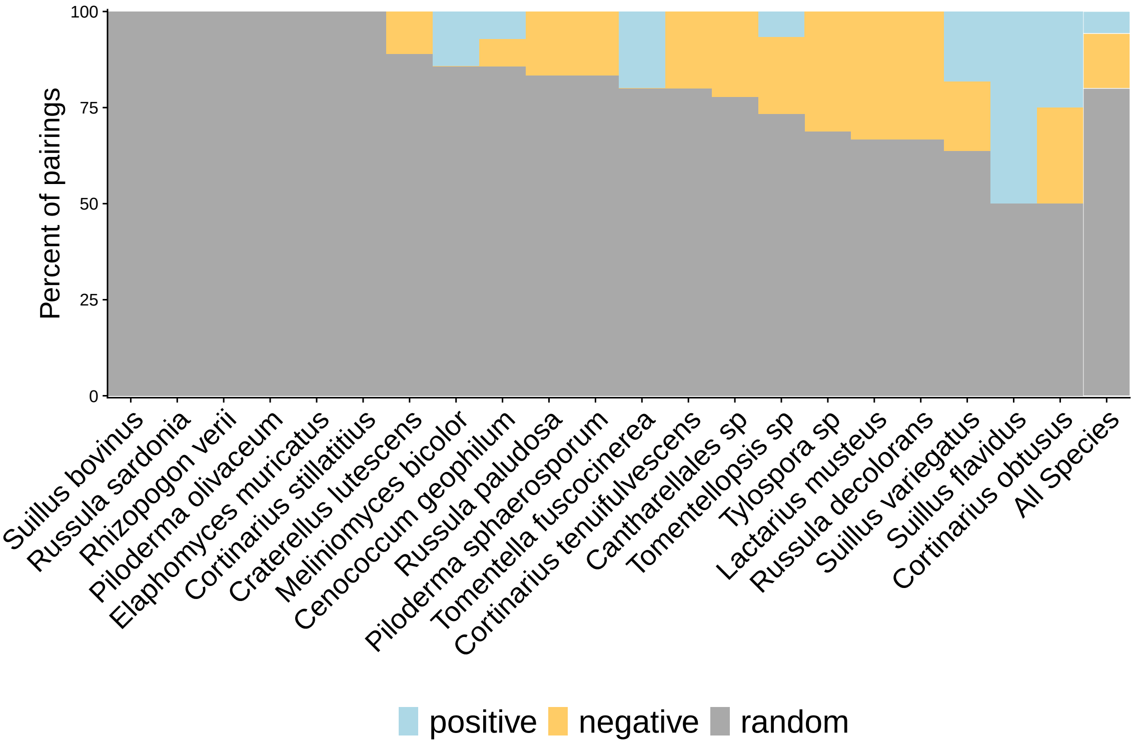

Figure 4.5.: The proportion (%) of positive, random and negative interactions that each EM fungal taxon is involved in. Yellow = negative co-occurrence, blue = positive co-occurrence, grey = co-occurrence no different than chance.

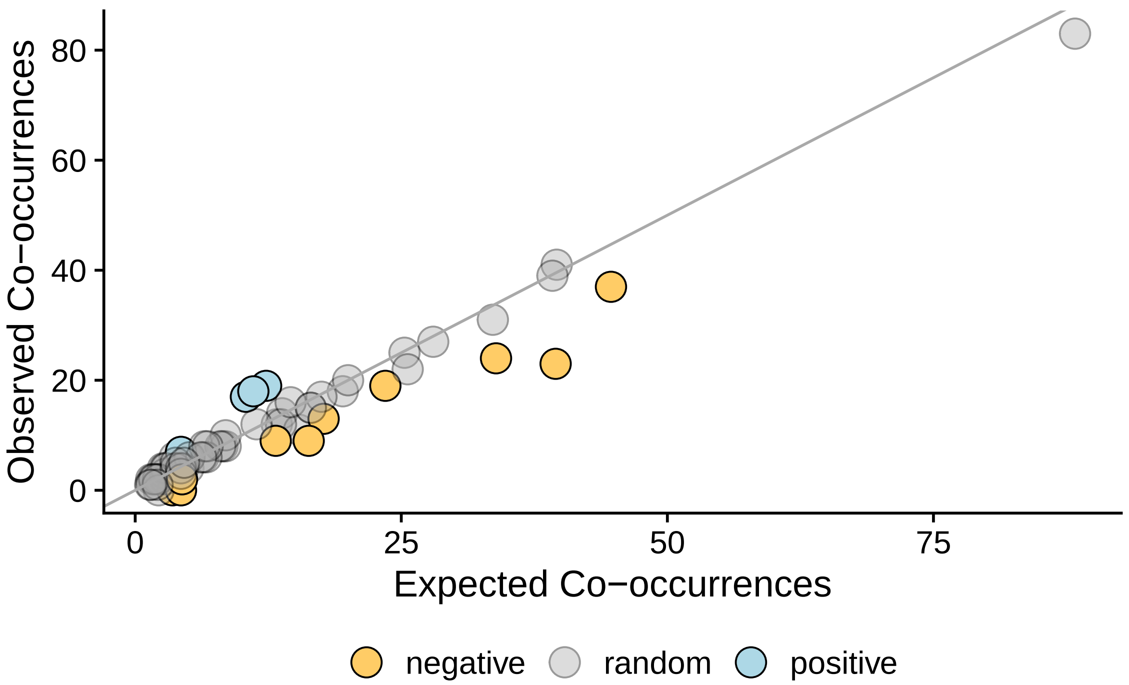

Figure 4.6.: Number of observed co-occurrences vs. the number of expected co-occurrences. Yellow = negative co-occurrence, blue = positive co-occurrence, grey = co-occurrence no different than chance.

4.4. Discussion

4.4.1. Spatial ecology

I found evidence for variation in EM fungal communities at both large and small scales. Between sites, I found that turnover in EM fungal communities was associated with environmental variables relating to rainfall and temperature (Fig. 4.2 A). These results are unsurprising; a previous study in this system found that rainfall was an important correlate with EM community composition (Jarvis et al. 2013), and more broadly, EM fungal communities have been shown to vary in response to climatic variables (Suz et al. 2014; van der Linde et al. 2018). However, I also found a lot of variation between grids within sites and between sites, particularly on axis 1, that was not explained by these variables, and found no environmental variables that correlated with these differences. EM fungal communities have also been shown to vary in response to nitrogen pollution and soil factors such as pH and organic matter (Cox et al. 2010; Jarvis et al. 2013;

Suz et al. 2014; van der Linde et al. 2018). Accordingly, I expect that if there were grid-level measurements of soil variables, these would be highly important in explaining variation in EM community composition.

I found evidence for spatial autocorrelation at the initial distance classes in less than half of the grids Fig. 4.3), suggesting that most grids were relatively homogeneous in their EM fungal communities. There are a couple of possible reasons that some but not all grids show this pattern. Root tips colonised by EM fungi have patchy distributions within the soil, with patch sizes ranging from a few tens of cm to many tens of metres (Sawyer et al. 1999; Anderson et al. 2001; Guidot et al. 2001; Pickles et al. 2010; Beiler et al. 2010). In some cases, autocorrelation here may be driven by patches of individual fungal species between 10 and 20 cm. Alternatively, by chance, some of the grids in this experiment may lie at the boundary of a larger patch of an individual fungus. An analysis of individual species correlograms within each grid (Appendix E) suggested that species in each of these grids showing autocorrelation at these distances were not the same in each grid. Patch sizes appear to be highly variable within as well as between species (Anderson et al. 1998, 2001; Riviere et al. 2006), and thus finding that different fungi contribute to this effect at different sites may not be surprising. If a grid was placed across the boundary of an individual genet, then the correlogram ought to show a steady decrease if the boundary of the patch exists as a gradient. Grid AB3 showed a pattern like this (Fig. 4.3, Abernethy dark line), which appeared to be driven by *Russula paludosa* (Moran's I correlogram, Appendix E). Thus, both of these processes may explain why only some grids showed spatial autocorrelation.

4.4.2. Competition and facilitation

Overall, I found evidence of a number of negative interactions between pairs of ectomycorrhizal species. In total, 10 of 70 (14%) of potentially interacting species pairs showed a negative pattern of co-occurrence, where species occurred less frequently than expected if they were distributed independently. Of these 10 negative interactions, 7 involved the two most common species across all sites, *Tylospora sp* and *Tomentellopsis sp*, including a negative interaction between these two species. Of the other negative interactions, the subsequent two most abundant species (*Russula paludosa* and *Cenococcum geophilum*) were involved in a further two negative interactions.

Such negative interactions, particularly between common species, may be a result of exploitation competition between fungal species. In EM interactions, host root tips are often considered as a resource for competition (Wu et al. 1999; Hortal et al. 2008; Kennedy et al. 2009), as they are required for nutrient exchange with the host plant. Assuming that the colonisation of many root tips requires an investment in fungal resources, and heavy colonisation of a single individual is more beneficial than diffuse colonisation of many individuals, there may be an opportunity cost in the ability of a fungus to colonise other nearby seedlings. This effect may be amplified if some of the resources used for colonisation are derived from the host being colonised and are therefore localised. This process may also explain why seedlings were unevenly colonised by fungal taxa; despite there being on average 5.2 species per grid, seedlings were colonised on average by two fungal taxa, which were not equally abundant.

It has been suggested that this kind of exploitation competition is important in interactions between EM fungi. These interactions are often subject to priority effects, where a seedling initially colonised by one species is unable, or only partly able, to be colonised by a subsequently colonising species (Kennedy et al. 2009). This effect is commonly reported when planting out inoculated nursery seedlings in the field, with inoculate fungi persisting in field conditions for long periods of time, sometimes tens of years (Fleming 1985; Villeneuve et al. 1991; Selosse et al. 1998), and has also been demonstrated in experimental settings (Kennedy et al. 2009). These competitive dynamics may be influenced by the exploration types (Agerer 2001) of the fungi in question. These exploration types are morphological syndromes which may represent different foraging strategies of the fungi in question, and are categorised by features such as the amount of extra-matricial mycelium produced and the presence or absence of rhizomorphs. Species that produce produce large amounts of extra-matricial mycelium, or produce long-distance rhizomorphs, may be better at finding and exploiting new root tips compared to those that produce few hyphae (Kennedy et al. 2007; Pickles et al. 2012). Although there was no explicit manipulation of exploration types in this experiment, the fungal species found in this experiment exhibited a range of morphologies which may explain some of the patterns of competitive dynamics seen in this experiment.

I also found evidence for positive interactions between some pairs of EM species. Such positive interactions may occur due to facilitative interactions between fungi, either directly, or indirectly through promotion of growth of the host plant (Kennedy et al. 2014). This may particularly be the case if different fungal species have complementary resource strategies (Jones et al. 2010). Alternatively, host strategies for maintaining co-operation, such as preferential allocation (Bever 2015) may mean that plants actively suppress colonisation by certain species, particularly if they are costly to maintain, which may create room for colonisation by other species. Alternatively, similarities in environmental preference at the micro-site level may mean that positive co-occurrences are found (Li et al. 2020), without requiring these species to interact. However, this seems unlikely in the present study given the homogeneity of the communities involved.

Curiously, three of the four positive interactions in this study involved members of the genus *Suillus*. Members of *Suillus* are specialists on members of the Pinaceae, sometimes to the sub-generic or individual species level (Liao et al. 2016), and *Suillus* ectomycorrhizas often develop into tubercles, dense clusters of root tips surrounded by a hyphal sheath (Paul et al. 2007). It has been shown that *Pinus contorta*/*S. tomentosus* tubercles host N-fixing bacteria, fixing N at a rate high above that of standard EM root tips (Paul et al. 2007). If *Suillus* ectomycorrhizas more generally facilitate nitrogen fixation, then the presence of *Suillus* tubercles on the seedlings in this experiment may have facilitated a larger benefit to their hosts. Assuming some of this benefit was then allocated for further growth, then new root tips may have become available for colonisation by other fungal species, facilitating co-occurrence. In one study experimentally manipulating the number and identity of species in the EM communities of seedlings, only the presence of *Suillus* was found to facilitate growth above the simple presence/absence of EM fungi (Kipfer et al. 2012).

Alternatively, *Suillus* may represent a poor competitor for root tips: on average, the most common species *S. variegatus* colonised only 15% of a seedling's root system, with each tubercle counted as a single root tip. Instead of colonising large sections of a root system, tubercle formation may allow *Suillus* species to maximise its ability to trade with its host plant from only a single point of colonisation. This would also result in positive estimates of co-occurrence, as *Suillus* would commonly be present despite exploitation competition from other EM species. This might also explain the specificity shown by *Suillus* to sub-genera - if the ability to form tubercles, and hence increase the number of root tips colonised from a single point, depends on the ability to manipulate host growth, then specificity may be a result of evolution to cause root tip proliferation on different host species. Further work investigating the competitive abilities of *Suillus* against generalist species would be helpful in exploring this hypothesis.

It is worth noting that these co-occurrence estimates were made using presence-absence data, which can be limited in terms of their ability to demonstrate ecological interactions (Blanchet et al. 2020). Negative interactions can only occur in such data when one species completely excludes another (Sander et al. 2017), and cases where negative interactions have occurred, but with one species only partially excluding another, are treated the same as truly neutral interactions. Furthermore, if competitive interactions are too strong, complete competitive exclusion of one species across most or all samples will preclude any ability to detect an interaction (Blanchet et al. 2020). Because of this, it is hard to quantify the true number of ecological interactions between mycorrhizal fungi in this system, with potential for underestimation. However, because of the high levels of replication within small ecologically homogeneous areas, combined with exhaustive characterisation of each sample, it seems reasonable that the interactions found here document at least some of the interactions between species of EM fungi.

Other studies of EM interactions in the field have also revealed evidence of both positive and negative interactions. Excavating and preserving roots in their in-situ positions, Agerer et al. (2002) found that root tips colonised by different fungal species segregated at the cm scale. In another study conducted within a 20 x 20 m grid, with sampling at both 1 m intervals and random transects at 5 cm intervals, ~30% of interactions between EM species pairs were significant, with strong positive and negative interactions between pairs of *Cortinarius* species (Pickles et al. 2010). A subsequent study at the same site found that EM communities within each sampling point were strongly structured by negative interactions, with the eight most abundant species involved in at least one competitive interaction (Pickles et al. 2012). Similarly, Koide et al. (2005) found that EM communities in both soil samples and root tips showed positive and negative associations, being particularly driven by a negative interaction between *Cenococcum geophilum* and a *Clavulina* species. It is worth noting, however, that field studies cannot prove the occurrence of competition or facilitation, as the processes determining the distributions of root tips or hyphae are typically not observed (Kennedy 2010). Thus, other processes, such as small-scale heterogeneity in soil conditions or resources leading to segregation of fungal species, cannot be discounted (Pickles et al. 2012).

One potential advantage of this study over previous studies is the whole sampling of seedling root systems at a small spatial extent. Although there was some evidence of patchiness in fungal distributions in a few grids, on the whole there was little autocorrelation within these small areas, suggesting that EM community composition in grids was relatively homogeneous. Within a more homogeneous area, individual seedlings thus function as individual replicates to test for EM interactions, and the effects of small-scale heterogeneity may be somewhat elided. However, the fungal communities on the roots of seedlings may differ from those of nearby mature trees, at least in frequency of association with different species (Twieg et al. 2007; Aučina et al. 2011). Because each seedling represents an individual potential source of C for ectomycorrhizal fungi, the dynamics of competition may differ compared to mature roots, with variation in photosynthetic capacity potentially changing the value of competitive interactions between fungi for specific seedlings. This is particularly true as young seedlings may initially represent a sink for fungal resources due to this poor photosynthetic capacity (Teste et al. 2009). Thus, the insights gained from seedling experiments may not be directly applied to interactions between fungi on mature trees.

4.4.3. Conclusions

Overall, I found that at large (between-site) scales, EM community composition was correlated with variation in annual precipitation and temperature range. At small scales, I found that EM community composition was homogeneous in most grids, but some grids showed evidence of patchy fungal distributions at scales of up to 20 cm. Within grids, I also found evidence for negative patterns of co-occurrence between pairs of EM fungi, particularly the two most common fungal species, which accounted for over half of the negative pairwise interactions found. These negative interactions could potentially be the result of competitive interactions between fungal species, leading to competitive exclusion. The presence of positive pairwise interactions, particularly those involving the genus *Suillus*, also suggests the possibility of facilitation amongst EM species, or differential competitive strategies that facilitate coexistence. These results underscore the importance of considering the ecology of fungal species when conducting analysis of fungal communities, as even spatially close samples can have divergent community composition due to interspecific interactions.

4.4.4. Contributions

The initial experimental work (experimental design, planting, harvesting, and some morphotyping) was carried out by Andy Taylor and Glenn Iason at the James Hutton Institute. I finished the morphotyping of all samples, conducted the molecular analyses, and carried out all of the analyses on the data set.

5

Discussion: Life history, evolution, ecology, and experimental considerations

5.1. Summary of key findings

In this thesis, I explored the extent to which genetic differentiation between populations or genotypes of Caledonian Scots pine affects interactions with mycorrhizal fungi. In chapter two, I showed that there is heritable variation in growth and root:shoot allocation of Scots pine from the Caledonian pinewoods of Scotland in response to inoculation with mycorrhizal fungi. Although I found evidence that these populations were differentiated in their responses to mycorrhizal fungi, there was no evidence that populations were adapted to local fungal communities. Taken together, these results suggest that although there is the potential for Scots pine to evolve in response to EM fungi, evolution in this system has not taken the form of local adaptation. Instead, the long lifespan of Scots pine trees may make other selective pressures, such as competition between conspecifics, more important. Long lifespans may also mean generalist interactions with mycorrhizal fungi more beneficial than specific ones if mycorrhizal fungi are more responsive to a changing environment.

In chapter three, I explored the relative importance of location versus host genotype in determining which EM fungi colonise seedlings from twelve maternal families. I found that the physical location of a seedling, both between sites, and within sites at the sampling grid level, explained the majority of the variation (~70%) in the composition of the fungal community on seedlings, but genotype, in terms of either maternal family or terpene phenotype, explained no variation at all. This again suggests that the long lifespan of Scots pine trees makes other selective pressures more important, and further suggests that any genetic variation between populations that does exist does not take the form of variation in preference for different fungal species.

In chapter four, I showed that variation in EM community composition between seedlings is driven by the ecology of the mycorrhizal fungi. EM community composition between sites was found to correlate with climatic variables, in particular rainfall and temperature. At small scales, I found that EM community composition was generally homogeneous within grids, but some grids did show evidence of patchy fungal distributions suggestive of small fungal genets. Furthermore, I found evidence of negative interactions between pairs of fungal species, suggesting that EM fungi compete for space on host roots. There was also evidence of potential facilitation between a small number of fungal species pairs. These results suggest that competitive interactions are determinants of mycorrhizal community composition at small scales. Overall, these results show the overriding importance of ecological processes within EM communities in determining community composition at any given location, which must be integrated into any hypothesis about coevolution between host plants and their symbionts.

5.2. The influence of life history on local adaptation

To date, much of the research into the drivers of coevolution between EM fungi and their hosts has focused on traits central to the regulation of the mutualism itself (Hoeksema 2010), such as signalling and recognition systems for the (ongoing) negotiation between symbiotic partners (Hoeksema et al. 2012; Piculell et al. 2019), the regulation of transportation of nutrients (Piculell et al. 2019), preferential allocation of nutrients to more beneficial partners (Bever et al. 2009), and host defence traits providing protection from fungal pathogens (Gehring et al. 2014; Piculell et al. 2018). However, there has been overall little focus on how life history traits, such as perenniality, long life spans, or dispersal mechanisms might act to determine the importance of individual traits such as symbiont preference, mycorrhizal dependence, or mycorrhizal investment, resulting in differences in the expected outcomes of coevolution.

Life history, as noted in chapters 2 and 3, is likely to be an important factor in determining the evolutionary response to mycorrhizal interactions. Particularly, it is likely that generation time is important as it can modify the encounter rates with different fungi. Shorter generation times might reduce the total number of strains or species encountered over the lifespan of a host plant (Hausmann and Hawkes 2009; Alguacil et al. 2012), and thus individual interactions with specific mycorrhizal species or strains might be important for the fitness of short-lived species. By comparison, long-lived species, such as trees or shrubs, may associate with a large number of fungal species over their lifespans, and likely also host a larger number of fungal species across their more expansive root systems (Hausmann and Hawkes 2009). Thus, the consequences for fitness of associating with any individual fungus that is a poor mutualist may be muted in comparison, particularly if other selective pressures, such as density-dependent mortality, grazing, or environmental stress pose stronger barriers to survival (Fig. 5.1).

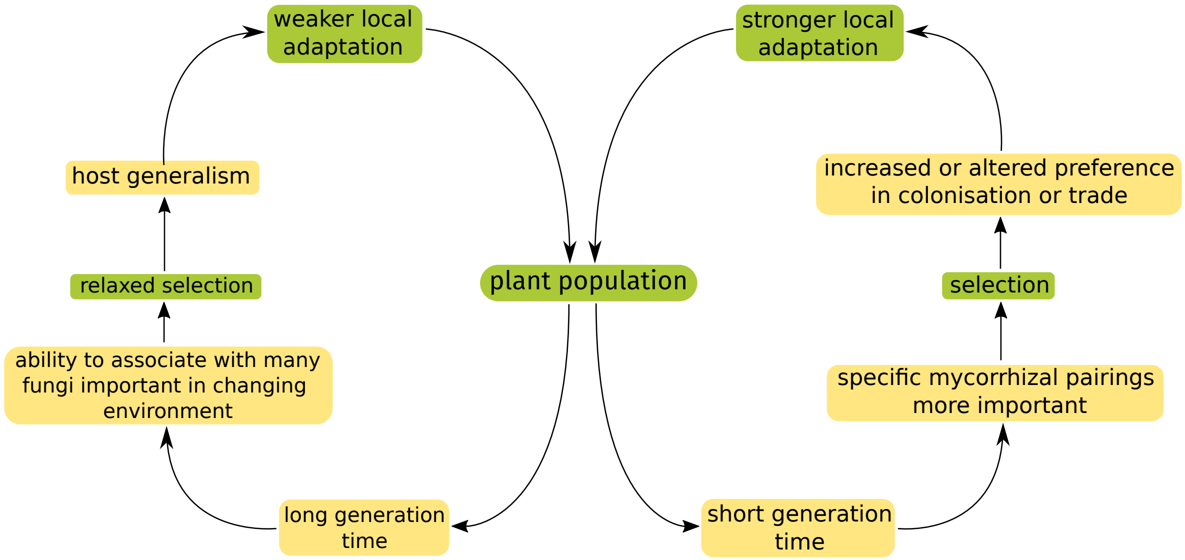

Figure 5.1.: The potential effects of long or short generation times on the specificity of mycorrhizal associations.

By comparison with EM fungi, AM associations are generally considered to be generalist (Smith and Read 2008), but despite this, the amount of benefit accrued by host plants with different fungal species is often highly species specific. This kind of specialism may be important in reducing competition with other species (Bever et al. 2010): if different AM fungal species provide different kinds of resources, and host plants have different nutritional requirements, then resource partitioning through preferential associations with different microbes may aid in the avoidance of competition with competitor species (Tedersoo et al. 2020). Furthermore, symbiont preference may also reduce the effects of negative plant-soil feedback (van der Putten et al. 2013) through the reduction in provision of C to mycorrhizal species more preferentially associated with competitor species. Given that the strength of interspecific competition can vary between communities (Callaway 2007; Pierce et al. 2016), selection for increased selectivity, either through symbiont preference or specificity in traded benefit, may be more common in communities where interspecific competition is strong.

Variation in these modes of association with mycorrhizal fungi among plants with different life history strategies may then affect patterns of host-mycorrhizal co-evolution. Local adaptation, where host plants perform better with fungi from local fungal communities compared to other fungal communities, is just one such pattern that may arise in the case of selection upon certain mycorrhizal traits in particular circumstances (Hoeksema 2010). Perennials have longer generation times and lower rates of evolution than annuals (Bousquet et al. 1992; Yue et al. 2010). If, as discussed above, forming the "correct" mycorrhizal associations is more important for short-lived species, we may expect that annuals or short-lived perennials are more likely to evolve symbiont preference to particular fungal species than long-lived species (Hausmann and Hawkes 2009) (Fig. 5.1). Furthermore, local adaptation may be more common in situations where symbiont preference is more likely to be important, such as in competition-dominated grasslands, which are simultaneously likely to be dominated by short-lived species.

To date, much of the available data on plant response to mycorrhizal inoculation is confined to arbuscular mycorrhizal interactions, and within this, there is a large focus on short-lived plants. In one meta-analysis database (Chaudhary et al. 2016), 48% of entries involved perennial or annual grasses and forbs interacting with AM fungi. Of the remaining 52% of data covering woody plants, only 17% of data covered AM hosts, while the remaining 35% covered EM hosts. A study utilising this dataset found strong evidence of local adaptation in AM plants, and though they found no effect of functional group (Rúa et al. 2016a), the lack of data means there may not be enough power to detect any differences between long-lived woody hosts and short-lived herbs in terms of their potential to show local adaptation.

It is worth noting that symbiont preference might also be related more generally to niche breadth (Batstone et al. 2018). The ability to associate with multiple symbiont species may be more beneficial to hosts due to complementarity between symbionts, providing more benefit to a host than any individual species due to functional differentiation. Furthermore, the ability to associate with a broad range of symbionts may facilitate survival in a wider variety of environments, if different symbiont species are adapted to function best in different conditions (Batstone et al. 2018). In support of this, Karst et al. (2018) found that tree species with small geographic range sizes tended to have stronger responses to mycorrhizal inoculation, suggesting a trade-off between widespread dispersal ability and dependence on the mycorrhizal mutualism, which may be mediated by a dependence of trees with short ranges on specific fungal species. The interaction between host niche breath more broadly, investment of resources in the mycorrhizal mutualism, and specialisation on particular fungal species may thus also be important in determining evolutionary trajectories.

This may in part explain some of the discrepancies found between the work in this thesis and previous work on local adaptation in pines to EM fungi. Most previous work to date has focused on pines with relatively small geographic ranges, such as *Pinus radiata*, *P. muricata*, *P. contorta* var. *contorta*, and *P. edulis* (Hoeksema and Thompson 2007; Hoeksema et al. 2009, 2012; Stone et al. 2018), and work in these species has shown a variety of effects of host and fungal genotype indicative of evolution. By comparison, *P. sylvestris* is found across much of the northern hemisphere, from Scotland to the Sea of Okhotsk in eastern Eurasia, and extending as far south as Spain and Turkey (Critchfield and Little 1966). This broad range, combined with long-range gene flow (Wachowiak et al. 2011), may also reduce selection for selectivity by hosts through homogenising gene flow, where small populations of trees with restricted ranges may become more selective through stronger local selection or drift if gene flow between populations is less common.

One consideration to be made when drawing these conclusions is that the studies conducted in this thesis involved hosts only at the seedling stage. Due to their life stage, seedlings may be under different ecological pressures compared to mature hosts. For example, due to the high levels of intraspecific competition at this stage, selectivity may be disadvantageous if it reduces overall colonisation, leading to a reduction in growth in a vital growth stage. Seedlings have previously been shown to host different communities of EM fungi compared to mature trees (Twieg et al. 2007; Aučina et al. 2011), which may be the result of this effect. Comparatively, mature trees may be able to afford to be selective in order to balance their nutritional requirements, and are not under such strong competition that this may put them at a disadvantage.

Overall, the current framework for understanding (co)-evolution in mutualisms, focusing on traits important to the outcome of mutualism itself (e.g. Hoeksema 2010), should be synthesised with broader frameworks, incorporating concepts around life history as well as plant-soil feedback (PSF) more broadly (terHorst and Zee 2016). Furthermore, it will be important to separate the various kinds of benefits provided by mycorrhizal fungi, as these are likely to have different responses to selection. For example, mycorrhizal fungi are now understood to provide protection against soil-borne pathogens, particularly in the case of ectomycorrhizal fungi (Teste et al. 2017). If these varying kinds of benefit can interact (for example, protection against pathogens in EM hosts reducing negative PSF, leading to increased monodominance (Tedersoo et al. 2020)), then this too will ultimately impact the trajectory of plant-fungal coevolution.

5.3. Mycorrhizal ecology and experimental considerations

The key finding from chapters 3 and 4 was the overriding importance of the ecology of mycorrhizal fungi, and not host genotype, in determining the composition of mycorrhizal communities on individual pine seedlings. At broad scales, mycorrhizal community composition is driven by changes in environment, particularly soil and climate (Suz et al. 2014), as well as neutral process such as dispersal limitation (van der Linde et al. 2018). Such broad-scale variation determines the potential pool of species a seedling can interact with. At small scales, patchy distributions of fungal individuals, and both positive and negative interactions between fungal individuals (Pickles et al. 2010, 2012), are likely to be important in determining the community composition at any sampling point.

While a number of experiments have demonstrated variation in the preference of hosts for different fungal species in glasshouse experiments (e.g. Hoeksema et al. (2012), Piculell et al. (2018)), such experiments consider host plants of known genotype growing individually in pots, interacting with an individual set of fungi. Glasshouse experiments are powerful tools that can disentangle effects that cannot be manipulated in the field, and can provide valuable insights into traits underlying these behaviours. However, such experiments must inherently overstate the effects of host genotype on selectivity, as selection by one genotype does not affect what selections are made by another (neighbourhood effects, for example Hausmann and Hawkes (2009)). Furthermore, EM fungi confined to a pot likely exhibit highly altered behaviour compared to their wild counterparts due to the small volume of soil space and increased homogeneity of the soil environment. In field settings, small-scale distributions of, and competitive interactions between fungi may counteract any selectivity displayed by host plants for mycorrhizal fungi. This is likely to have knock-on effects on co-evolutionary dynamics if genetic variation in traits mediating selectivity is not expressed in the wild. Because of this, future experimental work focusing on selectivity of different host genotypes, particularly if the focus is capturing the potential for evolution, must account for these confounding effects.

There are a number of potential ways that future experimental work could incorporate this real-life complexity. If the parameters of interest do not include the growth response of seedlings to inoculation by fungi, then the approach taken here, where different host genotypes are grown close together in grids in the field, might be an appropriate experimental design. If grids are small enough, they are more likely to avoid patchiness in fungal distributions, as patchiness appears to occur most commonly at the metre scale, though patchiness at the scale of tens of cm have also been found (Sawyer et al. 1999; Anderson et al. 2001; Guidot et al. 2001; Beiler et al. 2010). However, if different grids have different fungal communities by chance, as in the present experiment, then this could potentially reduce the available power to test interactions between host genotype and fungal species, and any effects may be masked by between-grid differences if genotype effects are weak. This could potentially be accounted for by increasing the number of grids at each site, perhaps even restricting the study to one location in order to more thoroughly sample the fungal community at one geographic location.

If a more holistic understanding of genotype-mycorrhizal associations is desired, it may also be possible to construct mesocosms in which multiple hosts are grown in a single growth vessel within a glasshouse. Mesocosms could be constructed to approximate soil conditions found in the field and could be inoculated with multiple species of mycorrhizal fungi. An additional conditioning stage, where hosts are grown in the soil before being removed, could assist in building a more realistic environment through plant-soil feedback. An experimental system like this might be able to more concretely link the mycorrhizal communities of individual hosts and performance-related traits such as growth.

Finally, it is worth considering which methods for the analysis of community data are most appropriate to explore these questions. In recent years, as a result of the increased availability of computing power, it has been possible to move away from traditional, distance-based methods of community analysis, such as ordination and variance partitioning, and shift to more powerful statistical methods (Hui 2016). In this thesis, I used Bayesian mixed models conducted in *MCMCglmm*, following the method of Shutt et al. (2020), to test for turnover in community composition between factors of interest. Similar models could be constructed in R-INLA (Lindgren and Rue 2015) if there was a strong spatial component to the data that needs accounting for. These methods allow the estimation of variance components for each term in the model, as well as the model parameters for each variable of interest, such as the response of individual fungal species to specific variables of interest. These methods are highly sensitive, and thus are potentially able to detect very small variations in association probabilities for specific fungal species (A. Phillimore, pers. comm.).

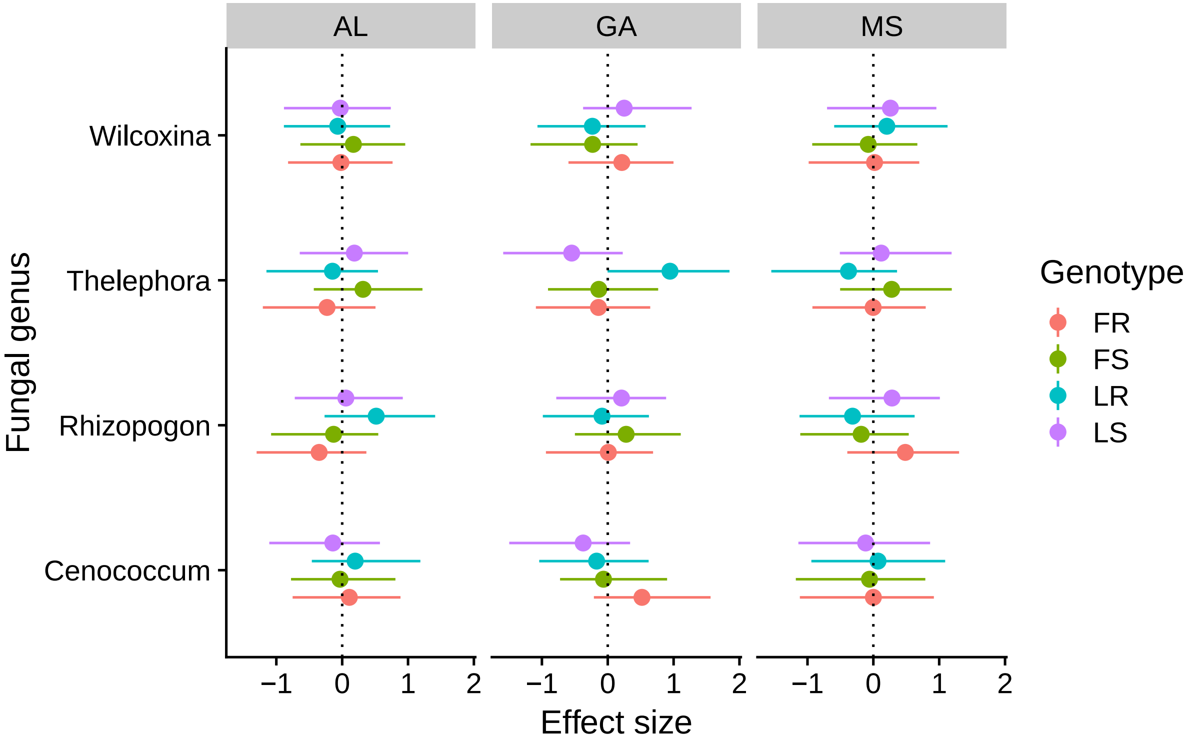

Figure 5.2.: Posterior distribution of model parameter estimates (linear predictor scale) from the reanalysis of data from Piculell et al. (2018). Columns indicate the three soil inocula from different geographic locations, and colours indicate pine genotype (FR = Fusiform rust resistant, FS = Fusiform rust susceptible, LR, = Pine decline resistant, LS = Pine decline susceptible).

To demonstrate the power of these methods, I re-analysed data from a paper by Piculell et al. (2018). In their glasshouse experiment, they explored the effects of soil origin and pathogen resistance genotype (categorised as the susceptibility or resistance to two pine fungal diseases) on the colonisation of four EM species on seedlings of loblolly pine (*Pinus taeda*). They showed a G x E interaction, with increased colonisation by the fungal genus *Thelephora* on seedlings resistant to pine decline. However, their analyses considered the proportional colonisation of each fungal genus individually in separate analyses, increasing the potential for Type I errors and perhaps requiring correction for multiple comparisons. I re-analysed the data using the methods outlined in Chapter 3 (methods in Appendix F). This reanalysis showed that soil origin explained 36% of the variation in fungal communities, with only 2% of variation in the model resulting from this soil x genotype effect (the rest of the variance was unexplained). The only non-zero parameter estimated in this G x E interaction was the same *Thelephora* effect found in the original analysis, but the estimated confidence intervals for the effect were wide and close to zero at the lower end (Fig. 5.2). Thus, re-analysis with this method calls into question the significance of one the conclusions of the paper, that there are differential interactions between pine genotypes and fungal species which could result in potential geographic selection mosaics. Future work should apply these modern statistical methods to make robust analyses of mycorrhizal colonisation data.

5.4. Regeneration of the Caledonian pinewoods

For decades, foresters have understood the importance of the inclusion of mycorrhizal fungi in forestry plantations (Chen et al. 2014; Domínguez-Núñez and Albanesi 2019). Trials dating back to the 1970s have demonstrated the potential benefits of mycorrhizal fungi to the growth and survival of nursery seedlings (Vozzo and Hacskaylo 1971). In one trial, inoculation of *Eucalyptus* seedlings in a nursery setting was found to increase seedling biomass by up to 41% (Chen et al. 2006), and benefits have been found for a range of tree species in survival, growth and nutrition (Chen et al. 2014). Although much of this research has focused on increasing the productivity of plantations for wood, these methods may also have potential uses in the reforestation of threatened woodlands for conservation purposes (Domínguez-Núñez and Albanesi 2019), such as the Caledonian pinewoods studied in this thesis.

For restoration, more than in plantation, it is important to consider the source of the mycorrhizal inoculum for use in the nursery. Inoculum can be sourced either as natural soil already colonised by fungi, or as spores (either commercially produced or from collected fruit bodies) (Domínguez-Núñez and Albanesi 2019). Commercial inoculum is likely to contain fungal species that are not native to the area in question, and given the ability for EM fungi on nursery seedlings to persist in the environment (Fleming 1985; Villeneuve et al. 1991; Selosse et al. 1998), and the increasing recognition of the dangers of fungal invasions (Dickie et al. 2016), may not be the wisest choice where ecosystem restoration is the goal. The collection of fungal fruit bodies may pose logistical difficulties, and germination success of spores is not guaranteed. Thus, despite the potential to collect other organisms (such as soil-borne pathogens), and the likelihood that any fungi colonising come from resistant spores (Taylor and Bruns 1999), natural soil may provide an easily accessible and minimally invasive inoculum source. Given the patchy distribution of mycorrhizal fungi at small scales, collecting this inoculum from multiple locations across a site may provide a more diverse fungal inoculum, which may potentially increase the benefit to seedlings as there is an increased probability of including a more beneficial species (Kipfer et al. 2012).

In this thesis, I found that using natural soil as an inoculum for seedlings from the Caledonian pinewoods generally resulted in an increase in biomass (Fig. 2.2). However, I found little evidence that using local inoculum would result in greater benefit to seedling growth than non-local soil. Despite this, using local soil as an inoculum may still be advisable, as it would use fungal species that are adapted to local soil conditions and reduce the potential for invasion of rare ecosystems and remove the risk of pathogen movement between sites. Where regeneration of the Caledonian pinewoods is occurring in areas that are not presently heavily afforested, inoculation of seedlings may provide a boost to growth, as well as resistance to pathogens, that may aid establishment of new woodland at a vulnerable stage.

5.5. Potential areas for future research

I explored the potential for local adaptation of Scots pine to mycorrhizal fungi in the Caledonian pinewoods, a geographically restricted set of populations with a maximum extent of only approximately 18,000 ha within the Highlands of Scotland (Salmela et al. 2010). Although local adaptation of these populations has previously been demonstrated to a variety of conditions (Salmela et al. 2011, 2013; Perry et al. 2016a; Donnelly et al. 2016, 2018), I found no evidence of local adaptation to mycorrhizal fungi. However, Scots pine has an extensive geographic range, from Scotland to the Sea of Okhotsk in eastern Eurasia, and extending as far south as Spain and Turkey (Critchfield and Little 1966). Given that the distributions of mycorrhizal fungi are determined by both environmental variables as well as dispersal limitation (van der Linde et al. 2018), local adaptation to mycorrhizal fungi may still occur over wider scales where mycorrhizal communities are more dissimilar. Alternatively, given that tree species that have wider ranges appear to respond less to mycorrhizal fungi (Karst et al. 2018), local adaptation may be unlikely in this tree species with long-distance gene flow (Wachowiak et al. 2011). Further studies which experimentally test the relationship the relationship between range size or geographic isolation and local adaptation to mycorrhizal fungi would be valuable in expanding our understanding of the landscape of adaptation to mycorrhizal fungi in tree species.

More broadly, further work on the effects of life history on evolution in mycorrhizal mutualisms would be warranted. To what extent do plant life history strategies, such as those defined in the C-S-R scheme (Grime 2001; Pierce et al. 2016), correlate with mycorrhizal traits, such as specificity, the obligateness of mycorrhizal associations, or the investment in mycorrhizal fungi? Is overall investment in mycorrhizal fungi correlated with traits such as host lifespan, and do factors such as interspecific competition between host plants affect the degree to which plants select for specific fungal species? With some additional information, it may be possible to answer some of these questions using the MycoDB database (Chaudhary et al. 2016), particularly those relating to questions of host response to inoculation. However, broader questions about the degree of host selectivity among potential mycorrhizal partners will require the collation of data from mycorrhizal communities in field conditions on different plant species, and will likely require estimates of selectivity based on beta diversity (mean number of mycorrhizal species colonising a host species out of the total fungal species richness in a study, or turnover-based estimates of beta diversity (Baselga 2010)) to be incorporated into a new meta-analysis database to approach an answer to these questions. Further basic research to generate more of this type of data may also be required.

5.6. Concluding remarks

Coevolution between host plants and mycorrhizal fungi is likely to be an important process that helps determine the outcomes of mycorrhizal interactions. While I found heritable variation in the response of Scots pine seedlings to mycorrhizal inoculation, I found no evidence of local adaptation, and found that the ecology of mycorrhizal fungi determined the communities of mycorrhizal fungi colonising individual seedlings in the field. These results raise questions about the importance of other factors that might influence the evolutionary trajectories of host plants, such as life history. The integration of mycorrhizal traits into more holistic theories of plant adaptation may provide more insight into coevolution between these important partners than can be understood from considering them solely in the context of mutualism.

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Heritable genetic variation but no local adaptation in a pine-ectomycorrhizal interaction

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ORIGINAL ARTICLE

Heritable genetic variation but no local adaptation in a pine-ectomycorrhizal interaction

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Abstract

Local adaptation of plants to mycorrhizal fungi helps determine the outcome of mycorrhizal interactions. However, there is comparatively little work exploring the potential for evolution in interactions with ectomycorrhizal fungi, and fewer studies have explored the heritability of mycorrhizal responsiveness, which is required for local adaptation to occur. We set up a reciprocal inoculation experiment using seedlings and soil from four populations of Scots pine (*Pinus sylvestris*) from Scotland, measuring seedling response to mycorrhizal inoculation after 4 months. We estimated heritability for the response traits and tested for genotype × environment interactions. While we found that ectomycorrhizal responsiveness was highly heritable, we found no evidence that pine populations were locally adapted to fungal communities. Instead, we found a complex suite of interactions between pine population and soil inoculum. Our results suggest that, while Scots pine has the potential to evolve in response to mycorrhizal fungi, evolution in Scotland has not resulted in local adaptation. Long generation times and potential for rapid shifts in fungal communities in response to environmental change may preclude the opportunity for such adaptation in this species, and selection for other factors such as resistance to fungal pathogens may explain the pattern of interactions found.

Keywords Local adaptation · Ectomycorrhizal fungi (EMF) · *Pinus sylvestris* (Scots pine) · Heritability · Mutualism

Introduction

Mycorrhizal fungi are near-ubiquitous across the plant kingdom, forming associations with approximately 80% of plant species (Smith & Read, 2008). Despite our understanding of their importance in terrestrial ecosystems in terms of both host performance and soil nutrient cycling, it is only relatively recently that we are beginning to understand the evolutionary landscape that helps shape the outcomes of these interactions. In particular, local adaptation (the differential success of a genotype in its home environment compared with a foreign environment) has been explored as a potentially important process in structuring some of the context-dependency in host benefit seen in many mycorrhizal studies (Rúa et al., 2016b; Hoeksema, 2010). Clear evidence of the importance of these adaptive processes has been shown for plants forming arbuscular mycorrhizal (AM) symbioses (Rúa et al., 2016a; Johnson et al., 2010), but there have been comparatively few studies exploring local adaptation in ectomycorrhizal (EM) systems. While there is some evidence suggesting the existence of genotype × genotype interactions in ectomycorrhizal hosts (Piculell et al., 2008; Hoeksema & Thompson, 2007; Hoeksema et al., 2012; Pickles et al., 2015); overall, there appears to be little evidence so far for local adaptation in host populations (Rúa et al., 2018).

For local adaptation to occur, populations must possess sufficient heritable variation in fitness-related traits and experience differential selection pressures in different places (Cheplick, 2015). Theoretical work on coevolution in mycorrhizal interactions has usually considered factors affecting the outcomes of mutualism, such as partner selection, variation in symbiont community composition, and environmental quality as the drivers of selection (Hoeksema, 2010), resulting in pockets of local adaptation or maladaptation depending on the outcomes of these processes (the “geographic mosaic of coevolution”) (Thompson, 2005). While much work has used reciprocal transplants to investigate the potential for genotype

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× genotype interactions with mycorrhizal fungi (e.g. Hoeksema et al., 2012; Johnson et al., 2010), there have been comparatively few studies that have explored the heritability of the traits in question.

In particular, the heritability of ectomycorrhizal host traits, including host performance, degree of colonization, or compatibility traits, which are potentially important for local adaptation, has received only cursory attention. Many tree populations show very high amounts of genetic variation (Petit & Hampe, 2006; Cavers & Cottrell, 2015), which means that robust estimates of heritability will often require large amounts of replication both at the family and individual level, which can be time consuming to achieve. An even greater degree of replication may be required in situations where cloning of the host is difficult, such as in *Pinus* species. To our knowledge, only one study has estimated the heritability of host performance in response to inoculation, reporting high levels of heritability in *Pinus elliotii* inoculated with a single genotype EM inoculum (Rosado et al., 1994). Other studies have reported heritability in other mycorrhizal traits, such as the number of mycorrhizal root tips and degree of colonization (Rosado et al., 1994; Tagu et al., 2005; Courty et al., 2011; Velmala et al., 2012), and the heritability of ectomycorrhizal community composition on hosts (Lamit et al., 2016; Velmala et al., 2012).

Whether and how heritable variation in traits arises and is acted on by selection can also depend on factors other than those determining mutualistic outcomes. For example, life history strategies employed by the host or symbiont can change the landscape of adaptation through their effects on the amount and distribution of genetic variation (Leimu & Fischer, 2008; Cheplick, 2015). Mycorrhizal plants exhibit a wide range of life histories, from short-lived annuals to herbaceous perennials to long-lived trees (Smith & Read, 2008). The vast majority of ectomycorrhizal hosts are woody perennials, often trees (Smith & Read, 2008). As such life history factors, including long generation times, large numbers of offspring, and long distance gene flow, will have significant effects on the ability of such species to evolve in response to symbiotic partners (Petit & Hampe, 2006). It has been suggested that long generation times reduce adaptive potential, but phenotypic studies in trees often show strong differentiation between populations for adaptive traits, often with high values of heritability (Cavers & Cottrell, 2015). This differentiation likely results from strong selective pressures operating in the early stages of the life cycle when there is heavy mortality (Petit & Hampe, 2006; Cavers & Cottrell, 2015). For hosts to adapt in response to local EM fungal communities, there must thus be both heritable variation in the outcome of mycorrhizal interactions, and mismatched associations with local EM fungi must be deleterious to the fitness of seedlings. In addition, such deleterious effects should occur on similar scales to other selective pressures such as competition, grazing, and environmental stress.

Even in cases where populations show heritability for mycorrhizal traits, local adaptation still may only occur in situations where the identity and quality of their mycorrhizal associates are stable over the course of generations. If fungal community structure is liable to shift within the lifespan of a host, either through neutral processes or in response to shifting environmental conditions, then this can potentially disrupt any local adaptation that has occurred on the part of the host (Iason et al., 2018). Community turnover in EM fungi occurs not only in response to distance but also in response to environmental variation, including climatic variables such as annual rainfall and temperature, soil factors including pH, and in response to atmospheric deposition of N and K (Linde et al., 2018; Cox et al., 2010; Jarvis et al., 2013; Suz et al., 2014). EM fungi also show functional specialization on specific soil resources (Agerer, 2001). Community turnover is thus likely in response to shifting climatic conditions and increasing pollution (Cox et al., 2010; Suz et al., 2014). Under such changing conditions, local adaptation of host populations is likely impossible, and, instead, shifts in EM community composition in response to environmental change may help facilitate host survival if they are more able to provide host benefit under new conditions (Batstone et al., 2018; Iason et al., 2018).

To measure the heritability of host performance and test for local adaptation in an ectomycorrhizal host, we set up a reciprocal inoculation experiment using seedlings and soil from populations of Scots pine (*Pinus sylvestris*) from the Caledonian pinewoods of Scotland. Established approximately 10,000 years ago, these forests have experienced gradual historic climatic warming (Salmela et al., 2010) and currently occur in a wide variety of climatic conditions and altitudes across their limited range, with a west to east rainfall gradient of approximately 3000 mm to 500 mm per year over little more than 150 km (Donnelly et al., 2016). Local adaptation of these populations has previously been demonstrated along this climatic gradient (e.g. Salmela et al., 2011, 2013; Donnelly et al., 2016), and heritable variation has also been found for both resistance to needle pathogen *Dothistroma septosporum* (Perry et al., 2016a,b) and fungal endophyte community composition [Cavers, unpublished]. The forests also have a well-documented ectomycorrhizal flora, and EM community composition at these sites has been shown to vary strongly in response to both rainfall and altitude (Jarvis et al., 2013, 2015).

We sought to answer the following questions: (1) Is there heritable variation within Scots pine for response to inoculation by EM fungi? (2) Does this response differ depending on the pine population of origin? (3) If so, do populations perform better when paired with their local EM community compared to others?

Methods

We set up a reciprocal cross-inoculation experiment to investigate the effects of soil biota on pine performance. The experiment used seedlings and soil from four populations of Scots pine across a longitudinal rainfall gradient in Scotland, resulting in a 4 × 4 factorial cross. To control for variability within populations and allow estimates of heritability, we used seed from six maternal families per population. Treatments consisted of seedlings grown in sterilized compost inoculated with field soil. Each treatment combination was replicated 8 times, with an additional 3 replicates containing sterilized inoculum to act as a control, resulting in a total of 1056 individuals. Plant performance was measured at the end of the experiment as aboveground and belowground biomass, and mycorrhizal colonization was measured for a subsample of root systems.

Source material

Cones were collected from four native stands of Scots pine in Scotland: Beinn Eighe and Strath Oykel in the west and Abernethy and Glen Tanar in the east (Fig. 1). These sites sit along a strong west-east rainfall gradient and additionally vary in both altitude and soil type (Table 1). Cones were collected from Beinn Eighe and Strath Oykel in late January 2018 and from Abernethy and Glen Tanar in late February of the same year. At each site, cones were collected from six open-pollinated mother trees. Seeds were cold-stratified at 4 °C for 3 weeks before being surface sterilized in 2% sodium hypochlorite for 10 min. They were then rinsed, imbibed in water for 3 h, and germinated on sterile agar before planting.

Field soil for use as inoculum was collected in late April 2018. At each site, soil from 4 small soil pits was collected to a depth of 30 cm from within the drip line of mature pine trees. These trees were chosen haphazardly, with a focus on collecting from beneath trees with low amounts of undergrowth, to allow for easy soil collection with minimal interaction with roots of other species. Collected soil for each site was sieved through a 6-mm mesh to remove stones and roots. The samples from each site were then homogenized to produce inocula for the experiment.

Glasshouse set-up

Soil from each site was mixed with sterile potting soil (a compost:sand mix, 2:1 Levington stock nursery compost: Royal Horticultural Society Sharp Sand, sterilized by steam autoclave at 121 °C for 30 min) in a ratio of 7 potting soil:1 inoculum, and placed into 0.46-L square pots (9 × 9 × 9.5 cm). Germinated seedlings were cut out of agar and planted in the centre of the pot on the 31st May 2018. Each set of replicates was arranged randomly in blocks on two greenhouse benches, for a total of 8 blocks, 4 per bench; controls using sterilized inoculum (also autoclaved at 121 °C for 30 min) were placed into the blocks at each end of one glasshouse bench and at one end for the other. Pots were placed directly onto an unmatted flood bench base to limit root growth outside of the pots. After a week, any seedlings that had not taken hold were replaced with spares. Seedlings were watered in by hand following potting and subsequently were watered two to three times a day (depending on moisture levels) by an overhead sprinkler system. Seedlings were grown in this way for 4 months.

Seedling analysis

Seedlings were harvested between the 18th of September and 1st of October 2019 in block order to capture any additional growth in the block effect. Seedlings were first cut at the soil level to separate above- and belowground biomass. Pots were then emptied, and the contents were carefully sieved and then washed to extract the roots with minimal damage. Root systems were inspected by eye for visible presence of mycorrhizal colonization—evident as thickened, coloured, and/or bifurcated root tips—and were scored for presence or absence. Subsequently, 5+ root systems per population:soil treatment were randomly selected from among 4 families per population for detailed analysis of root colonization. Colonization was assessed as the number of colonized ectomycorrhizal root tips per cm root length. Root length was measured following the gridline intersect method as described by Tennant (1975) using 5 randomizations. The shoots and roots for all seedlings were dried in an oven at 50 °C for 48 h, before being weighed to obtain a measure of above- and belowground biomass.

Statistical analysis

In order to separate out the component of growth that was due to the soil biota, we used mycorrhizal response ratios instead of raw seedling measurements. These were calculated as the ratio of a seedling's biomass or root:shoot ratio to the mean value of the sterile control seedlings for a given soil treatment and maternal family. In addition, we grouped soil treatments into two categories: western soils (Beinn Eighe and Strath Oykel) and eastern soils (Abernethy and Glen Tanar). This allowed us to simplify the interpretations of our model interactions to account for the major axis of variation between our sites. All statistical models were conducted in R using the *lmerTest* package.

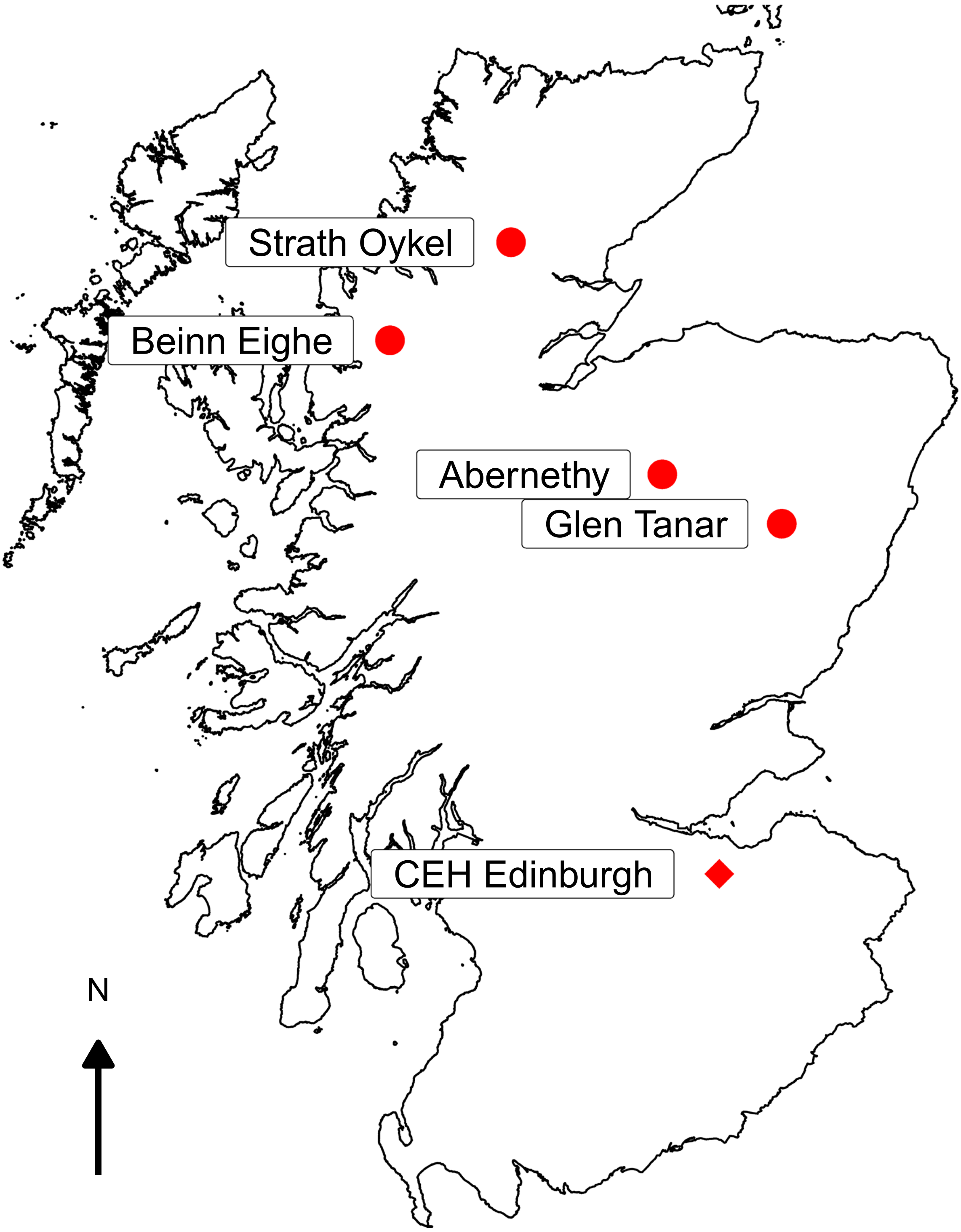

Fig. 1 Map of each pine population used in the experiment (circles) and the location of the glasshouses (diamond) where the experiment was conducted

Heritability and coefficient of genetic variation

We estimated narrow-sense heritability (h^2), the proportion of phenotypic variance (V_P) that can be explained by additive genetic effects, by fitting models for each trait in which maternal family and block were included as random effects Eq. 1, where μ is the mean and ε is the residual error. These models were fitted for each trait both within each inoculum treatment and for the full dataset.

$$\text{Trait} = \mu + \text{Family} + \text{Block} + \varepsilon \qquad (1)$$

From these models, h^2 was estimated using Eq. 2, where V_A is the additive genetic variance; V_P is the phenotypic variance;

Table 1 Coordinates of each population used in the trial and the trial site, as well as climatic variables (Met Office) and soil types (Soil Information For Scottish Soils, James Hutton Institute). MAP = mean annual precipitation

Forest	Latitude	Longitude	Altitude (m)	MAP (mm)	Soil type
Beinn Eighe	57.63	− 5.35	21	2476	Peaty gleyed podzol
Strath Oykel	57.98	− 4.61	67	1234	Peaty gleyed podzol
Abernethy	57.21	− 3.61	362	1060	Humus-iron podzol
Glen Tanar	57.05	− 2.86	177	801	Humus-iron podzol
Common garden	55.86	− 3.21	190		

and V_{fam}, V_{block}, and V_{res} are the among family, among block, and residual variances, respectively; and R is the relatedness of individuals. As the relatedness of the seedlings was unknown, we estimated heritability for three values of R: seedlings are all half-siblings ($R = 4$), seedlings are 50% half-siblings and 50% full siblings ($R = 3$), and seedlings are all full siblings ($R = 2$).

$$h^2 = \frac{V_A}{V_P} = \frac{RV_{\text{fam}}}{V_{\text{fam}} + V_{\text{block}} + V_{\text{res}}} \tag{2}$$

Standard errors were calculated following Visscher (1998) (Eq. 3), where s is the mean number of offspring per family, and f is the number of families.

$$SE_{h^2} = R\frac{\sqrt{2\left(1-\frac{h^2}{4}\right)^2\left[1+(s-1)\frac{h^2}{4}\right]^2}}{s(s-1)(f-1)} \tag{3}$$

We also calculated the coefficient of genetic variation (CV_a) (Houle, 1992) (Eq. 4), a measure of genetic variation normalized by the trait mean. Heritability is an estimate only of the proportion of trait variance attributable to genetic components, so, by accounting for the total amount of phenotypic variance, CV_A provides a more realistic measure of the ability of a trait to respond to selection.

$$CV_A = \frac{\sqrt{V_A}}{\mu_{\text{Trait}}} \times 100 \tag{4}$$

Genotype × environment interactions

To investigate whether there were interactions between pine genotype and soil treatment, we used linear mixed modelling to test models structured as in Eq. 5. We included the specific soil origin as a random effect to control for variation between sites in our east/west soil categorization. In order to test for local adaptation, we used the same model structure but replaced the soil term with a term indicating sympatry or allopatry of the pine population and soil inoculum.

$$\begin{aligned}&\text{Response}\sim\text{Population} \times \text{Soil(east/west)}\\&\quad + \text{random(Family)} + \text{random(Block)}\\&\quad + \text{random(Soil)}\end{aligned} \tag{5}$$

Results

In total, 1045 of 1056 seedlings survived through to the end of the experiment. Of these, 93% of inoculated seedlings showed signs of successful EM colonization, and 89% of sterile control seedlings remained uncolonized at the end of the experiment. Of the 81 seedlings for which we measured the degree of colonization, we found no effect of the degree of colonization on host biomass response ($F_{1,73} = 1.2$, $p = 0.28$). However, we did find that the presence of EM fungi on seedlings, regardless mycorrhizal treatment, had a net positive effect on growth (Mycorrhizal presence: $F_{1,1018.9} = 25.9$, $p < 0.001$; Mycorrhizal treatment: $F_{1,1013.5} = 0.004$, $p = 0.95$) (Fig. 2). For this reason, we assumed growth benefits to the seedlings were likely due to the presence of EM fungi rather than the other biotic components of the soil inocula. For all further analysis, we removed all seedlings from the data set that did not meet treatment standards. This left 960 seedlings in the dataset in total (709 inoculated seedlings, 251 control seedlings).

On average, seedlings inoculated with mycorrhizal fungi grew an additional 62.6 (SE ± 3.9) mg larger than non-inoculated seedlings. However, the range of responses was wide, with 29.3% of seedlings across all treatments showing reduced growth compared with the control. Additionally, inoculated seedlings allocated relatively less biomass to roots than shoots, with root:shoot ratios on average 7.5% (SE &plusmn 0.88%) smaller than those of control seedlings. Again, however, there was a wide range in the response of this trait, with 40.6% of all seedlings increasing their root:shoot ratio in response to inoculation.

Heritability

Although the amount of within- and between-family variation was very large (Fig. 3), we found evidence for significant

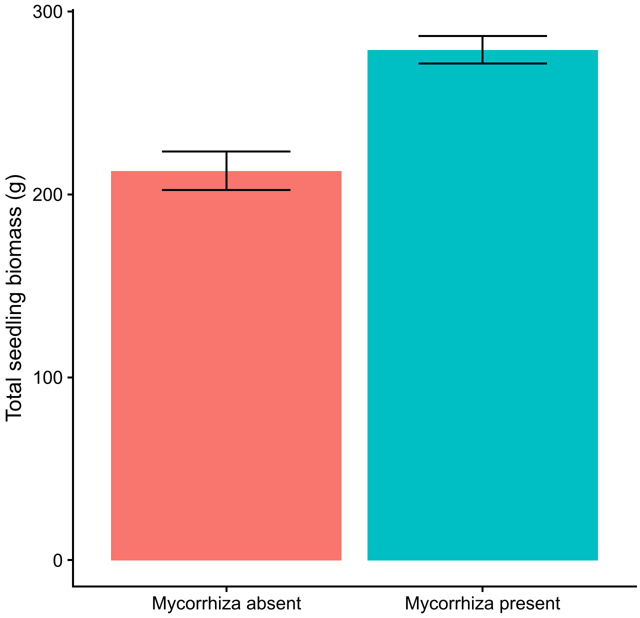

Fig. 2 Bar chart showing the response of seedlings to the presence or absence of mycorrhizal fungi, regardless of mycorrhizal treatment. Error bars show 95% confidence intervals

heritability of both mycorrhizal response traits under all soil conditions (Table 2). Estimates varied from 0.13 to 1.05, although, in many cases, standard errors were large. Variation in traits due to block effects was low for all estimates; however, in all cases, residual variance accounted for the vast proportion of error.

Genotype × environment interactions

We found evidence for a significant G × E interaction between seedling populations and soil treatments for biomass response to inoculation (Population × Soil: $F_{3,673.6} = 12.6$, $p < 0.001$), but no individual effects of population or soil (Fig. 4). In particular, we found that eastern inocula had a uniformly beneficial effect on growth for all populations, whereas western inocula had markedly different growth benefits depending on the population of origin for a seedling. Pines from either extreme of the longitudinal range in this study (Beinn Eighe in the west and Glen Tanar in the east) grew better with western inocula than eastern inocula, whereas pines from the middle

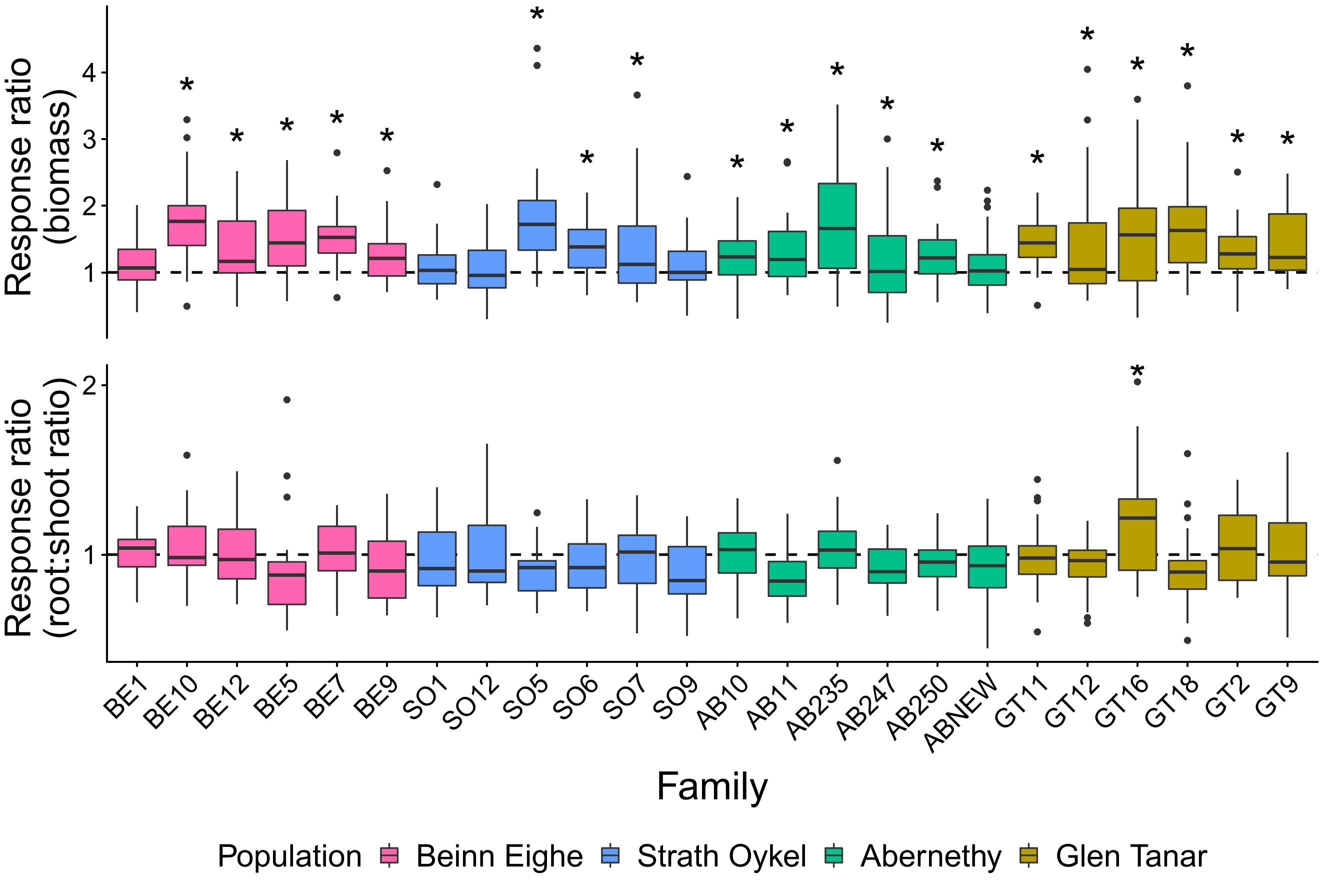

Fig. 3 Box plots showing the range of response ratios of seedling families for biomass (top) and root:shoot ratio (bottom). Middle line shows the median, box shows the first and third quantiles, and the whiskers show no more than 1.5× the interquantile range. Colours indicate the population of origin for the seedling. Dashed line indicates no change compared with a non-inoculated seedling, with an asterisk indicating a significant difference compared with non-inoculated controls. Populations Beinn Eighe and Strath Oykel are located in the west, and Abernethy and Glen Tanar in the east

Table 2 Heritabilities (SE) of mycorrhizal response traits in each of the three soil categories (western soils only, eastern soils only, and all soils). Each R column shows heritability estimated under different relatedness assumptions ($R = 2$: seedlings are full siblings; $R = 3$: seedlings are 50% full siblings and 50% half-siblings; $R = 4$: seedlings are all half-siblings). V columns: proportions of variance attributable to family (V_f) and block (V_b). CV_A: Coefficient of genetic variation. We considered heritabilities to be significant when the standard errors did not overlap with zero

Trait	Soil origin	h^2 ($R = 2$)	h^2 ($R = 3$)	h^2 ($R = 4$)	V_f (%)	V_b (%)	CV_A
Biomass response	West	0.38 (0.09)	0.57 (0.16)	0.76 (0.24)	19.11	2.74	41.50
	East	0.52 (0.1)	0.78 (0.19)	1.05 (0.28)	26.17	2.89	41.36
	Both	0.2 (0.05)	0.3 (0.09)	0.4 (0.14)	10.06	2.51	28.24
Root:shoot response	West	0.32 (0.08)	0.47 (0.14)	0.63 (0.22)	15.79	3.31	17.89
	East	0.13 (0.06)	0.2 (0.1)	0.27 (0.15)	6.73	2.21	10.77
	Both	0.15 (0.04)	0.23 (0.07)	0.3 (0.12)	7.50	2.42	11.89

two populations showed no difference in growth when inoculated with either inoculum.

We also found evidence for a significant G × E interaction between population and soil treatment for a shift in root:shoot ratio following inoculation (Population × Soil: $F_{3,674.2} = 2.7$, $p = 0.042$), but no individual effect of soil or population (Fig. 5). More specifically, we found a complex pattern of interactions between soil types and populations: one population (Strath Oykel) allocated more biomass to shoots over roots when grown with western inocula, while the others (Abernethy, Beinn Eighe, and Glen Tanar) showed no differentiation in root:shoot response between inocula.

Local adaptation

Assuming that the locally adapted phenotype would show increased biomass accumulation when grown with its local soil inoculum, we found no evidence for any main effect of growing in home soil vs away soil, either considering soils as four individual treatments (effect of sympatry on biomass response: $F_{1,674.8} = 0.95$, $p = 0.33$; effect of sympatry on root:shoot response: $F_{1,675} = 3.07$, $p = 0.08$) or on an east-west basis; effect of sympatry on biomass response: $F_{1,673.8} = 0.06$, $p = 0.81$; and effect of sympatry on root:shoot response: $F_{1,674.3} = 2.1$, $p = 0.14$).

Discussion

Overall, we found evidence for heritability in both host performance and change in root shoot ratio in response to inoculation in all of our soil treatments. In line with previous studies in this system, we expected to find little overall differentiation between populations, but to find variation that fell along the major east-west climatic axis of Scotland. Although we did find evidence for G × E interactions between pine populations and soil inocula, suggesting some degree of genetic differentiation between populations in their response to specific fungi,

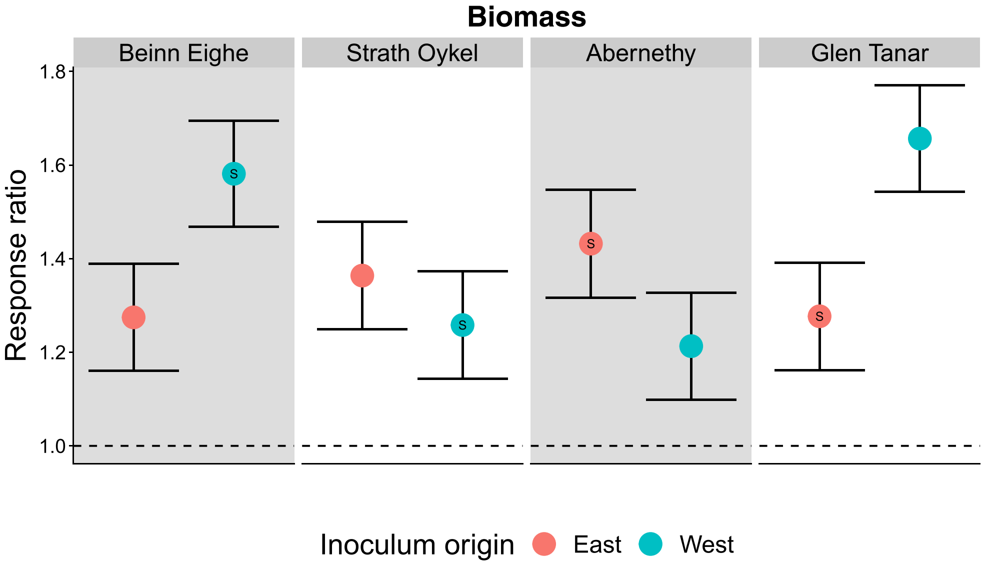

Fig. 4 Response ratio of total seedling biomass ± SE from model predictions. Red points = eastern soil inoculum (Abernethy, Glen Tanar). Blue points = western soil inoculum (Beinn Eighe, Strath Oykel). S = sympatric combination. Pine populations are ordered from most western to most eastern. There were no significant differences apart from a significant interaction between Strath Oykel seedlings grown in western soil. Dashed line = no change compared with a non-inoculated seedling. Model predictions and SE were fitted using R package glmmTMB

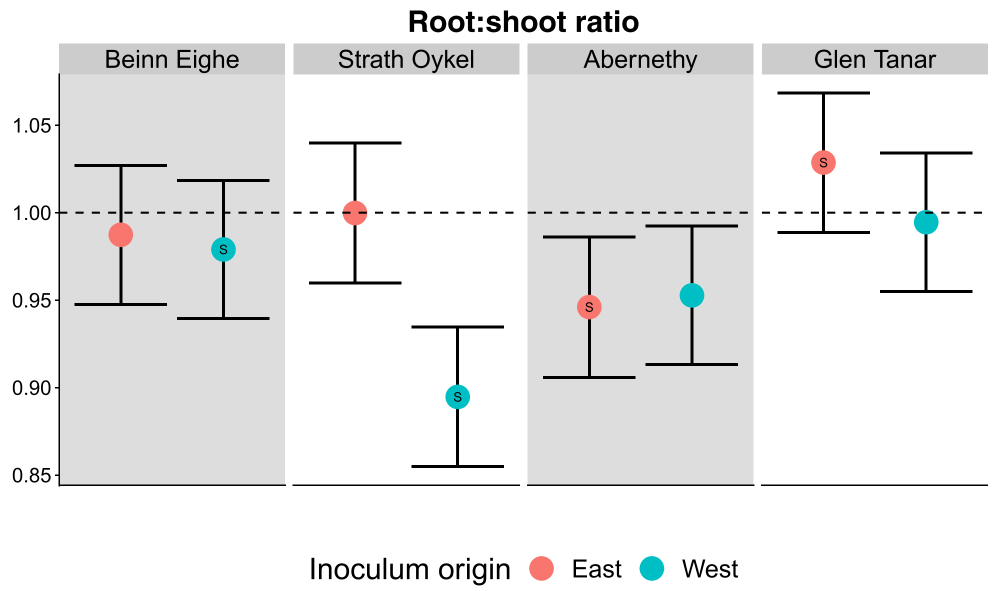

Fig. 5 Percentage change in seedling root:shoot ratio following inoculation ± SE from model predictions. Red points = eastern soil inoculum (Abernethy, Glen Tanar). Blue points = western soil inoculum (Beinn Eighe, Strath Oykel). S = sympatric combination. The only significant interaction was a decrease in root:shoot ratio when Strath Oykel populations were grown in western soil. Pine populations are ordered from most western to most eastern. Dashed line = no change compared to a non-inoculated seedling. Model predictions and SE were fitted using R package glmmTMB

we were unable to detect any clear pattern of geographic variation, either along the east-west gradient or in terms of local adaptation.

Heritability

For both growth in response to inoculation and shift in root:shoot ratio, we found significant heritability within as well as across all soil treatments. Estimates of heritability were high under the most realistic relatedness scenario ($R = 4$, all half-sibs), but had wide estimated errors. Previously reported values for plant performance were broadly comparable with those found here for plant growth. Rosado et al. (1994) reported values of heritability for plant growth between 0.55 and 1.2 in inoculated seedlings, higher than in non-inoculated seedlings (0.26 to 1), in *Pinus elliottii* inoculated with *Pisolithus tinctorius*, suggesting a greater degree of variability in these traits in response to EM inoculation than without. However, the authors reported no errors for their estimates. A few other studies have shown heritability in other EM-associated host traits, including the number of EM root tips, root enzyme activity, and species-specific compatibility (Lamit et al., 2016; Tagu et al., 2005; Courty et al., 2011; Velmala et al., 2012), suggesting that host genotype is an important component of EM interactions. Understanding how these lower-level traits integrate to produce higher-level responses in host or fungal growth would be valuable to further our understanding of the process of adaptation in these organisms (Hoeksema, 2010).

Values of heritability are hard to accurately estimate and require large amounts of replication at the family and population level. Estimates can also be influenced by maternal effects; particularly, in the case of young seedlings, these effects can inflate measures of variance between families, leading to overestimation of heritability. Differences in environment between populations can also affect seed development, which may lead to additional differences in performance that are conflated with genetic variance (Falconer & Mackay, 1996). These latter effects are particularly difficult to control for without multigenerational trials, which are very time consuming when working with trees. We did not control for such non-genetic factors in this study, which may explain why our estimates of heritability were particularly high for our estimates of biomass response. It is also worth noting that the measurements of heritability in both this study and in Rosado et al. (1994) were conducted on seedlings grown in individual pots under glasshouse conditions. Seedlings in natural environments are normally connected to a common mycelial network, such that they are connected to fungal partners that are maintained by nearby mature trees (Teste et al., 2009). It has also been suggested that many of the negative outcomes seen in mycorrhizal studies are a result of such laboratory conditions and that experiments conducted in field conditions should produce more positive results (Frederickson, 2017). It would be instructive for future work to test for heritability of mycorrhizal response under field conditions as well.

Local adaptation

In Scotland, mycorrhizal communities have been shown to vary strongly in response to the W-E rainfall gradient (Jarvis et al., 2013). Because of this, we assumed that community composition in the two western and two eastern inocula would be broadly similar. Our results here suggest that populations of Scots pine within Scotland have diverged in their interactions with EM fungi in terms of both growth and biomass allocation. This effect does not appear to be a simple effect of pine population, but instead depends on the specific combination of both population and inoculum in question. However, these G × E interactions show no clear geographic pattern. In particular, we can find no effect of either local adaptation or variation along the east-west climate gradient. Instead, the seemingly random pattern of interactions suggests that other unmeasured traits, such as resistance to fungal pathogens, may play a role determining the divergence of these populations.

Previous studies have reported mixed results for evidence of local adaptation in EM interactions. A previous study using 5 pine populations at a wider geographic scale found similar evidence for G × G interactions, but some host traits (relative growth rate and short root length) were smaller in sympatric plant-soil combinations (Hoeksema et al., 2012), while another study on the same system found a clinal gradient of colonization based on the distance between the host population and fungal population (Hoeksema & Thompson, 2007). A study explicitly incorporating climate, altitude, and soil effects also found that soil fungi mediated the adaptation of Douglas fir seedlings in over half of their treatment combinations (Pickles et al., 2015). However, a recent meta-analysis found no overall effect of host-fungal sympatry on host performance in EM systems, though this conclusion was based on a small number of studies (Rúa et al., 2018).

Because we sampled at a small spatial scale, we cannot exclude the possibility that gene flow swamped local selection. However, between the most eastern and most western populations, there is a lag of over 2 weeks in peak pollen production, which would limit gene flow between the geographic extremes (Whittet et al., 2017). Local adaptation has been demonstrated in these populations along the west-to-east gradient for a number of traits, including needle morphology (Donnelly et al., 2016), response to waterlogging (Donnelly et al., 2018), photochemical capacity (Salmela et al., 2011), and spring phenology (Salmela et al., 2013). Because mycorrhizal fungal communities in this pinewood system have also been shown to vary longitudinally along a rainfall gradient (Jarvis et al., 2013), we believe that the absence of local adaptation we found is due to a lack of selection for mycorrhizal response rather than the homogenizing effects of gene flow.

Instead, this overall lack of adaptation may result from life history traits of EM hosts. Over the course of a long lifespan, the importance of mycorrhizal associations for host fitness may vary strongly. For example, trees face varying strengths of intraspecific competition depending on their life stage (Petit & Hampe, 2006), and, thus, for a seedling, a boost in growth made by associating with preferred fungi may be much more important for survival than for a mature canopy tree. Alternatively, host preference in seedlings may be disadvantageous, as associations with whatever fungi are available may provide a better chance of survival than being selective. Additionally, if EM community composition is liable to shift within the lifespan of a host, then even if EM fungal associations pose a barrier to survival, then any host adaptation is likely to be quickly disrupted. Instead, the reassortment of EM community composition in response to environmental change may facilitate host survival by providing fungal species or genotypes that are better adapted to current conditions. EM fungi can manipulate host growth, for example by modifying the allocation of biomass to roots or shoots, which may facilitate host survival in a changing environment.

If local adaptation is unlikely in long-lived hosts, host-fungal genotype × genotype interactions may still be maintained through other traits that modify biotic interactions. Recent research is beginning to indicate that host resistance to pests and pathogens can influence mycorrhizal compatibility. For example, *Pinus edulis* individuals resistant to attack by a stem boring moth were found to harbour different EM communities (Sthultz et al., 2009), which provided differing amounts of host benefit under increasing drought (Patterson et al., 2018; Gehring et al., 2014). Similarly, genotypes of *P. taeda* resistant to two fungal pathogens showed both variation in compatibility with specific fungal genera and variation in relative growth rate (Piculell et al., 2018). Evidence of similar effects has also been hinted at in angiosperms, with fungal leaf pathogen communities strongly correlating with EM fungal communities (Lamit et al., 2015). Selection for resistance to these pathogens may thus help maintain the complex G × G interactions found in studies of local adaptation to EM fungi.

It is worth noting that the soil treatments applied here are likely to have included non-EM organisms, such as bacteria and pathogenic fungi. Although we found that the presence of mycorrhizal fungi generally resulted in an increase in plant growth, the G × E interactions found here could be influenced by negative interactions with soil-borne pathogens. If host pathogen resistance and EM response traits are indeed linked, then further work to separate these independent selective effects will be required. In the Caledonian pinewoods, resistance to the foliar needle pathogen *Dothistroma septosporum* has been found both among maternal families (Perry et al., 2016b) and among provenances (Perry et al., 2016a). Additionally, constitutive secondary defensive compounds, particularly monoterpenes, have been shown to influence herbivory by a variety of organisms (Iason et al., 2011), as well as the ground vegetation around a tree (Iason et al., 2005).

Variation in the monoterpene $\delta^3-carene$ shows a geographic bias within Scotland, with high $\delta^3-carene$ levels uncommon in north-west sites. These or other genetic factors may play a role in determining response to mycorrhizal inoculation and could explain some of the G × E interactions found in this study. Further work investigating the effects of both $\delta^3-carene$ and monoterpene chemotype more broadly on Scots pine-EM interactions are underway at present.

Conclusions

The complex set of host-soil interactions found here continues to underscore the complexity of geographic patterns in host-EM interactions. Although we were unable to ascribe a clear geographic pattern to the host-soil interactions found here, we demonstrated the heritability of mycorrhizal response in Scots pine within a relatively confined geographic area. Given the lack of data on the heritability of ectomycorrhizal responsiveness in the literature and the importance of heritability to the development of local adaptation, we would recommend that future studies on local adaptation to symbionts explicitly incorporate host genetic structure, such as clones or maternal families, in order generate robust estimates of heritability.

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Author contributions All authors contributed to the design of the study. J.D. performed the experimental work and carried out the analysis with input from the other authors. J.D. wrote the manuscript, with revisions from the other authors.

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B

Maximum likelihood trees for assessment of taxonomic consistency

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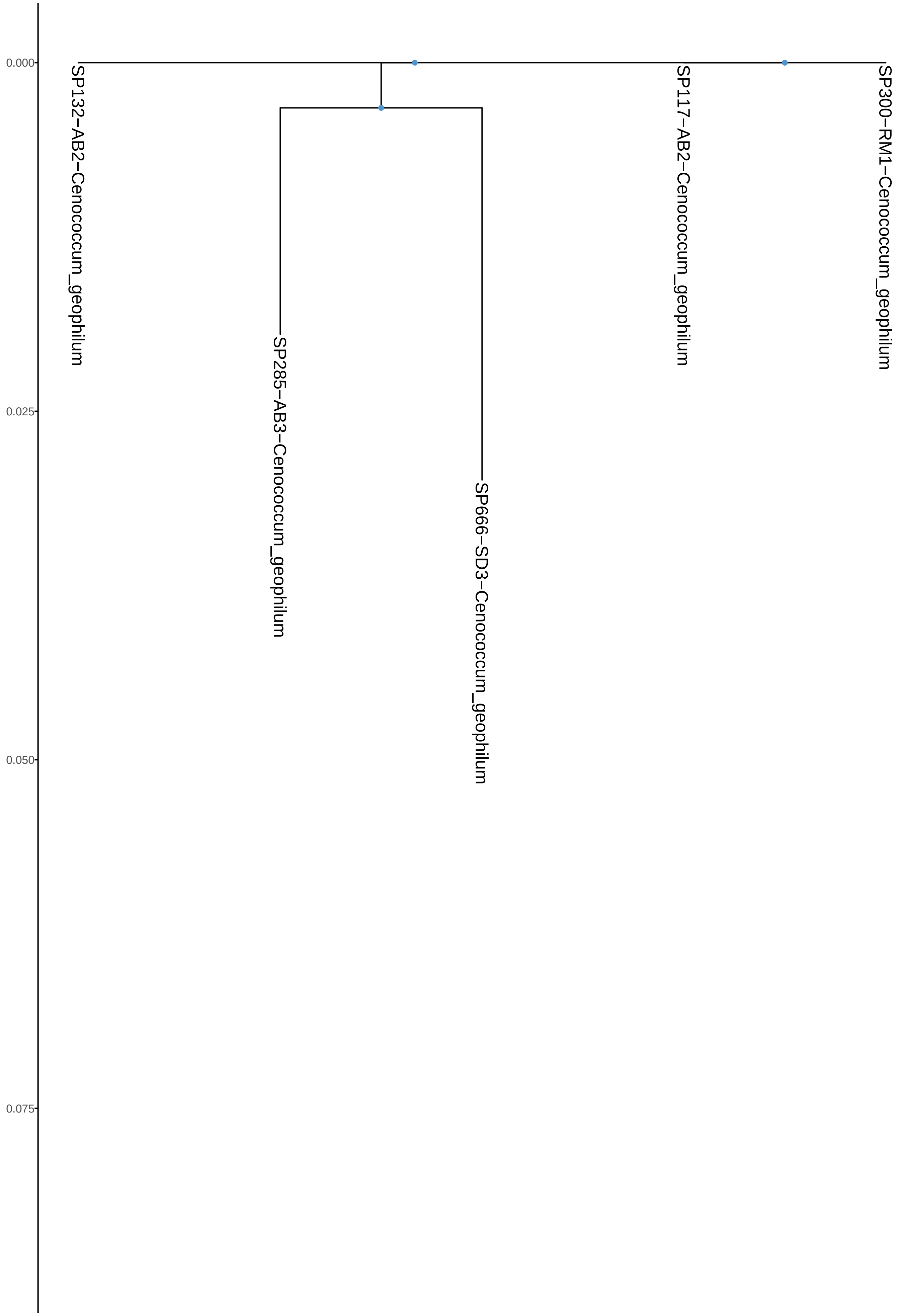
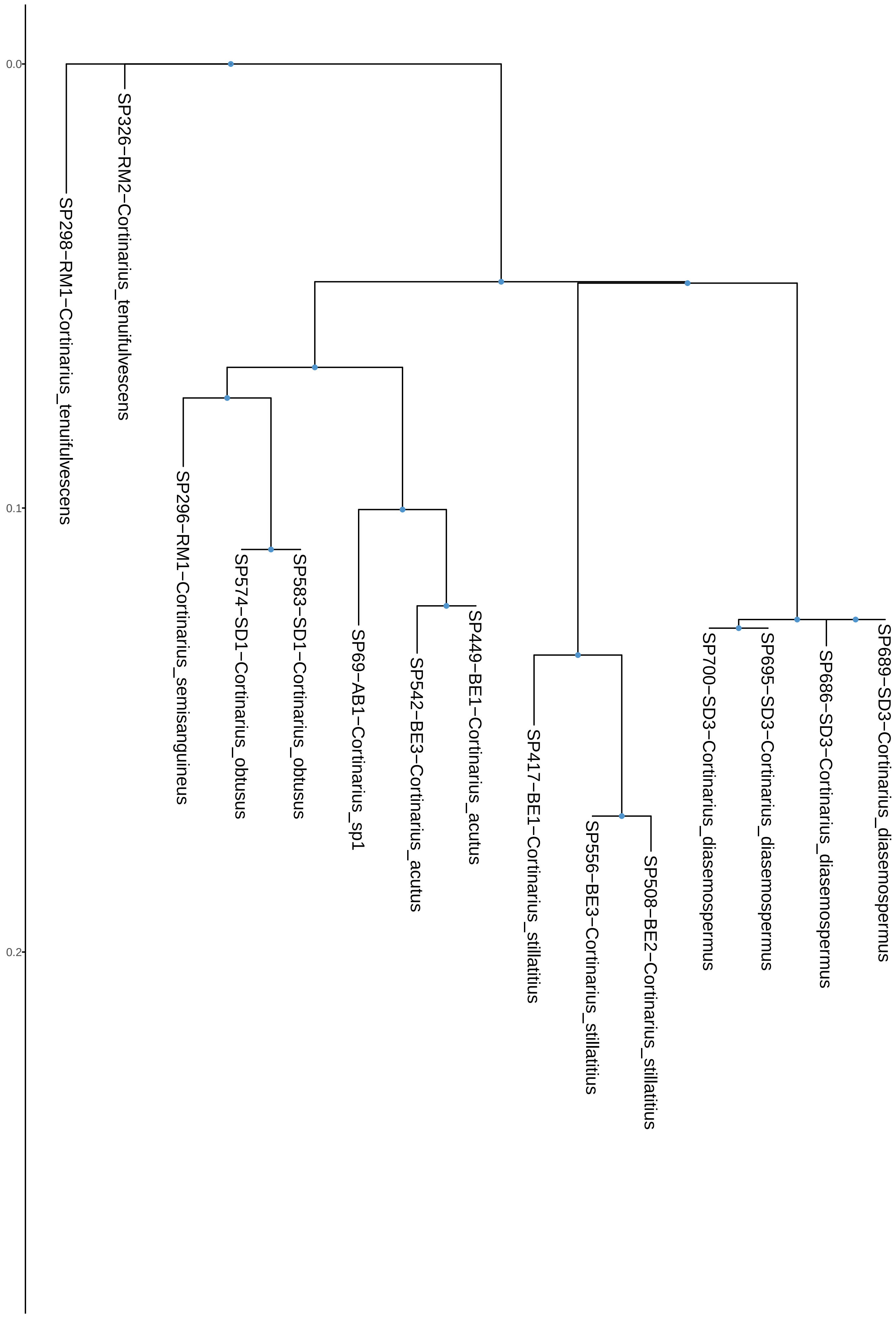
These are the maximum likelihood trees for each fungal genus identified, calculated using *RAxML* (Stamatakis (2014)). Scale bars show the genetic distance between sequences in the number of expected changes per locus.

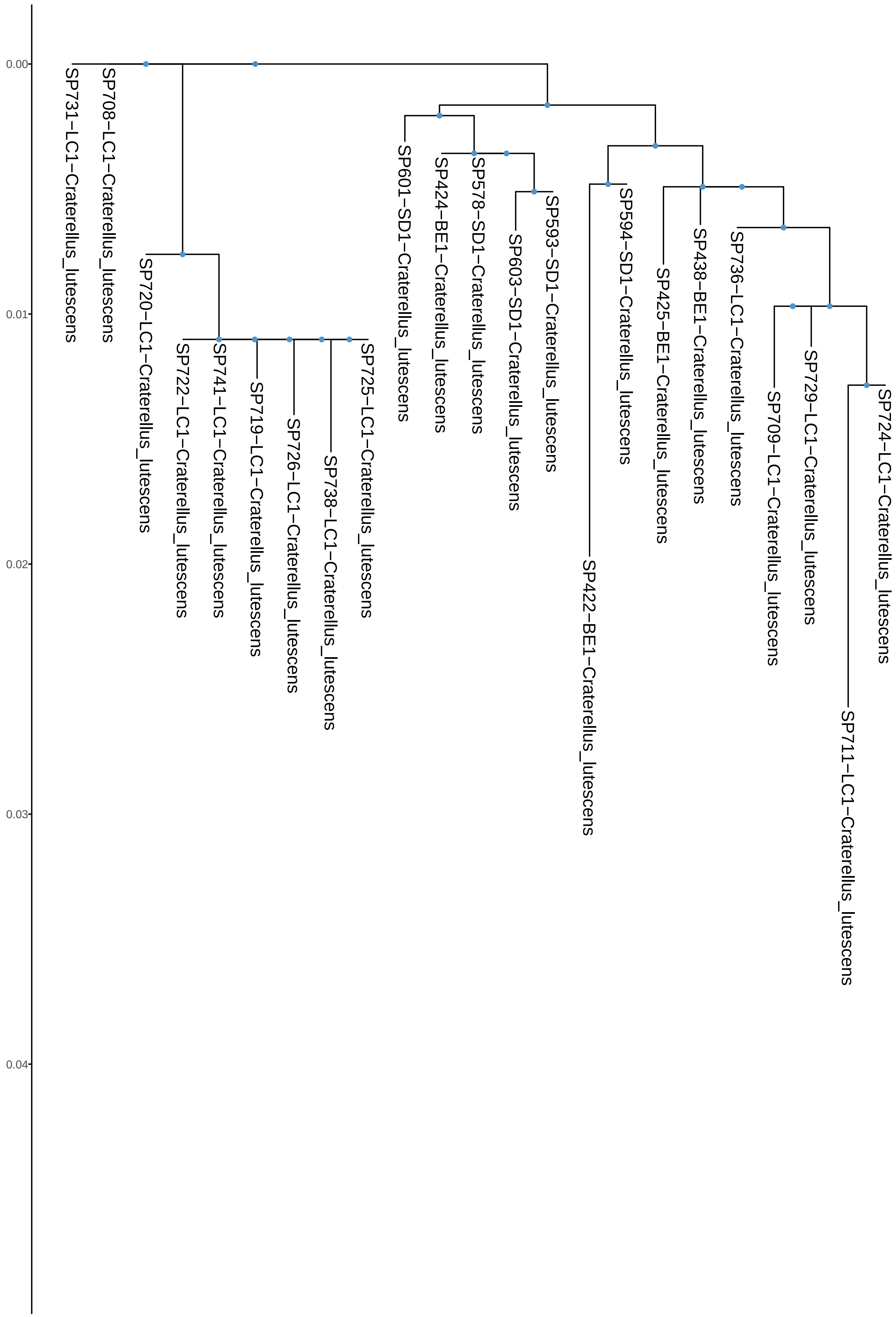
Sequence labels on tips show the morphotype ID (SPXXX), the originating grid, and the BLAST-identified taxon.

Cenococcum


0.000
0.025
0.050
0.075
SP132−AB2−Cenococcum_geophilum
SP285−AB3−Cenococcum_geophilum
SP666−SD3−Cenococcum_geophilum
SP117−AB2−Cenococcum_geophilum
SP300−RM1−Cenococcum_geophilum

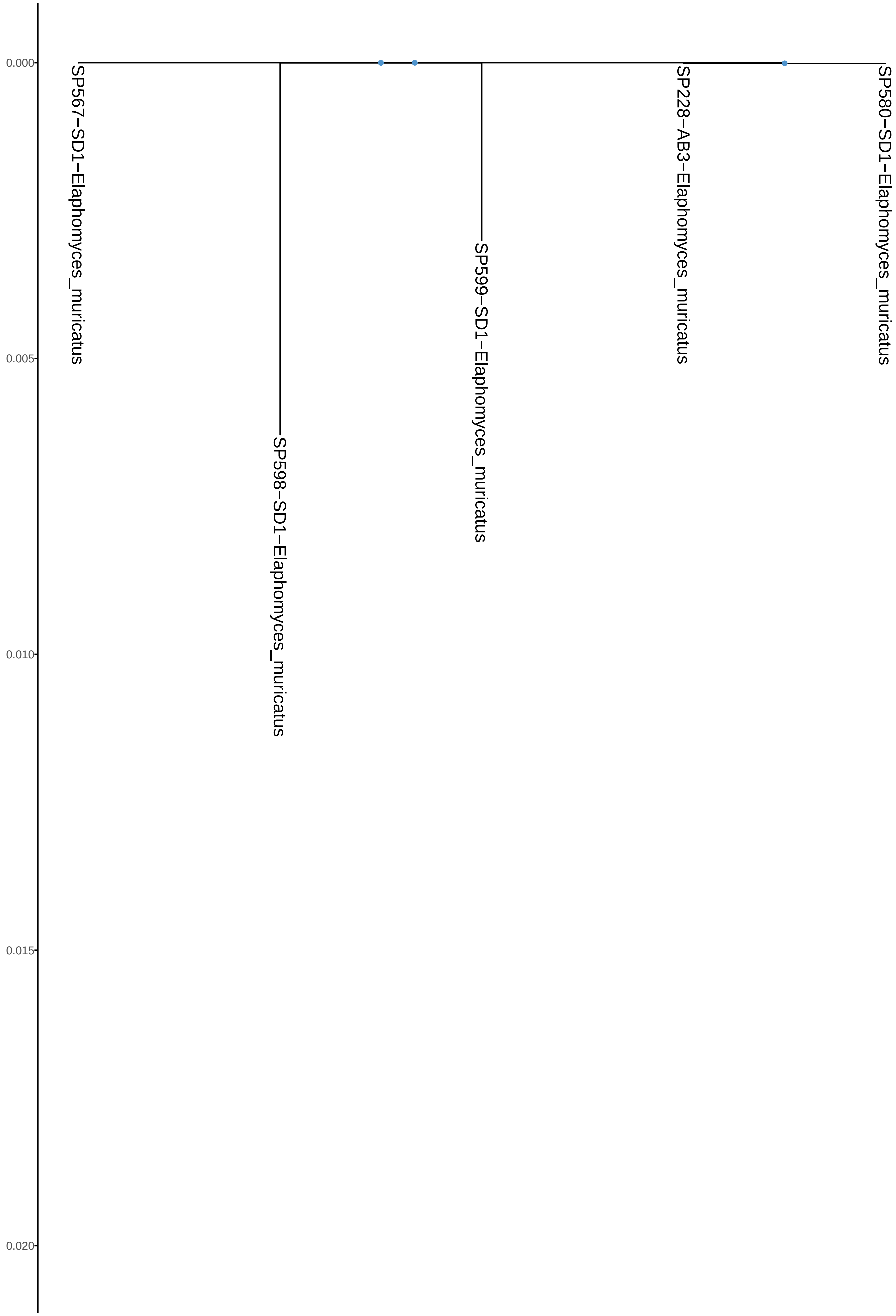


Cortinarius

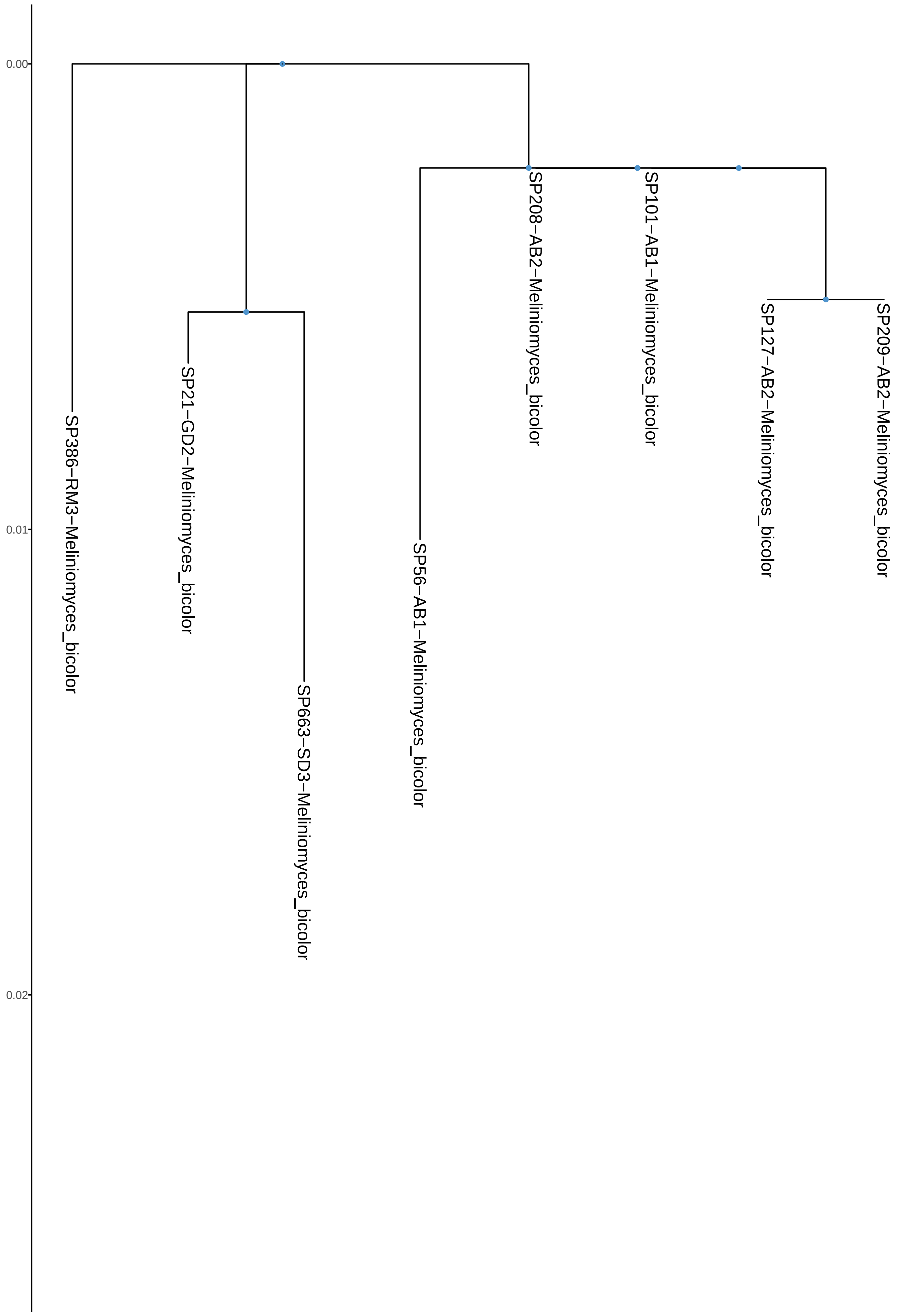


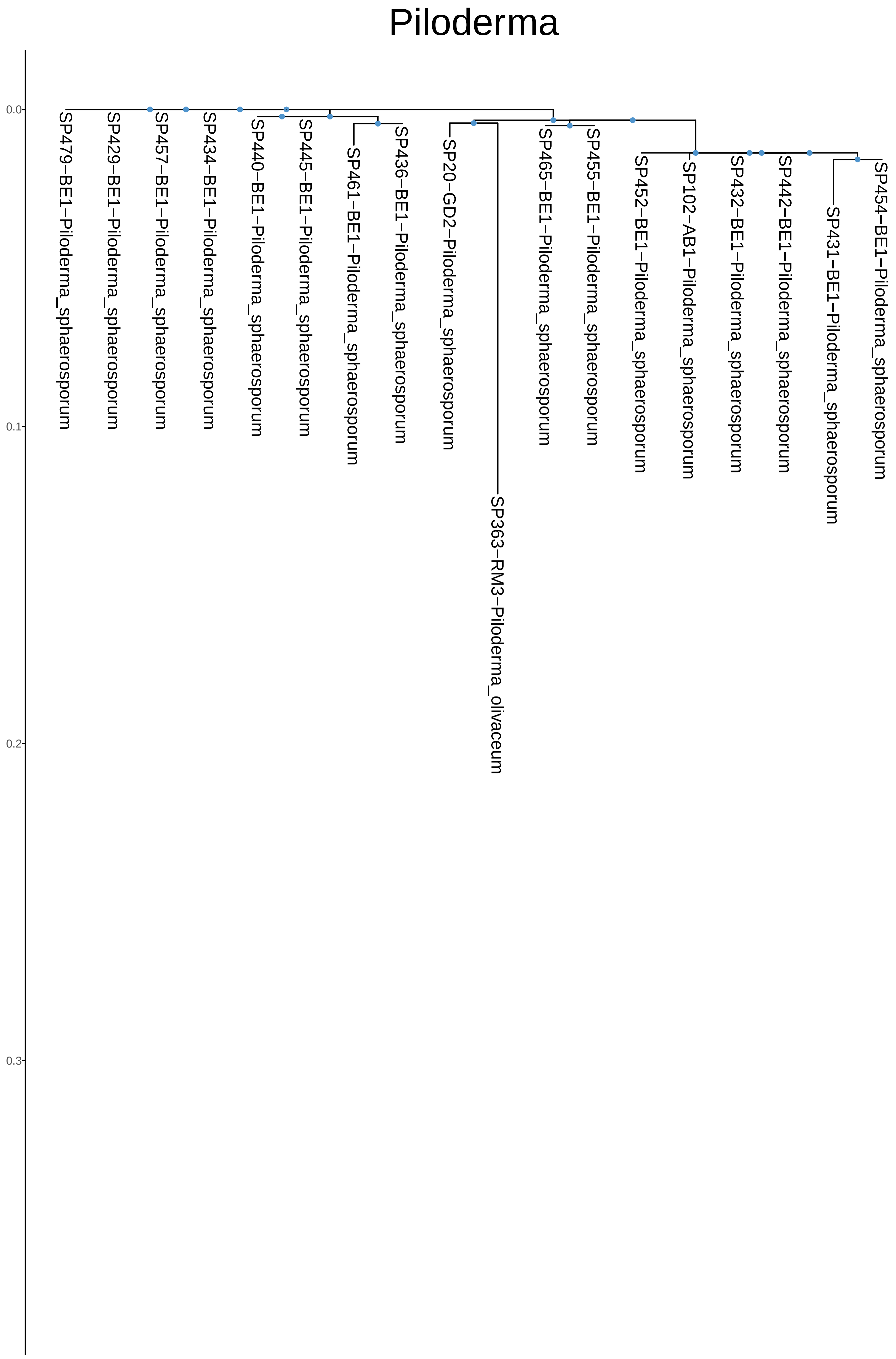
Craterellus

Elaphomyces

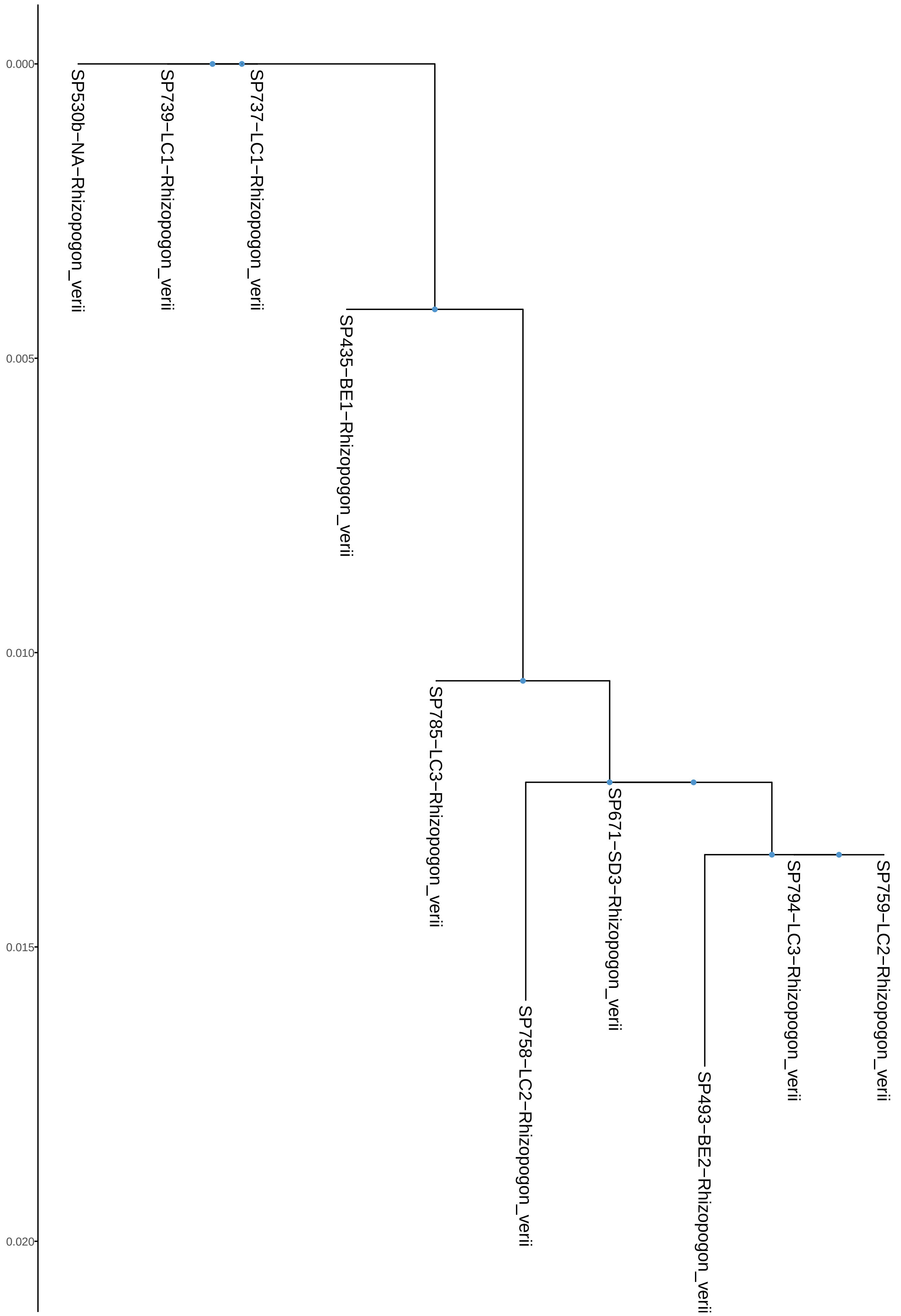


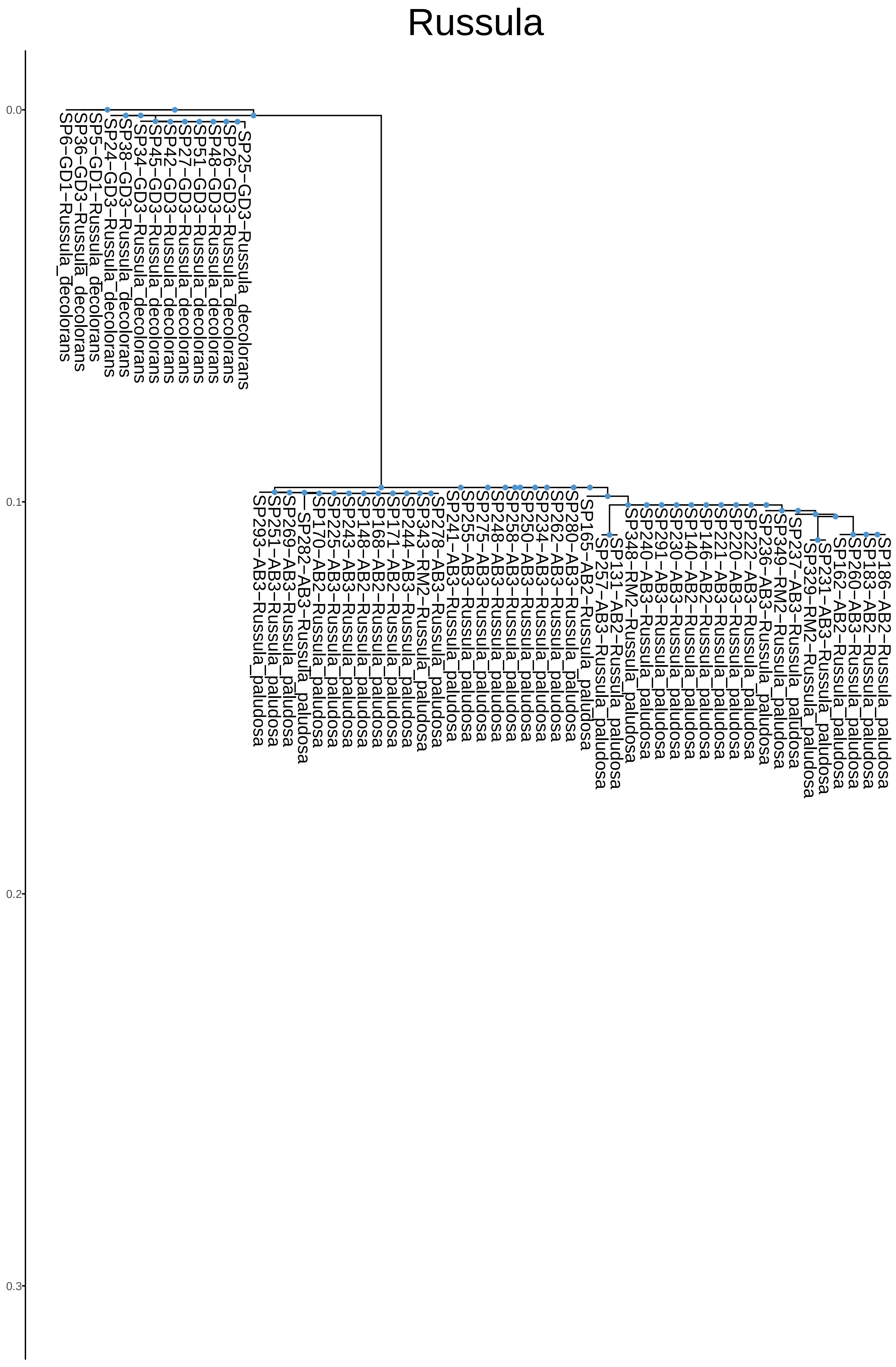
Meliniomyces




Piloderma
0.0
0.1
0.2
0.3
SP479–BE1–Piloderma_sphaerosporum
SP429–BE1–Piloderma_sphaerosporum
SP457–BE1–Piloderma_sphaerosporum
SP434–BE1–Piloderma_sphaerosporum
SP440–BE1–Piloderma_sphaerosporum
SP445–BE1–Piloderma_sphaerosporum
SP461–BE1–Piloderma_sphaerosporum
SP436–BE1–Piloderma_sphaerosporum
SP20–GD2–Piloderma_sphaerosporum
SP363–RM3–Piloderma_olivaceum
SP465–BE1–Piloderma_sphaerosporum
SP455–BE1–Piloderma_sphaerosporum
SP452–BE1–Piloderma_sphaerosporum
SP102–AB1–Piloderma_sphaerosporum
SP432–BE1–Piloderma_sphaerosporum
SP442–BE1–Piloderma_sphaerosporum
SP431–BE1–Piloderma_sphaerosporum
SP454–BE1–Piloderma_sphaerosporum

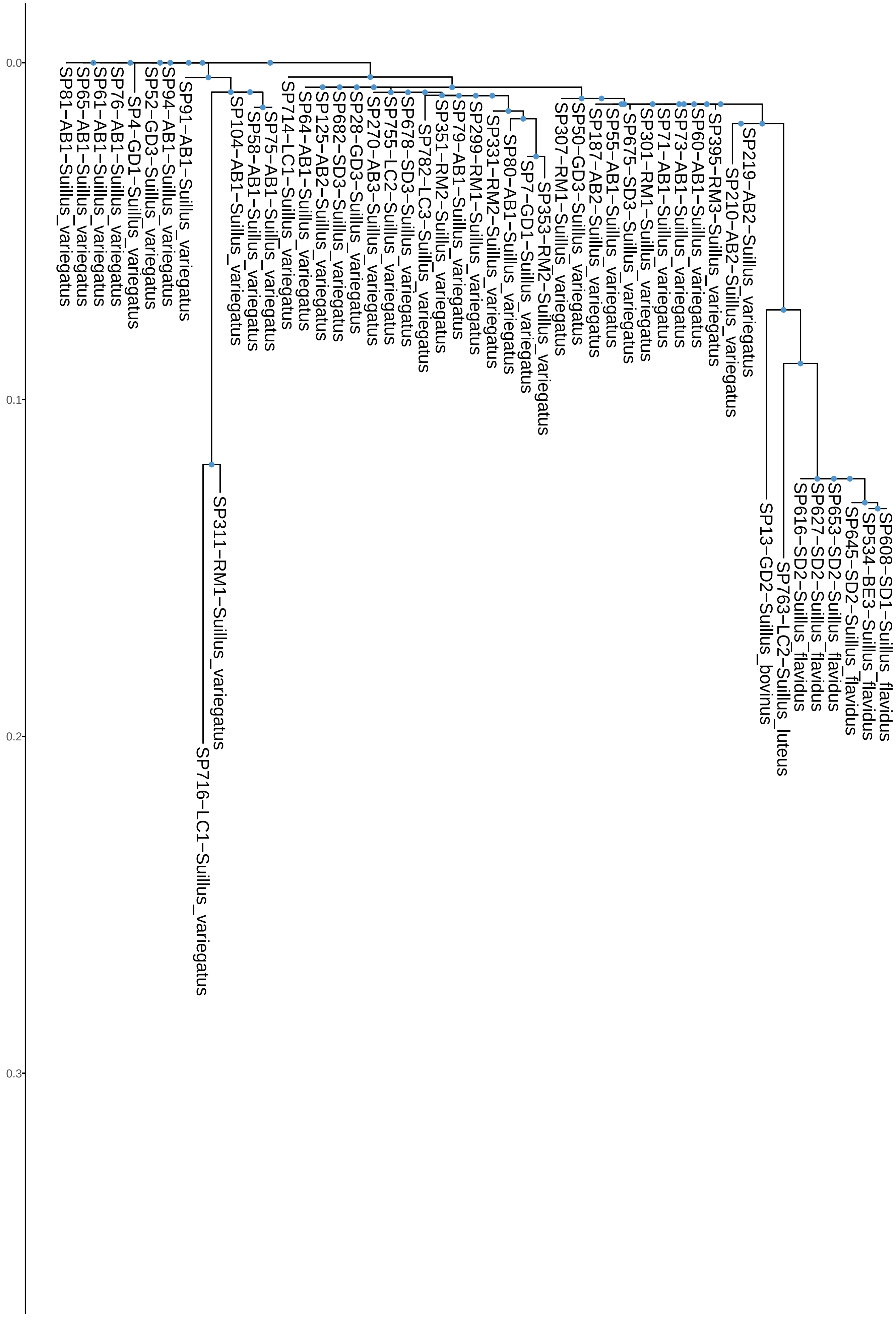
Rhizopogon


0.000
0.005
0.010
0.015
0.020
SP530b–NA–Rhizopogon_verii
SP739–LC1–Rhizopogon_verii
SP737–LC1–Rhizopogon_verii
SP435–BE1–Rhizopogon_verii
SP785–LC3–Rhizopogon_verii
SP758–LC2–Rhizopogon_verii
SP671–SD3–Rhizopogon_verii
SP493–BE2–Rhizopogon_verii
SP794–LC3–Rhizopogon_verii
SP759–LC2–Rhizopogon_verii

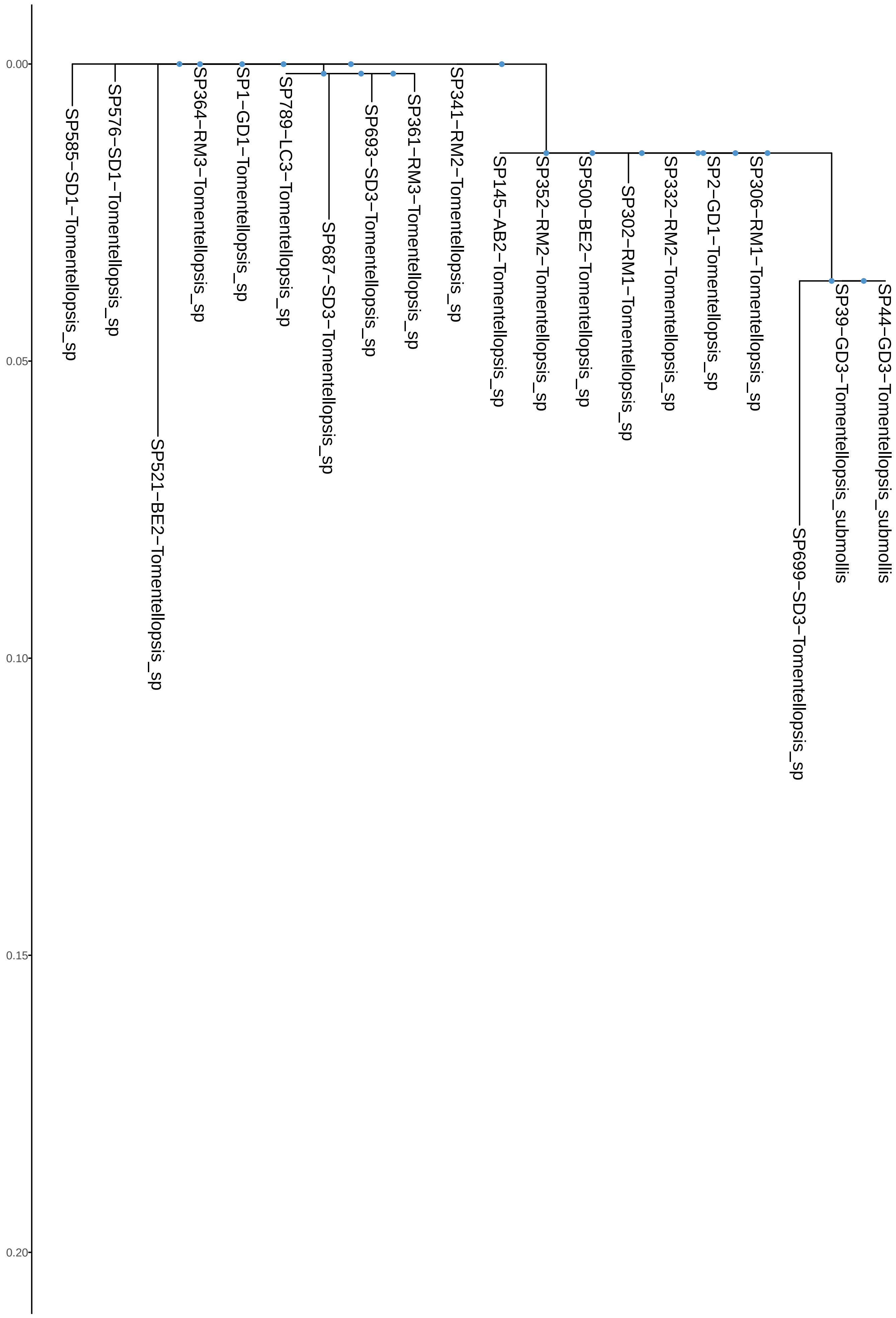


Russula
0.0
0.1
0.2
0.3
SP6–GD1–Russula_decolorans
SP36–GD3–Russula_decolorans
SP5–GD1–Russula_decolorans
SP24–GD3–Russula_decolorans
SP38–GD3–Russula_decolorans
SP34–GD3–Russula_decolorans
SP45–GD3–Russula_decolorans
SP42–GD3–Russula_decolorans
SP27–GD3–Russula_decolorans
SP51–GD3–Russula_decolorans
SP48–GD3–Russula_decolorans
SP26–GD3–Russula_decolorans
SP25–GD3–Russula_decolorans
SP293–AB3–Russula_paludosa
SP251–AB3–Russula_paludosa
SP269–AB3–Russula_paludosa
SP282–AB3–Russula_paludosa
SP170–AB2–Russula_paludosa
SP225–AB3–Russula_paludosa
SP243–AB3–Russula_paludosa
SP148–AB2–Russula_paludosa
SP168–AB2–Russula_paludosa
SP171–AB2–Russula_paludosa
SP244–AB3–Russula_paludosa
SP343–RM2–Russula_paludosa
SP278–AB3–Russula_paludosa
SP241–AB3–Russula_paludosa
SP255–AB3–Russula_paludosa
SP275–AB3–Russula_paludosa
SP248–AB3–Russula_paludosa
SP258–AB3–Russula_paludosa
SP250–AB3–Russula_paludosa
SP234–AB3–Russula_paludosa
SP262–AB3–Russula_paludosa
SP280–AB3–Russula_paludosa
SP165–AB2–Russula_paludosa
SP257–AB3–Russula_paludosa
SP131–AB2–Russula_paludosa
SP348–RM2–Russula_paludosa
SP240–AB3–Russula_paludosa
SP291–AB3–Russula_paludosa
SP230–AB3–Russula_paludosa
SP140–AB2–Russula_paludosa
SP146–AB2–Russula_paludosa
SP221–AB3–Russula_paludosa
SP220–AB3–Russula_paludosa
SP222–AB3–Russula_paludosa
SP236–AB3–Russula_paludosa
SP349–RM2–Russula_paludosa
SP237–AB3–Russula_paludosa
SP329–RM2–Russula_paludosa
SP231–AB3–Russula_paludosa
SP162–AB2–Russula_paludosa
SP260–AB3–Russula_paludosa
SP183–AB2–Russula_paludosa
SP186–AB2–Russula_paludosa

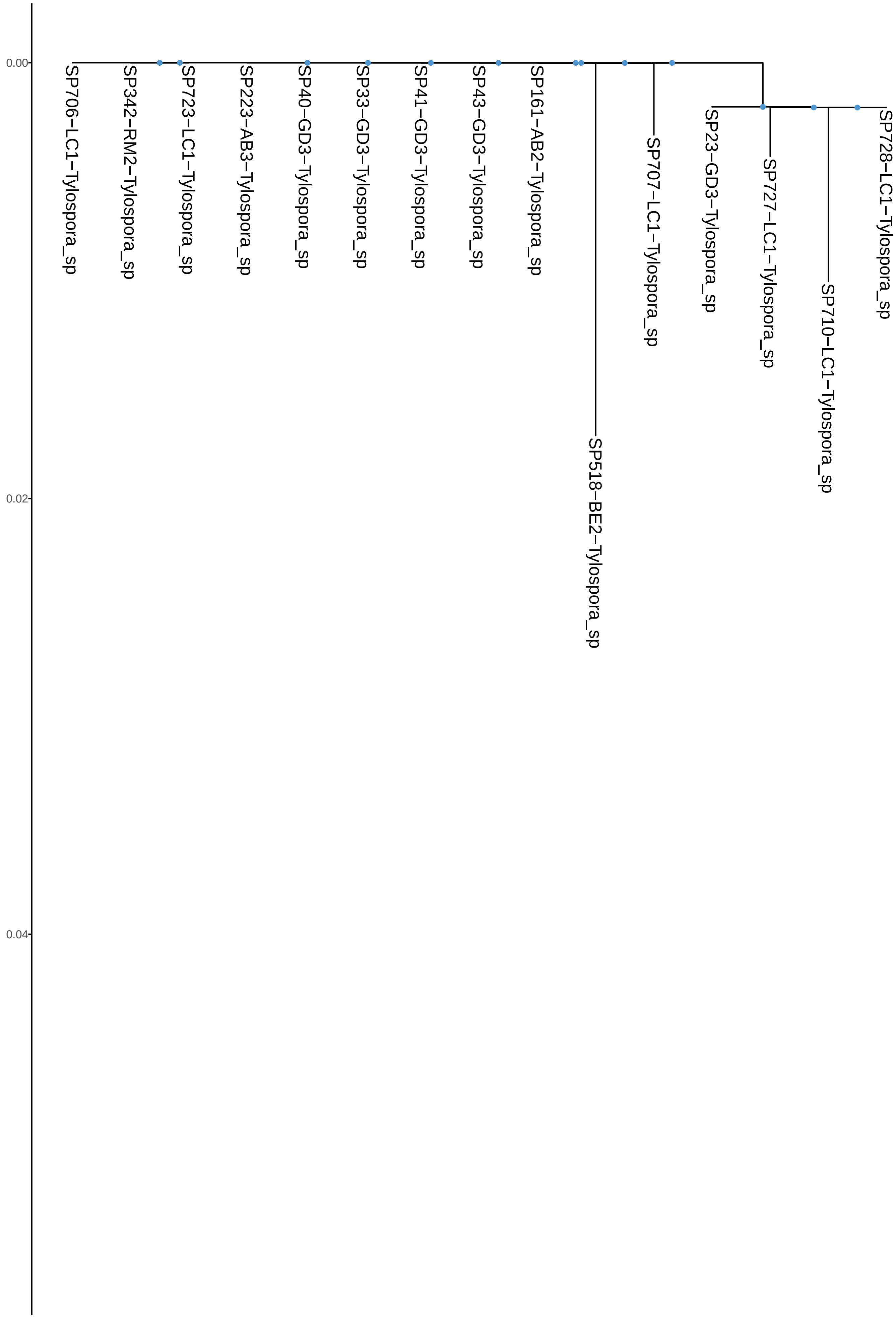
Suillus


0.0
0.1
0.2
0.3
SP81–AB1–Suillus_variegatus
SP65–AB1–Suillus_variegatus
SP61–AB1–Suillus_variegatus
SP76–AB1–Suillus_variegatus
SP4–GD1–Suillus_variegatus
SP52–GD3–Suillus_variegatus
SP94–AB1–Suillus_variegatus
SP91–AB1–Suillus_variegatus
SP311–RM1–Suillus_variegatus
SP716–LC1–Suillus_variegatus
SP104–AB1–Suillus_variegatus
SP58–AB1–Suillus_variegatus
SP75–AB1–Suillus_variegatus
SP714–LC1–Suillus_variegatus
SP64–AB1–Suillus_variegatus
SP125–AB2–Suillus_variegatus
SP682–SD3–Suillus_variegatus
SP28–GD3–Suillus_variegatus
SP270–AB3–Suillus_variegatus
SP755–LC2–Suillus_variegatus
SP678–SD3–Suillus_variegatus
SP782–LC3–Suillus_variegatus
SP351–RM2–Suillus_variegatus
SP79–AB1–Suillus_variegatus
SP299–RM1–Suillus_variegatus
SP331–RM2–Suillus_variegatus
SP80–AB1–Suillus_variegatus
SP7–GD1–Suillus_variegatus
SP353–RM2–Suillus_variegatus
SP307–RM1–Suillus_variegatus
SP50–GD3–Suillus_variegatus
SP187–AB2–Suillus_variegatus
SP55–AB1–Suillus_variegatus
SP675–SD3–Suillus_variegatus
SP301–RM1–Suillus_variegatus
SP71–AB1–Suillus_variegatus
SP73–AB1–Suillus_variegatus
SP60–AB1–Suillus_variegatus
SP395–RM3–Suillus_variegatus
SP210–AB2–Suillus_variegatus
SP219–AB2–Suillus_variegatus
SP13–GD2–Suillus_bovinus
SP763–LC2–Suillus_luteus
SP616–SD2–Suillus_flavidus
SP627–SD2–Suillus_flavidus
SP653–SD2–Suillus_flavidus
SP645–SD2–Suillus_flavidus
SP534–BE3–Suillus_flavidus
SP608–SD1–Suillus_flavidus

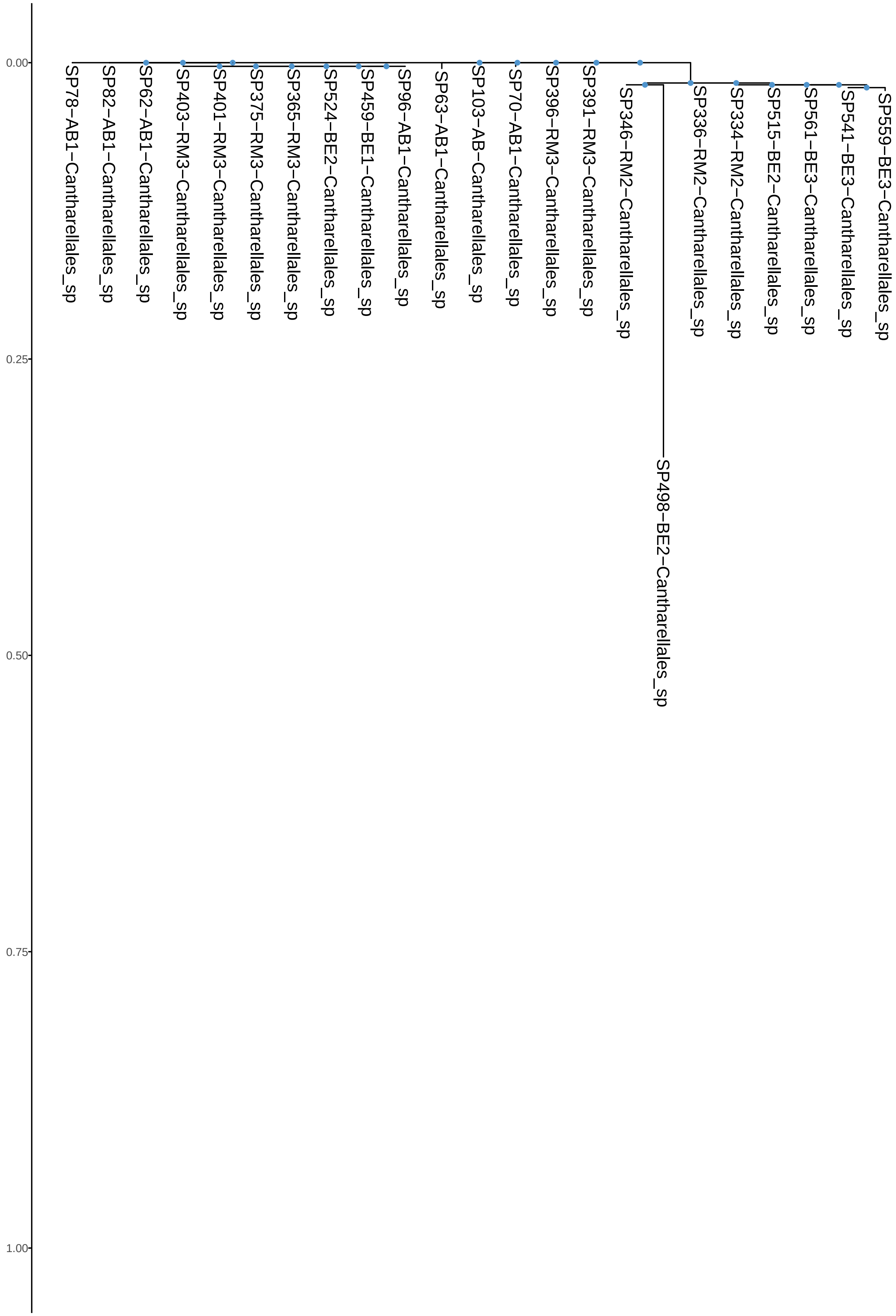
Tomentellopsis



Tylospora


0.00
0.02
0.04
SP706–LC1–Tylospora_sp
SP342–RM2–Tylospora_sp
SP723–LC1–Tylospora_sp
SP223–AB3–Tylospora_sp
SP40–GD3–Tylospora_sp
SP33–GD3–Tylospora_sp
SP41–GD3–Tylospora_sp
SP43–GD3–Tylospora_sp
SP161–AB2–Tylospora_sp
SP518–BE2–Tylospora_sp
SP707–LC1–Tylospora_sp
SP23–GD3–Tylospora_sp
SP727–LC1–Tylospora_sp
SP710–LC1–Tylospora_sp
SP728–LC1–Tylospora_sp

Unidentified Cantharellales



C

Ordination stress plots for Chapter 3

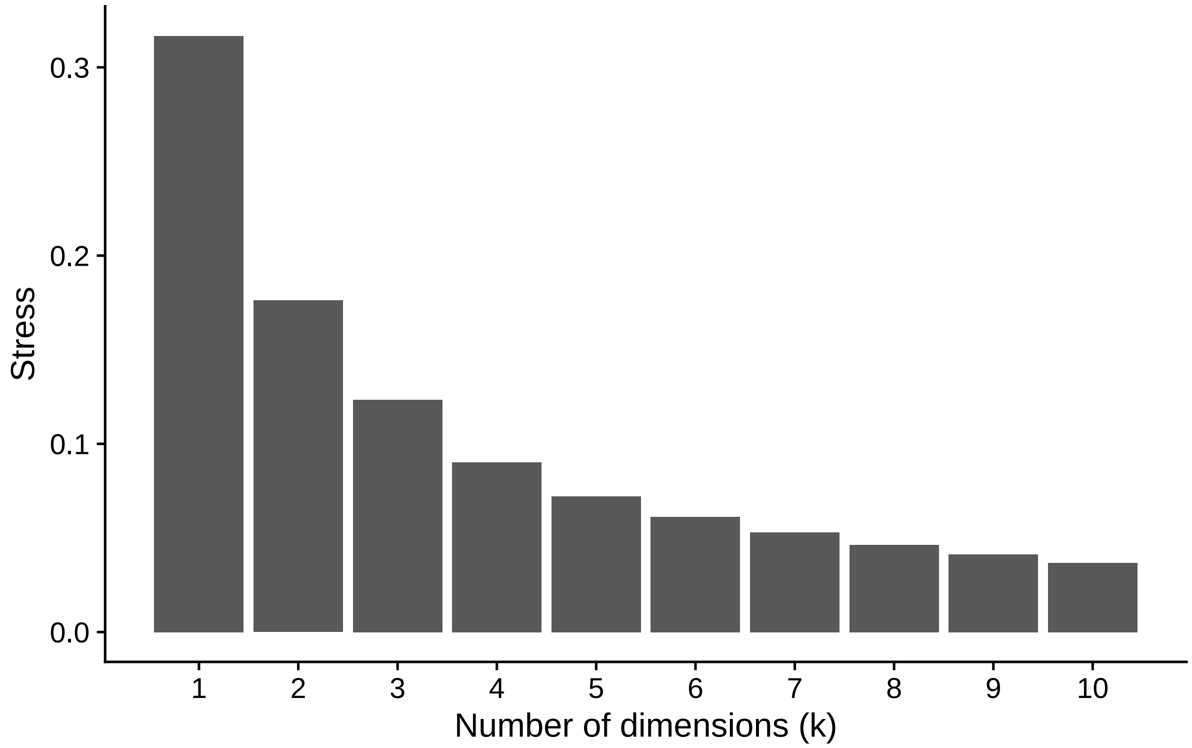


Figure C.1.: Plot of NMDS stress against the number of dimensions.

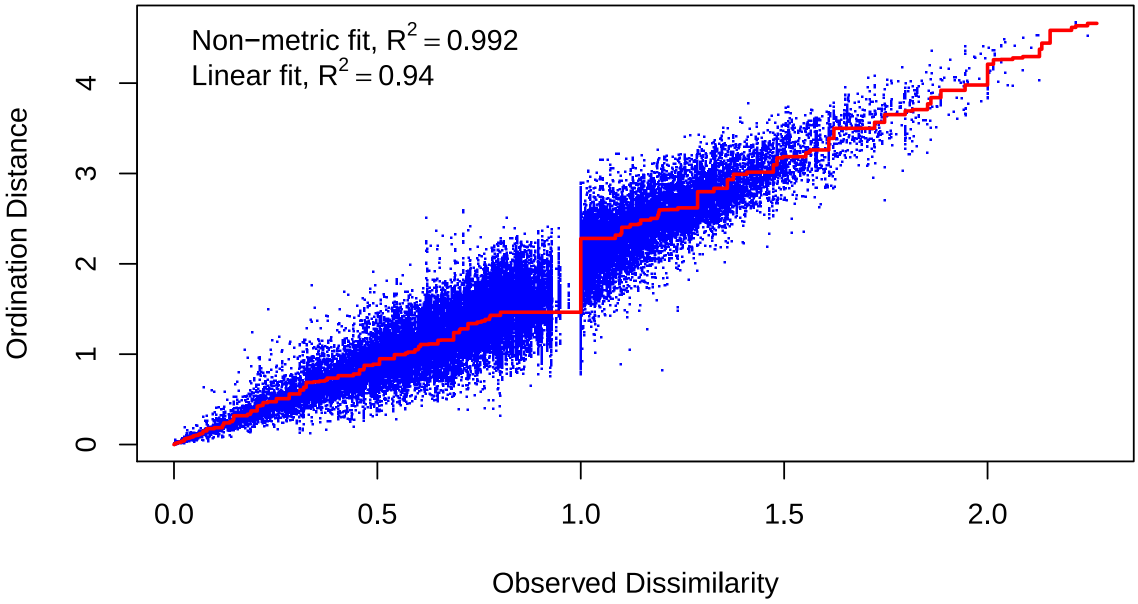


Figure C.2.: Shepard Diagram for ordination k = 4.

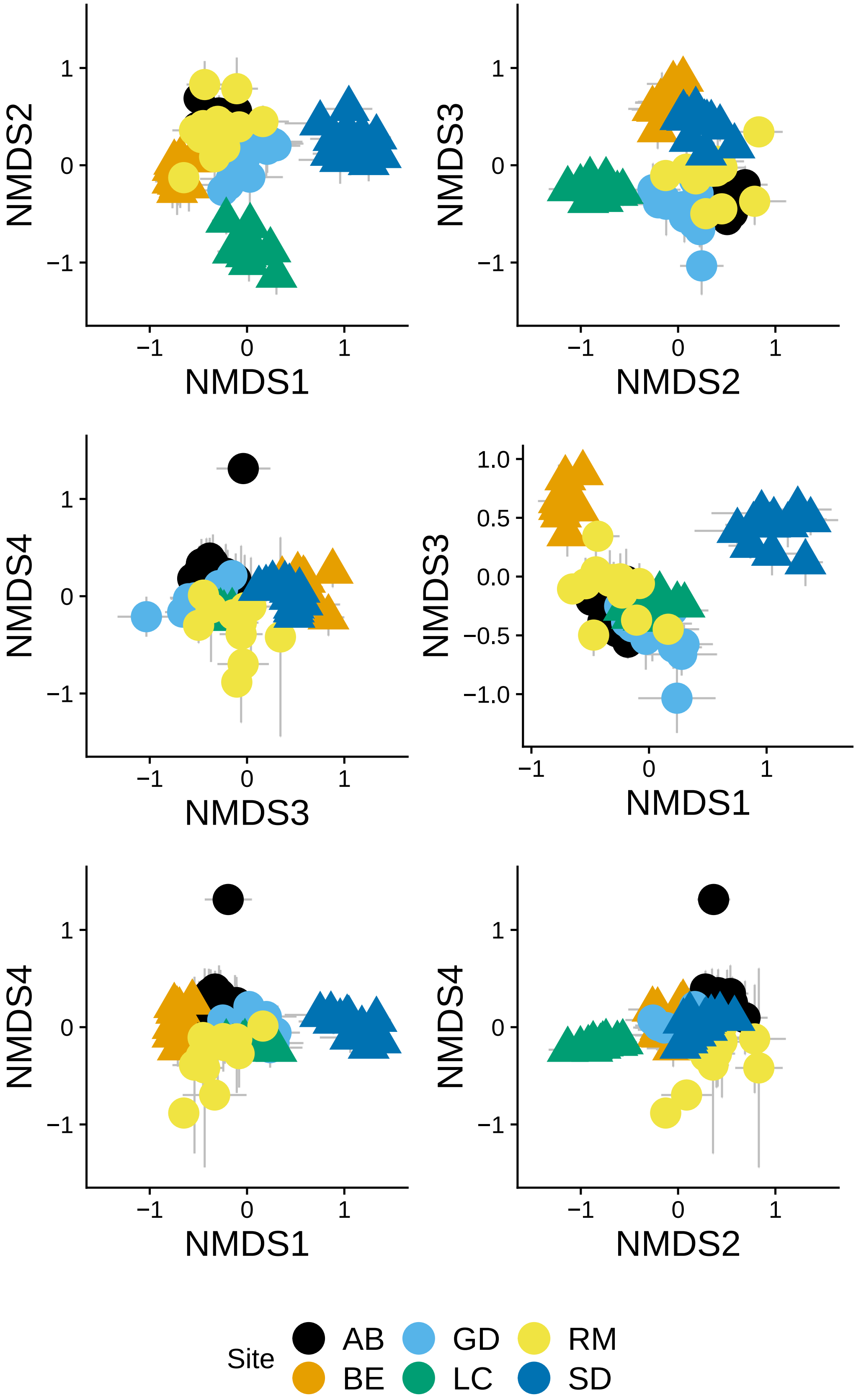


Figure C.3.: Plots of each NMDS axis from the final NMDS against each other..

D

Ordination stress plots for Chapter 4

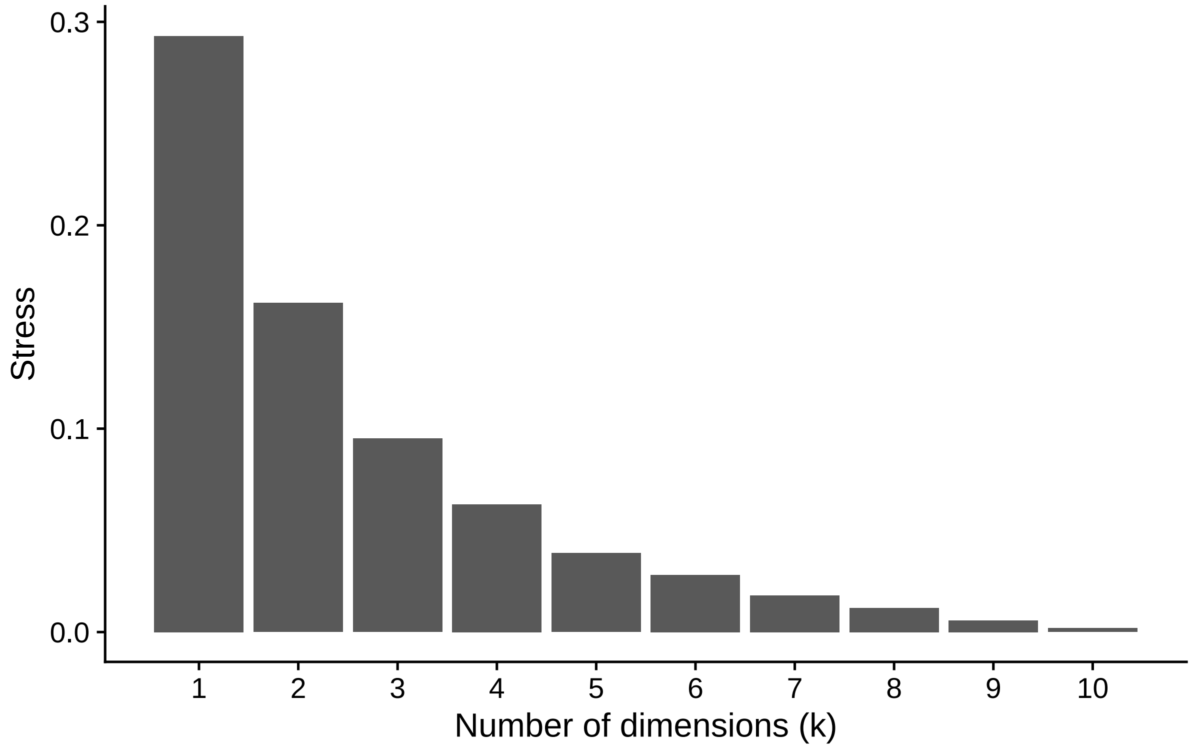


Figure D.1.: Plot of NMDS stress against the number of dimensions.

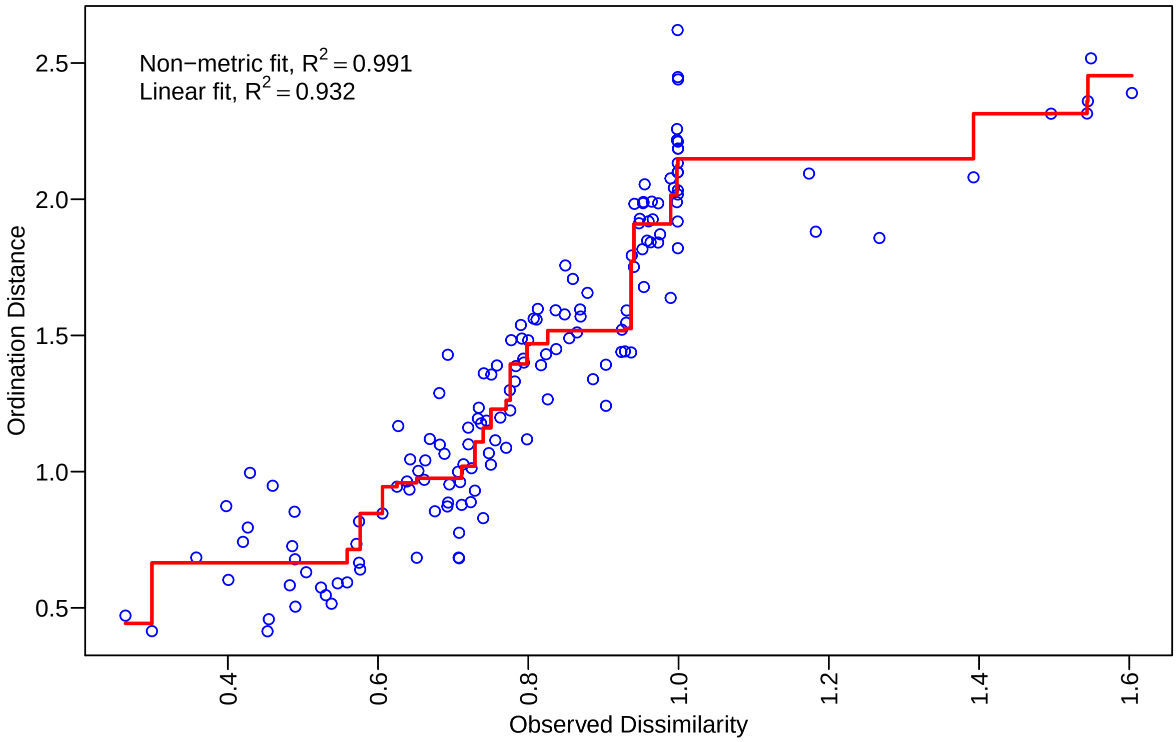


Figure D.2.: Shepard Diagram for ordination k = 4.

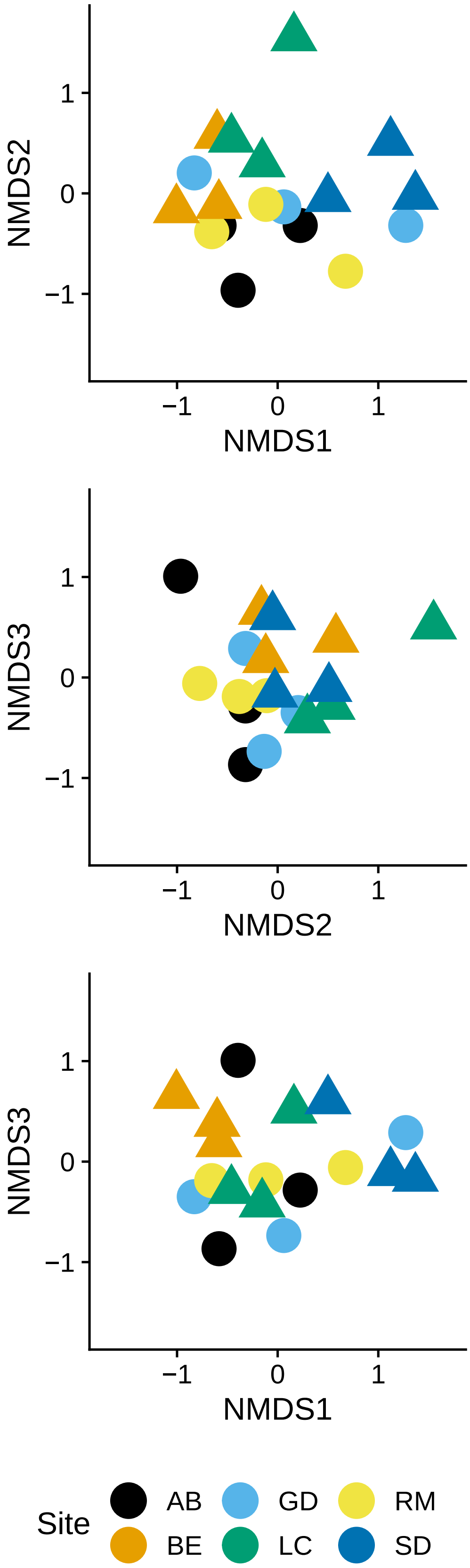


Figure D.3.: Plots of each NMDS axis from the final NMDS against each other.

E

Moran's I for individual species

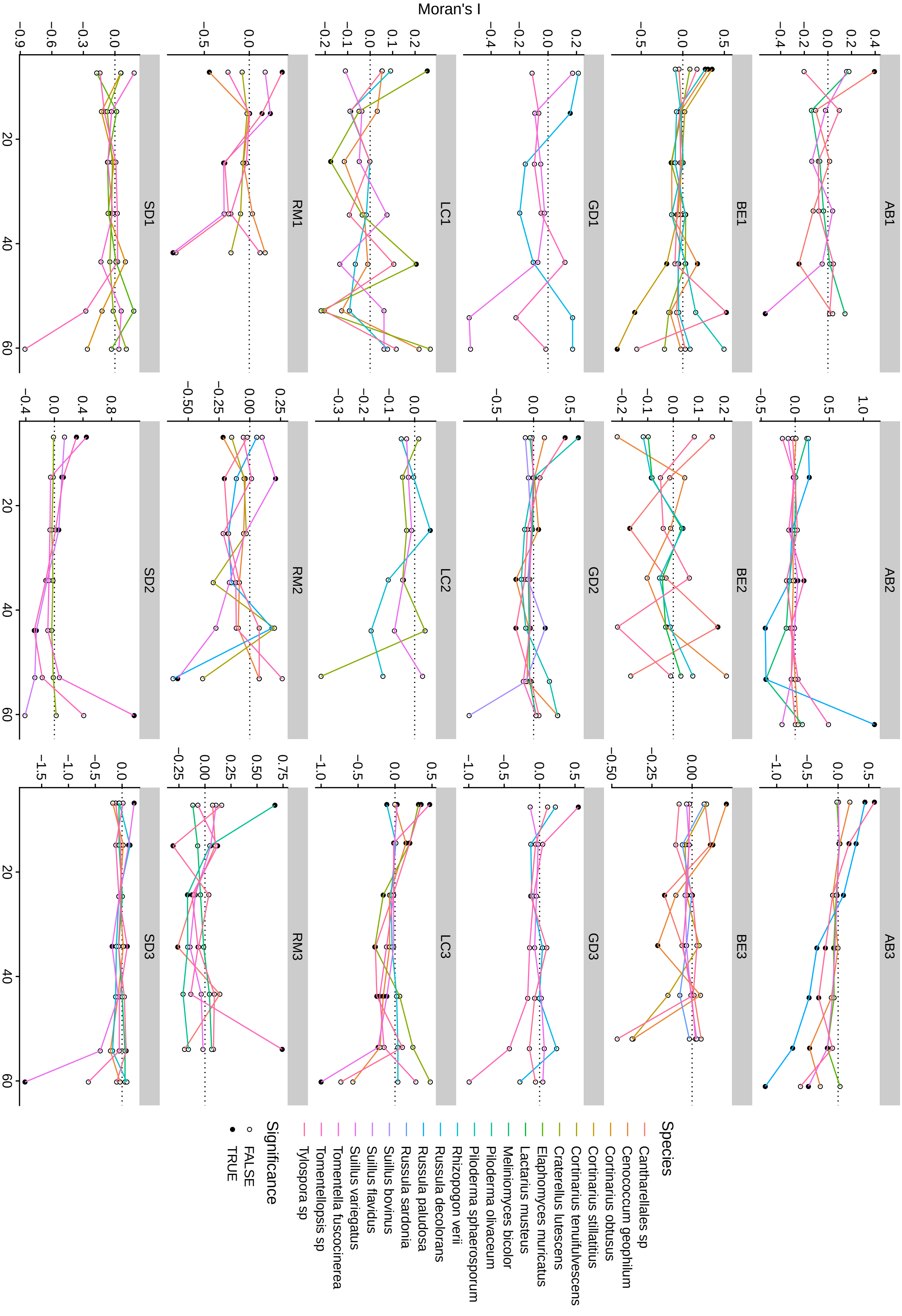


Figure E.1.: Moran's I correlograms for each fungal taxon in each grid. Colours indicate different fungal taxon. Black points = significant autocorrelation, white points = no autocorrelation.

F

Methods for re-analysis of Piculell et al. (2018) data

To re-analyse the data using the methods described in Chapter 3, I downloaded the mycorrhizal community data from the supplemental information in the paper (Piculell et al. 2018). I transformed the data into long format using the *melt* function in the R package *reshape2*, such that the dataframe was organised with each row giving the abundance of a single fungal species on an individual seedling (each seedling thus having four rows in the dataset).

I constructed a model using the structure outlined in Chapter 3 to repeat the analysis carried out in the paper. I constructed a model in *MCMCglmm* in R (Hadfield 2019) with a poisson error structure. The response variable was the number of root tips of each fungal species on each seedling, and I included the total number of root tips as an offset to model colonisation as a rate-related process. I estimated the amount of turnover in community composition between the variables of interest (Soil origin, genotype, and Soil origin:Genotype) by modelling their interaction with fungal species (OTU) (Eq. F.1). I also included OTU as a term by itself to control for variation in the overall abundance of different fungal species.

$$\begin{aligned}\text{Number of root tips} \sim\ & log(\text{Total root tips}) \ +\ random(\text{OTU}) \ + \\ & random(\text{Soil origin} : \text{OTU}) \ + random(\text{Genotype} : \text{OTU}) \ + \\ & random(\text{Soil origin} : \text{Genotype} : \text{OTU})\end{aligned} \tag{F.1}$$

Proportions of explained variance were calculated using the mode of the posterior distribution for each variance estimate. Overall, I found that soil origin explained 34% of the total variance in the model, while genotype explained no variance. The interaction between soil origin and genotype explained 2.1% of the total variance, but it is worth noting that while the posterior distribution was fairly normally distributed, the lower 95% credible interval was close to zero (0.03), so it cannot be discounted that there is in fact little to no GxE effect at all.