Universitat de Lleida

Soil fungal communities in Mediterranean forest soils and their relationships with soil parameters

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DOCTORAL THESIS

Soil fungal communities in Mediterranean forest soils and their relationships with soil parameters.

IRENE ADAMO

Memòria presentada per optar al grau de Doctor per la Universitat de Lleida
Programa de Doctorat en Gestió Forestal i del Medi Natural

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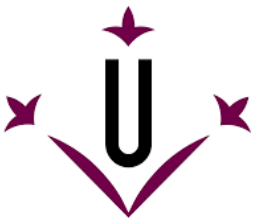

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RELATED WORKS AND MANUSCRIPTS

The following manuscripts are derived from this thesis:

1. Adamo I, Piñuela Y, Bonet JA, Castaño C, Martínez de Aragón J, Parladé J, Pera J & JG Alday (2021). Sampling forest soils to describe fungal diversity and composition. Which is the optimal sampling size in Mediterranean pure and mixed pine oak forest? Fungal Biology 125(6): 469-476. doi: https://doi.org/10.1016/ j.funbio.2021.01.005
2. Adamo I, Castaño C, Y, Bonet JA, Colinas C, Martínez de Aragón J, JG Alday (2021). Soil physicochemical properties have a greater effect on soil fungi tan host species in Mediterranean pure and mixed pine forests. Soil Biology and Biochemistry, 160: 108320. doi: https://doi.org/10.1016/j.soilbio.2021.108320.
3. Adamo I, Castaño C, Y, Bonet JA, Colinas C, Martínez de Aragón J, JG Alday (2021). Lack of phylogenetic differences in ectomycorrhizal fungi among distinct Mediterranean pine forest habitats. Journal of fungi 7: 793. doi: https://doi.org/10.3390/jof7100793.
4. Adamo I, Alday JG, Bonet JA, Clemmensen K, Coll LL, Martínez de Aragón J, Castaño C (2023). Distinct nutrient economy within ectomycorrhizal types relates to dominance by microbial groups with contrasting ecologies in a Mediterranean forest. Manuscript for review.

Contribution to other manuscripts:

1. Santana VM, Alday JG, Adamo I, Alloza JA, Baeza MJ (2020). Climate, and not fire, drives the phylogenetic clustering of species with hard-coated seeds in Mediterranean Basin communities. Perspectives in Plant Ecology, Evolution and Systematics 45: 125545. https://doi.org/10.1016/j.ppees.2020.125545.
2. Adamo I, Dashevskaya S & JG Alday (2022). Fungal perspective of pine and oak colonization in Mediterranean degraded ecosystems. Forests 13(1): 88. https://doi.org/10.3390/f13010088.

Congress proceedings, scientific posters, or educational manuscripts:

1. Adamo I, Morera Marra A, De Miguel S, Alday G. J. Random Forest modelling for fugal diversity analyses in Mediterranean forest ecosystems. V Reunión del Grupo de Trabajo de Modelización Forestal. Spain, November 2019.

2. Adamo I, Dasveshkaya S, Alday G. J. Fungal perspective of pine and oak colonization in Mediterranean mined areas. The 12th European Conference on Ecological Restoration. Online, September 2021.
3. Adamo I, Castaño C, Piñuela Y, Martínez de Aragón J, Bonet JA, Dashevskaya S, Alday JG. Changes in tree species affect litter colonizing saprophytic communities in Mediterranean pure pine, mixed pine-oak and pure oak forests. XV Congreso Nacional de la AEET. Spain, October 2021.
4. Adamo I, Castaño C, Bonet JA, Colinas C, Martínez de Aragón J, Alday JG. Soil physico-chemical properties effects on soil fungi in species in Mediterranean pure and mixed pine forests. Sibecol meeting 2022. Portugal 2022.
5. Análisis de redes para explorar los patrones de ensamblaje ectomicorrícico en pinares puros y mixtos mediterráneos. Congreso Forestal Español. Spain 2022.

ABSTRACT

Forests play a critical role in maintaining global biodiversity and ecosystem functioning. Within forest ecosystems, soil fungi serve as vital components, shaping nutrient cycling, plant growth, and overall ecosystem stability. Recent studies have shed light on the significance of fungal communities and their intricate relationships with various factors, such as soil physico-chemical properties, host species, and habitat types, within Mediterranean pine forests. However, still little is known about the impacts that a global climate change will have on soil fungal community dynamics and on nutrient cycling in drought-prone Mediterranean forests ecosystems. The main objective of this thesis was to describe the effect of forest structure and soil physic-chemical properties changes on soil fungal community and nutrient cycling dynamics. In addition, we identified minimum number of pooled samples needed to obtain a reliable description of fungal communities in terms of diversity and composition in three different Mediterranean forests.

This doctoral thesis was carried out in two experimental set-ups. The first of 5 triplets representing a mixed *Pinus sylvestris- Quercus. robur* stand, and two monospecific stands of each species, respectively located in in Pre-pyrenees. The second set up consisted of 42 forests dominated by either pure *Pinus. nigra, Pinus halepensis or Pinus sylvestris* or a *Pinus nigra– Pinus. halepensis or Pinus nigra– Pinus sylvestris* mixture located in pre-Pyrenees region of Catalonia in north-eastern Spain where set of long-term monitoring plots in which fungal fruit- ing has been recorded for ~20 years. Using molecular techniques such as high-throughput DNA sequencing (PacBio RS II, Illumina MiSeq), ergosterol extraction and real-time PCR (qPCR), we have described d fungal composition and biomass dynamics of these forest soils, and we assessed the effects of changes in forest structure and soil physio-chemical properties.

First, we observed that pooling three soil samples in *Pinus* and *Quercus* stands provided consistent richness estimations, while at least six samples were needed in mixed-stands. On the other hand, ß-diversity decreased with increasing sample pools in monospecific-stands, while there was no effect of sample pool size on mixed-stands and on species composition. Then, we observed that along a regional gradient in the Mediterranean Pre-Pyrenees fungal communities were primarily affected by soil properties, with pH, P and the C:N ratio the strongest predictors shaping fungal communities in these forest ecosystems. Importantly, mycorrhizal communities are significantly affected by P but not N, therefore an important nutrient trader in these

ecosystems. Similarly, ectomycorrhizal communities at phylogenetic were not different among pine tree hosts neither in phylogenetic. Moreover, we observed that pH was the only significant variable influencing phylogenetic ectomycorrhizal community, while phylogenetic structure was slightly influenced by the shared effect of stand structure, soil and geographic distance. Lastly, we detected changes in the nutrient economy among ectomycorrhizal types relates to shifts in microbial communities with contrasting ecologies. Therefore, replacement of conifers by broadleaf species may promote shifts towards inorganic N-cycling, while ectomycorrhizal fungi in conifers may retain N and slow C cycling.

Based on these results, it can be concluded that 1.- The sample pool size significantly influences the estimation of soil fungal diversity, and increasing the number of soil sample pools improves diversity predictions. 2.- Soil physicochemical properties have a greater influence on soil fungi than host species, and environmental filtering plays a significant role in shaping fungal communities. 3.- Phylogenetic composition, structure, and diversity of ectomycorrhizal fungi are affected by local abiotic conditions and soil pH act as important factors in shaping ectomycorrhizal phylogenetic communities. 4. -The replacement of conifers by broadleaf species can lead to shifts in microbial communities and nutrient cycling, promoting inorganic nitrogen cycling and potentially altering soil carbon and nitrogen fluxes.

RESUMEN

Los bosques juegan un papel fundamental en el mantenimiento de la biodiversidad mundial y el funcionamiento de los ecosistemas. Dentro de los ecosistemas forestales, los hongos del suelo sirven como componentes vitales, dando forma al ciclo de nutrientes, el crecimiento de las plantas y la estabilidad general del ecosistema. Estudios recientes han arrojado luz sobre la importancia de las comunidades fúngicas y sus intrincadas relaciones con diversos factores, como las propiedades físico-químicas del suelo, las especies huésped y los tipos de hábitat, dentro de los bosques de pino mediterráneo. Sin embargo, todavía se sabe poco sobre los impactos que tendrá un cambio climático global en la dinámica de la comunidad de hongos del suelo y en el ciclo de nutrientes en los ecosistemas de bosques mediterráneos propensos a la sequía. El objetivo principal de esta tesis fue describir el efecto de los cambios en la estructura del bosque y en las propiedades físico-químicas del suelo sobre la comunidad fúngica del suelo y la dinámica del ciclo de nutrientes. Además, identificamos el número mínimo de muestras agrupadas necesarias para obtener una descripción fiable de las comunidades fúngicas en términos de diversidad y composición en tres bosques mediterráneos diferentes.

Esta tesis doctoral se llevó a cabo en dos zonas experimentales. El primero constaba de 5 tripletes que representaban un bosque mixto de *Pinus. sylvestris*-*Quercus robur*, y dos bosques monoespecíficos de cada especie, ubicados respectivamente en el Prepirineo. La segunda zona constaba de constaba de 42 bosques dominados por *Pinus nigra* puro, *Pinus halepensis* o *Pinus sylvestris* o un bosque mixto de *Pinus nigra– Pinus halepensis* o *Pinus nigra– Pinus sylvestris* ubicados en la región prepirenaica de Cataluña, en el noreste de España, donde se ha registrado un conjunto de parcelas de seguimiento a largo plazo en las que se ha registrado fructificación fúngica durante ~20 años. Utilizando técnicas moleculares como secuenciación de ADN de alto rendimiento (PacBio RS II, Illumina MiSeq), extracción de ergosterol y PCR en tiempo real (qPCR), describimos la composición fúngica y la dinámica de la biomasa de estos suelos forestales, y evaluamos los efectos de cambios en la estructura del bosque y en las propiedades fisicoquímicas del suelo.

Primero, observamos que la combinación de tres muestras de suelo en rodales de Pinus y Quercus proporcionó estimaciones de riqueza consistentes, mientras que se necesitaban al menos seis muestras en rodales mixtos. Por otro lado, la diversidad ß disminuyó con el aumento de los grupos de muestras en rodales monoespecíficos, mientras que no hubo efecto del tamaño

del grupo de muestras en rodales mixtos y en la composición de especies. Luego, observamos que a lo largo de un gradiente regional en los Prepirineos mediterráneos, las comunidades fúngicas se vieron afectadas principalmente por las propiedades del suelo, siendo el pH, P y la relación C:N los predictores más fuertes que dan forma a las comunidades fúngicas en estos ecosistemas forestales. Es importante destacar que las comunidades de micorrizas se ven significativamente afectadas por P pero no por N, por lo tanto, un importante comerciante de nutrientes en estos ecosistemas. Del mismo modo, las comunidades ectomicorrízicas a nivel filogenético no fueron diferentes entre los pinos hospedantes. Además, observamos que el pH fue la única variable significativa que influyó en la comunidad ectomicorrícica filogenética, mientras que la estructura filogenética estuvo ligeramente influenciada por el efecto compartido de la estructura del bosque, el suelo y la distancia geográfica. Por último, detectamos cambios en la economía de nutrientes entre los tipos de ectomicorrícicos relacionados con cambios en las comunidades microbianas con ecologías contrastantes. Por lo tanto, el reemplazo de coníferas por especies de hoja ancha puede promover cambios hacia el ciclo del N inorgánico, mientras que los hongos ectomicorrícicos en las coníferas pueden retener el N y retardar el ciclo del C.

En base a estos resultados, se puede concluir que 1.- El tamaño del pool de muestras influye significativamente en la estimación de la diversidad fúngica del suelo, y aumentar el número de pools de muestras de suelo mejora las predicciones de diversidad. 2.- Las propiedades fisicoquímicas del suelo tienen una mayor influencia sobre los hongos del suelo que sobre las especies hospederas, y la filtración ambiental juega un papel importante en la formación de comunidades fúngicas. 3.- La composición filogenética, la estructura y la diversidad de los hongos ectomicorrícicos se ven afectadas por las condiciones abióticas locales y el pH del suelo actúa como un factor importante en la conformación de las comunidades filogenéticas de ectomicorrízicos. 4. -La sustitución de coníferas por especies de hoja ancha puede provocar cambios en las comunidades microbianas y el ciclo de nutrientes, promoviendo el ciclo del nitrógeno inorgánico y alterando potencialmente los flujos de carbono y nitrógeno del suelo.

RESUMEN CATALAN

Els boscos tenen un paper fonamental en el manteniment de la biodiversitat mundial i el funcionament dels ecosistemes. Dins dels ecosistemes forestals, els fongs del sòl serveixen com a components vitals, donant forma al cicle de nutrients, el creixement de les plantes i l'estabilitat general de l'ecosistema. Estudis recents han donat llum sobre la importància de les comunitats fúngiques i les seves intricades relacions amb diversos factors, com les propietats fisicoquímiques del sòl, les espècies hoste i els tipus d'hàbitat, dins dels boscos de pi mediterrani. Tot i això, encara se sap poc sobre els impactes que tindrà un canvi climàtic global en la dinàmica de la comunitat de fongs del sòl i en el cicle de nutrients en els ecosistemes de boscos mediterranis propensos a la sequera. L'objectiu principal d'aquesta tesi va ser descriure l'efecte dels canvis a l'estructura del bosc ia les propietats fisicoquímiques del sòl sobre la comunitat fúngica del sòl i la dinàmica del cicle de nutrients. A més, identifiquem el nombre mínim de mostres agrupades necessàries per obtenir una descripció fiable de les comunitats fúngiques en termes de diversitat i composició a tres boscos mediterranis diferents.

Aquesta tesi doctoral es va dur a terme a dues zones experimentals. El primer constaba 5 triplets que representaven un bosc mixt de *Pinus sylvestris-Quercus robur*, i dos boscos monoespecífics de cada espècie, ubicats respectivament al Prepirineu. La segona zona constava de constava de 42 boscos dominats per *Pinus nigra pur, Pinus halepensis o Pinus sylvestris* o un bosc mixt de *Pinus nigra– Pinus halepensis o Pinus nigra–Pinus sylvestris* ubicats a la regió prepirinenca de Catalunya, al nord-est d'Espanya, on s'ha registrat un conjunt de parcel·les de seguiment a llarg termini on s'ha registrat fructificació fúngica durant ~20 anys. Utilitzant tècniques moleculars com a seqüenciació d'ADN d'alt rendiment (PacBio RS II, Illumina MiSeq), extracció d'ergosterol i PCR en temps real (qPCR), descrivim la composició fúngica i la dinàmica de la biomassa d'aquests sòls forestals i n'avaluem els efectes de canvis a l'estructura del bosc ia les propietats fisicoquímiques del sòl.

Primer, observem que la combinació de tres mostres de sòl a rodals de Pinus i Quercus va proporcionar estimacions de riquesa consistents, mentre que es necessitaven almenys sis mostres en rodals mixtes. D'altra banda, la diversitat ß va disminuir amb l'augment dels grups de mostres a rodals monoespecífics, mentre que no hi va haver efecte de la mida del grup de mostres a rodals mixtes i en la composició d'espècies. Després, observem que al llarg d'un gradient regional als Prepirineus mediterranis, les comunitats fúngiques es van veure afectades

principalment per les propietats del sòl, sent el pH, P i la relació C:N els predictors més forts que donen forma a les comunitats fúngiques en aquests ecosistemes forestals. És important destacar que les comunitats de micorizes es veuen significativament afectades per P però no per N, per tant, un important comerciant de nutrients en aquests ecosistemes. De la mateixa manera, les comunitats ectomicorríziques a la filogenètica no van ser diferents entre els pins hospedants ni en la filogenètica. A més, observem que el pH va ser l'única variable significativa que va influir a la comunitat ectomicorrícica filogenètica, mentre que l'estructura filogenètica va estar lleugerament influenciada per l'efecte compartit de l'estructura del rodal, el terra i la distància geogràfica. Finalment, detectem canvis en l'economia de nutrients entre els tipus d'ectomicorrícics relacionats amb canvis a les comunitats microbianes amb ecologies contrastants. Per tant, el reemplaçament de coníferes per espècies de fulla ampla pot promoure canvis cap al cicle del N inorgànic, mentre que els fongs ectomicorrícics a les coníferes poden retenir el N i retardar el cicle del C.

En base a aquests resultats, es pot concloure que 1.- La mida del pool de mostres influeix significativament en l'estimació de la diversitat fúngica del sòl i augmentar el nombre de pools de mostres de sòl millora les prediccions de diversitat. 2.- Les propietats fisicoquímiques del sòl tenen més influència sobre els fongs del sòl que sobre les espècies hostaleres, i la filtració ambiental juga un paper important en la formació de comunitats fúngiques. 3.- La composició filogenètica, l'estructura i la diversitat dels fongs ectomicorrícics es veuen afectades per les condicions abiòtiques locals i el pH del sòl actua com un factor important en la conformació de les comunitats filogenètiques d'ectomicorrízics. 4. -La substitució de coníferes per espècies de fulla ampla pot provocar canvis a les comunitats microbianes i el cicle de nutrients, promovent el cicle del nitrogen.

INTRODUCTION

Fungi inhabiting soils are fundamental drivers of the main forest ecosystems processes (Bardgett and van der Putten, 2014), such as organic matter decomposition, soil nutrient release and plant nutrient uptake (Bardgett and Wardle, 2010). Through multiple trophic guilds, fungi mediate carbon and nitrogen dynamics (Baldrian 2016). For instance, mycorrhizal fungi are important regulators of soil organic matter dynamics by accessing to organically bound nitrogen (Read and Perez-Moreno, 2003; Smith & Read, 2008). On the other hand, saprotrophic fungi acquire carbon by degrading the soil organic material, which precipitates CO_2 release (Boddy et al., 2007). Moreover, mycorrhizal fungal mycelium contributes importantly to microbial biomass in forest soils (Hogberg and Hogberg 2002; Joergensen and Wichern, 2008, Ekblad 2013) and contribute to C and N retention through their mycelial biomass (Clemmensen et al., 2013; Ekblad et al., 2013; Näsholm et al., 2013). However, we still lack comprehensive studies describing different facets of soil fungi especially in less-studied drought-prone ecosystems such as Mediterranean forests: i) what is the most efficient soil sampling strategy? ii) how the soil fungal communities assembly (e.g. compositionally and phylogenetically), and iii) how they respond to niche processes, such as environmental filtering (e.g., soil parameters, host and forest stand drivers), and neutral processes, such as distance decay similarity (e.g., geographical distance, Bahram et al., 2013).

The characterization of fungal communities has become crucial to disentangle forest soil microbial community dynamics and related ecological processes (Lindahl et al., 2013). High-throughput sequencing (HTS) methods have become a powerful tool to quantify fungal diversity in soils and have provided new information regarding the ecology of fungi in forests ecosystems (Hibbett et al. 2009; Lindahl et al., 2013; Hibbett et al., 2016; Nilsson et al., 2016). Previous studies provided laboratory protocols (Clemmensen et al., 2016; De Filippis et al., 2017; Dopheide et al., 2019) or guidance on the multiple bioinformatic and taxonomic identification pipelines to prepare and assess high-throughput sequencing data (Gweon et al., 2015; Nguyen et al., 2016, Rognes et al., 2016; Somervuo et al. 2016; Bjørnsgaard et al., 2017; Anslan et al., 2017; Pauvert et al., 2019). Despite few attempts to optimize soil sampling protocols in high-throughput sequencing studies (Dickie et al., 2018), we still lack optimal soil sampling protocols to study fungal diversity and composition in Mediterranean soils. In addition, it is crucial to understand how these communities are structured in Mediterranean forests. Therefore, assessing the optimal sample pooling size in Mediterranean ecosystems is

fundamental since it could affect the observed diversity and community composition, which can potentially be detrimental to understand nutrient cycling and resistance against drought in these ecosystems (Mohan et al., 2014).

Soil fungal communities are highly influenced by differences in soil physio-chemical properties and vegetation features such as host type or stand structure (i.e., niche processes; Větrovský et al., 2019). However, previous studies have found that geographical distance (i.e., neutral processes; Green and Bohannan, 2006) can have a primary role in shaping fungal community structure (Peay et al., 2012; Bahram et al., 2013; Peay and Bruns, 2014). Nevertheless, both niche and neutral processes have been described as extremes of a continuum, whereas biological communities are usually located somewhere between these two theoretical extremes (Gravel et al., 2006), raising the need to determine the relative importance of each process on soil fungal community assembly in different ecosystems (Cao et al., 2019). For example, we still lack comprehensive studies on soil fungal community assembly dynamics that simultaneously describe the relative importance of niche processes, such as environmental filtering (e.g., soil parameters and forest stand drivers), and neutral processes, such as distance decay similarity (e.g., geographical distance, Bahram et al., 2013), especially in less-studied drought-prone ecosystems such as Mediterranean forests.

It is well known that pH has a strong role in regulating fungal communities worldwide (Tedersoo et al., 2014; Goldmann et al., 2015; Zhang et al., 2016; Glassman et al., 2017; Tedersoo et al., 2020), and often affects nutrient cycling (Adamczyk et al., 2016), determining the availability of soil nutrients such as nitrogen, phosphorus and potassium (Awad et al., 2019; Guo et al., 2020). Nitrogen is often the main limiting nutrient in soils, especially in colder terrestrial ecosystems where decomposition is limited (Read and Perez-Moreno, 2003; Kyaschenko et al., 2017). However, the primary productivity of Mediterranean terrestrial plants is generally limited by P and not N (Du et al., 2020). This suggests that P could be a more important nutrient trader than N in these ecosystems in fungal–tree interactions. Thus, given that soil conditions and water limitation during summer months are factors that determine plant communities (Thullier et al., 2008), soil parameters may shape fungal communities in Mediterranean soils differently than in other forest ecosystems. However, this issue still needs to be explored.

Mixed forests are of interest because they are more adaptable to climate change or disturbances than monocultures (Bravo-Oviedo et al., 2014). The coexistence of tree species may be supported by complementary niches for tree growth and nutrient uptake, increasing forest resistance to disturbances (Bello et al., 2019). Thus, due to these complementary niches,

mixed forests are expected to harbour higher levels of taxonomical richness in ecosystem niches (Ishida et al., 2007; Cavard et al., 2011). In this regard, previous studies comparing soil fungal communities under distinct tree hosts in boreal and temperate ecosystems have reported higher levels of soil fungal richness in mixed stands than in pure stands (Ishida et al., 2007; Nagati et al., 2018). Although some mycorrhizal fungi are known to have relatively broad host ranges and, therefore, are rarely specific to a tree host genus (Molina et al., 1992), clear differences in soil fungal communities between pure and mixed forests have been found in studies comparing host trees with contrasting traits (i.e., deciduous vs conifers) (Ishida et al., 2007). However, it is still unclear whether these differences also occur when host trees have similar traits or belong to the same genus (i.e., *Pinus*).

Simultaneously, there is a lack of knowledge on how ectomycorrhizal fungi are phylogenetically structured among Mediterranean pine host species and whether they respond to similar abiotic factors at both taxonomic and phylogenetic levels. Previous studies showed that ectomycorrhizal responses to climate warming are modulated by host plant performance and nutrient availability (Mohan et al., 2014; Fernandez et al., 2016). Therefore, it is crucial to disentangle whether these drivers influence ectomycorrhizal phylogenetic composition and structure, to better understand forest ecosystem functioning (Sardans & Peñuelas, 2013). It is well known that phylogenetic analyses are useful tools to estimate the relative importance of evolutionary and ecological forces structuring communities (Webb, 2002; Maherali et al., 2007, Peay & Bruns, 2010). In this regard, phylogenetic indices have been implemented to calculate phylogenetic relatedness of an observed community and compare the value to expectations of community assembly under neutral processes from a regional species pool (Maherali et al., 2014). Therefore, these indices enable us to characterize whether communities are more phylogenetically related (phylogenetic clustering) or less phylogenetically related (phylogenetic over dispersion) than expected by chance (Webb, 2000; Webb, 2002). In general, habitat filtering is the dominant assembly process when closely related species that share similar traits are selected to co-exist within the community (i.e., phylogenetic clustering). In contrast, competition processes occur when distantly related species with dissimilar traits are selected to co-occur within a community (i.e., phylogenetic over dispersion, (Webb, 2002), while random phylogenetic structure is detected when none of the above processes are inferred (Vamosi et al., 2009; Webb, 2002). Although the ecological processes filtering communities have recently received criticism (Kraft et al., 2015), investigating the communities' phylogenetic responses to environment in different ecosystems is fundamental to understand the mechanisms that structure communities (Emerson & Gillespie, 2008; Tucker et al., 2017).

However, how ectomycorrhizal communities are phylogenetic structured in Mediterranean pine forests has not been studied yet.

In forests, fungal communities with contrasting ecologies are often compartmentalized along a soil depth gradient, where free-living saprotrophs dominate the litter layer and ECM dominate more decomposed layers (Lindahl et al., 2015; Clemmensen 2021; Voriskova et al., 2013). However, fungal communities with contrasting ecologies, particularly ectomycorrhizal fungi and fungal saprotrophs, can also coexist and compete for soil N (Fernandez et al. 2016). Outcomes of these competitive interactions can alter C and N cycling, with dominance of ectomycorrhizal fungi over fungal saprotrophs resulting in the suppression of organic matter decomposition (Fernandez et al., 2014; 2016; 2018; Sterkenburg et al., 2018; Van der Wal et al., 2013). In addition, mycorrhizal-associated nutrient economy (MANE) framework predict that dominance by microorganisms with contrasting ecologies affect the mode of N cycling between mycorrhizal types (Phillips et al. 2013). Particularly, dominance by fungal saprotrophs in AM types and high plant litter qualities promote fast organic matter decomposition which can fuel N mineralization and nitrifiers, supporting inorganic-N economy. By contrast, in ECM types, lower litter qualities and dominance by ECM fungi promote organic N-cycling (Phillips et al., 2013; Lin et al., 2017), potentially slowing soil C cycling (Averill et al. 2014; Averill and Hawkes 2016). However, microbial communities and soil fungal dynamics and abundances also vary within tree species of the same mycorrhizal type (Fernandez et al. 2018; Hagenbo et al. 2020). Similarly, litter qualities can also differ among species of the same mycorrhizal type, particularly between conifers and angiosperms (Hobbie, 2010). This leads to hypothesize that mode of N cycling and microorganisms between tree species belonging to the same mycorrhizal type could also differ. Although it is known that ectomycorrhizal communities differ between coniferous and broadleaf forests (Ishida et al., 2007, Tedersoo et al., 2013), the implications for N and C cycling and the relationship with distinct microbial groups still needs to be explored.

Changes in land use and warmer temperatures are predicted to induce shifts in vegetation types worldwide. Particularly in the Mediterranean basin, a shift from conifers to broadleaf species have been predicted because of abandonment of forest management activities. For example, drought and higher temperatures are expected to cause latitudinal shifts in *Pinus sylvestris*, which could potentially be replaced by broadleaf species (e. g. *Quercus spp*.). Although belowground consequences of shifts from ECM to AM types have been explored (Steidinger et al. 2019; Soudzilovskaia et al. 2019), similar assessments are needed to predict microbial-driven shifts in belowground processes between different tree species.

OBJECTIVES

The overall objective of the thesis is to advance the understanding of soil fungal communities dynamics in Mediterranean forest ecosystems and ecosystem processes, describing their intricate relationships with various factors, such as soil physicochemical properties, host species, and habitat types (forest structure). Within this general scope, the four main objectives are:

1. To identify the minimum number of pooled samples needed to detect a non-biased soil fungal diversity and compositional values, which are fundamental to answer ecological questions in Mediterranean forest ecosystems. It must be considered that for soil DNA quantification there are no studies about species-volume relationships in Mediterranean forest.

2. To characterize the soil fungal community composition and diversity in pure and mixed pine forest, as well as, to identify the main soil type, spatial and forest structural factors that accounted for the highest proportion of fungal community variation between different pine forest types. Here, we also investigated to what extent niche processes (i.e., soil physico-chemical parameters and forest structural factors) vs a neutral process (i.e., distance decay similarity measured as spatial distance) shape the overall, mycorrhizal and saprotrophic soil community assemblages in different pine forests.

3. To characterize the ectomycorrhizal phylogenetic composition and phylogenetic structure in pure and mixed Mediterranean pine forests. Here, it should consider that *P. halepensis*, *P. nigra*, and *P. sylvestris* are phylogenetically closely related, therefore, ectomycorrhizal phylogenetic composition, structure, and diversity will not be different among them due to co-evolutionary processes. Here, it is also hypothesized that soil physio-chemistry will act as the main habitat filter on ectomycorrhizal phylogenetic composition (i.e. pH, P, N and CN).

4. To assess differences in C and N cycling among the three vegetation types (i.e., pure *Q. robur* and *P. sylvestris* forest and a mixed forest with *Q. robur* and *P. sylvestris*), and test whether differences are linked to microbial communities with contrasting ecologies. Here, a higher potential organic N-mining mode in *P. sylvestris* would correspond to an increase in ECM fungi with high foraging strategies. By contrast, in *Q. robur* we expect shifts toward open, inorganic N-cycling, paralleling lower abundances of ECM fungi or higher dominance by ECM fungi with lower foraging strategies.

Briefing of the methodology applied and thesis structure.

A brief explanation of the methodology applied in each of the four chapters will be described:

Chapter I.

Sampling forest soils to describe fungal diversity and composition. Which is the optimal sampling size in Mediterranean pure and mixed pine oak forests?

Current literature on methodological soil fungal community sampling and optimization of soil sampling protocols in high-throughput sequencing studies still lack optimal soil sampling protocols to study fungal diversity and composition in Mediterranean soils. Thus, out first objective was to identify the minimum number of pooled samples needed to reach diversity plateau, i.e., optimal sample pooling size, for a set of distinct forest types in Mediterranean area. This might help us to detect reliable diversity and compositional values for a given area to answer subsequent ecological questions in forest ecosystems using appropriate sampling effort (Chapter I).

This study employed soil sampling, molecular techniques, statistical analysis, and diversity metrics to investigate the optimal sampling size and characterize fungal diversity and composition in Mediterranean pure and mixed pine oak forests. Primarily, Mediterranean pure and mixed pine oak forests were selected as study sites to represent different ecological conditions and habitat types. Within the study sites, soil samples were collected from multiple locations, ensuring spatial representation across the forest ecosystems. Soil cores were systematically collected from various depths to capture vertical distribution. Molecular techniques such as DNA extraction, PCR amplification, and high-throughput sequencing were used to analyse fungal diversity and composition. These techniques allowed the identification and characterization of fungal taxa in the soil samples. Afterwards, bioinformatics tools were used to process and analyse the DNA sequences obtained. Operational taxonomic units (OTUs) were generated based on sequence similarity, and alpha and beta diversity indices were calculated. Then, the different sampling sizes were compared to determine the minimum number of soil samples required to accurately capture the majority of fungal taxa present using rarefaction analysis and non-parametric estimators. The results obtained from the data analyses were interpreted in relation to the optimal sampling size required to accurately describe fungal diversity and composition in Mediterranean pine oak forests.

Chapter II.

Soil physico-chemical properties have a greater effect on soil fungi than host species in Mediterranean pure and mixed pine forests.

There is still a lack of comprehensive studies describing simultaneously the relative importance of niche processes, such as environmental filtering (e.g., soil parameters and forest stand drivers), and neutral processes, such as distance decay similarity (i.e., geographical distance), in driving soil fungal community assemblages, especially in less-studied drought-prone ecosystems such as Mediterranean forests. Therefore, our second aim was to assess the main soil type and spatial and forest structural factors that accounted for the highest proportion of fungal community variation in five different Mediterranean pine forests: i.e., pure forests dominated by *P. nigra*, *P. halepensis*, and *P. sylvestris*, and mixed forests of *P. nigra*-*P. halepensis* and *P. nigra*-*P. sylvestris* (Chapter II).

This study employed soil sampling, physicochemical analyses, host species assessment, molecular techniques, statistical analysis, and diversity metrics to investigate the influence of soil physicochemical properties and host species on soil fungi in Mediterranean pure and mixed pine forests. Primarily, Mediterranean pure and mixed pine forests, representing different ecological conditions and habitat types were selected. Within the selected forest, soil samples were collected from multiple locations to capture spatial variation. The sampling strategy involved collecting soil cores from various depths to assess the vertical distribution of soil fungi. Soil physicochemical properties, including pH, organic matter content, nutrient availability, and moisture levels, were measured using standard laboratory techniques. These properties serve as indicators of the soil's physical and chemical characteristics. The identity and characteristics of host tree species present in the study sites were documented. This information allowed the evaluation of potential host species effects on soil fungi. Molecular techniques such as DNA extraction, PCR amplification, and high-throughput sequencing were used to analyse fungal diversity and composition. Multivariate and regression analyses were employed to assess the relationship between soil physicochemical properties, host species, and fungal community composition. These analyses aimed to determine the relative importance of soil properties compared to host species in shaping the soil fungal community. The results obtained from the data analysis were interpreted to determine the relative influence of soil physicochemical properties and host species on soil fungi in Mediterranean pine forests.

Chapter III.

Lack of Phylogenetic Differences in Ectomycorrhizal Fungi among Distinct Mediterranean Pine Forest Habitats

It is known that ectomycorrhizal responses to climate warming are modulated by host plant performance and nutrient availability and studies on community assembly mainly focused on ectomycorrhizal taxonomic community dynamics. Therefore, investigating the phylogenetic community responses to the environment in different ecosystems is fundamental to understand the mechanisms that structure communities. Thus, our aim was to characterize the ectomycorrhizal phylogenetic composition and phylogenetic structure in five different Mediterranean pine forests: i.e., pure forests dominated by *P. nigra*, *P. halepensis*, and *P. sylvestris*, and mixed forests of *P. nigra*-*P. halepensis* and *P. nigra*-*P. sylvestris* (Chapter III). This study used soil sampling, DNA extraction, PCR amplification, high-throughput sequencing, bioinformatic tools, phylogenetic analysis, and statistical analysis to investigate the phylogenetic differences among ectomycorrhizal fungi in distinct Mediterranean pine forest habitats. Primarily, Mediterranean pure and mixed pine forests, representing different ecological conditions and habitat types were selected. Within the selected forest, soil samples were collected from multiple locations to capture spatial variation. Afterwards, DNA extraction techniques were employed to isolate DNA from the collected root samples. PCR amplification was then performed using specific fungal DNA markers to target the fungal community. The amplified fungal DNA samples were subjected to high-throughput sequencing, allowing for the generation of large amounts of sequencing data. The obtained DNA sequences were analyzed using bioinformatics tools and compared with existing reference databases to assign taxonomic identities to the fungi. Phylogenetic analyses, such as constructing phylogenetic trees, were performed to examine the relatedness and differences among the identified fungal taxa. Statistical methods, including multivariate analysis and non-parametric tests, were employed to assess the phylogenetic differences among the ectomycorrhizal fungal communities in different Mediterranean pine forest habitats. The results obtained from the data analysis were interpreted to determine whether there were significant phylogenetic differences among the ectomycorrhizal fungi in the studied habitats.

Chapter IV.

Distinct nutrient economy within ectomycorrhizal types relates to dominance by microbial groups with contrasting ecologies in a Mediterranean forest.

Lastly, changes in land use and warmer temperatures are predicted to induce shifts in vegetation types worldwide, particularly in the Mediterranean basin. Therefore, belowground consequences are expected and shifts from ECM to AM types have been explored but not in Mediterranean forest ecosystems. Thus, our aim was to assess differences in C and N cycling among the three vegetation types and assess whether differences are linked with microbial communities with contrasting ecologies; pure forests dominated by *P. sylvestris*, and *Quercus robur*, and mixed forests of *P. sylvestris*-*Q. robur* (Chapter IV).

This study used soil sampling, DNA extraction, PCR amplification, high-throughput sequencing, bioinformatic tools, soil physicochemical analyses and statistical analyses, to investigate the differences in C and N cycling among the three distinct forest habitats. Primarily, a *P. sylvestris* and *Q. robur* pure and mixed forest between both species were selected. Within the selected forest, soil samples were collected from multiple locations to capture spatial variation. Afterwards, DNA extraction techniques were employed to isolate DNA from the collected root samples. PCR amplification was then performed using specific fungal DNA markers to target the fungal community. Soil physicochemical properties, including pH, P, C and N, were measured using standard laboratory techniques. Molecular techniques such as DNA extraction, PCR amplification, and high-throughput sequencing were used to analyse fungal diversity and composition. Multivariate and regression analyses were employed to assess the relationship between soil physicochemical properties, and fungal community composition and forest habitats. These analyses aimed to determine how soil fungal communities influence soil nutrient processes differently depending on host species.

CHAPTER I

"Sampling forest soils to describe fungal diversity and composition. Which is the optimal sampling size in Mediterranean pure and mixed pine oak forest?"

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Sampling forest soils to describe fungal diversity and composition. Which is the optimal sampling size in Mediterranean pure and mixed pine oak forests?

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Abstract

Soil sampling is a critical step affecting perceived fungal diversity, however sampling optimization for high-throughput-DNA sequencing studies have never been tested in Mediterranean forest ecosystems. We identified the minimum number of pooled samples needed to obtain a reliable description of fungal communities in terms of diversity and composition in three different Mediterranean forests (pine, oak, and mixed-pine-oak). Twenty soil samples were randomly selected in each of the three plots per type. Samples were pooled to obtain mixtures of 3, 6, 10, 15, 20 samples, and sequenced using Illumina MiSeq of fungal ITS2 amplicons. Pooling three soil samples in *Pinus* and *Quercus* stands provided consistent richness estimations, while at least six samples were needed in mixed-stands. ß-diversity decreased with increasing sample pools in monospecific-stands, while there was no effect of sample pool size on mixed-stands. Soil sample pooling had no effect over species composition. We estimate that three samples would be already optimal to describe fungal richness and composition in Mediterranean pure stands, while at least six samples would be needed in mixed stands.

Keywords: Fungal communities, DNA metabarcoding, number of pooled samples, mixed forests, Mediterranean forest, beta-diversity

1. Introduction

Soil fungi are drivers of fundamental ecosystems processes (Bardgett and van der Putten, 2014) such as soil carbon cycling and mineral nutrition of plants (Smith and Read, 2008; Bardgett and Wardle, 2010). Due to the enormous diversity of fungi and their fundamental roles as decomposers, mutualists, or pathogens of plants and animals (Mueller and Schmit, 2007; Tedersoo et al., 2014), the characterization of fungal communities has become crucial to disentangle soil microbial community dynamics and related ecological processes (Lindahl et al., 2013). High-throughput sequencing (HTS) methods have become a powerful tool to quantify fungal diversity in soils and have provided new information regarding the ecology of fungi in forests ecosystems (Hibbett et al. 2009; Lindahl et al., 2013; Hibbett et al., 2016; Nilsson et al., 2016). Previous studies have provided laboratory protocols (Clemmensen et al., 2016; De Filippis et al., 2017; Dopheide et al., 2019) or guidance on the multiple bioinformatic and taxonomic identification pipelines to prepare and assess high-throughput sequencing data (Gweon et al., 2015; Nguyen et al., 2016, Rognes et al., 2016; Somervuo et al. 2016; Bjørnsgaard et al., 2017; Anslan et al., 2017; Pauvert et al., 2019). Despite few attempts to optimize soil sampling protocols in high-throughput sequencing studies (Dickie et al., 2018), we still lack optimal soil sampling protocols to study fungal diversity and composition in Mediterranean soils. In addition, it is crucial to understand how these communities are structured in Mediterranean forests because of their potential important role in tree resistance against drought (See Mohan et al., 2014). For instance, Castaño et al. (2018) studied seasonal dynamics of these communities and how they respond to changing moisture and temperature, however lacked optimal sampling scheme to properly capture soil fungal diversity. Therefore, assessing the optimal sample pooling size in Mediterranean ecosystems is fundamental since it could affect the observed diversity and community composition, which can potentially be detrimental to understand nutrient cycling and resistance against drought in these ecosystems (Mohan et al., 2014).

Most of the methodological studies have been performed in boreal and temperate ecosystems, but soil fungal communities in Mediterranean forest ecosystems seem to differ compared to boreal or temperate ecosystems (Castaño et al., 2018; Pérez-Izsquierdo et al., 2019). For instance, Mediterranean communities described in Castaño et al., (2018) were highly dominated by ectomycorrhizal species, and these were mainly species with short/contact exploration types (i.e. *Inocybe spp*; Castaño et al., 2018), which contrasts with many other boreal ecosystems, where medium-fringe or long exploration types may be more dominant

(Sterkenburg et al., 2015). Since differences in exploration types determine how fungi explore spatially the soil (Agerer, 2001), distinct sampling approaches may be used depending on the dominating community. Therefore, sampling effort may be distinct for each community or habitat type, since fungal community members can have distinct growth, morphologies and trophic strategies, and mycelia can grow from few cm. to up to several meters (Agerer, 2001; Smith et al., 1992).

Correct assessment of soil fungal diversity or community composition using HTS methods requires an efficient soil sampling strategy, due to the species soil-area relationships and the complexity of the soil matrix (Grundmann and Debouzie, 2000; Ranjard et al., 2003). For instance, the heterogeneous distribution of fungi in the soil matrix has been recently highlighted (Ranjard et al. 2003), with fungal communities often distributed in forest soils in a patchy manner (Cairney, 2005). Fungal communities also operate in a distinct scale than other microbes such as bacteria, with a single genet often occupying distances between <1 m and >5 m. (Dunham et al., 2003; Murata et al., 2005), up to 20 m, (Bonello et al., 1998; Sawyer et al., 1999). In addition, the amount of soil used to profile these communities employing molecular methods is typically limited to few grams or even < 1 g. Therefore, subsampling large amounts of soil to few grams is a common practice in fungal ecology studies dealing with soils (Kang and Mills, 2006). Moreover, the patchy distribution of fungi require that several samples are taken in a given site/plot, which are then often typically pooled before DNA analyses (Kang and Mills, 2006) or after DNA extraction (Dickie et al., 2018). If distinct soil samples are taken in a given area, it is crucial that samples are freeze-dried and grind to fine powder to facilitate homogenization (Lindahl et al., 2013). However, how the different number of pools (i.e. sampled volumes) and the number of samples taken in a given area may affect soil fungal diversity and community composition in samples with distinct ecological traits inhabiting distinct host species has not been tested yet in Mediterranean forest ecosystems.

It is well known that the observed number of plant and animal species increases with sampling area and volume (Arrhenius, 1921; McArthur, 1965; MacArthur and Wilson, 1968). For instance, Duarte et al. (2017), assessed the diversity of aquatic fungi across graded size of alder leaves and found that alpha diversity was positively influenced by increasing leaf area. Likewise, for microbes, Song et al. (2015) detected an increase in fungal OTU richness with increasing soil sample size from 0.25 g to 10 g in both prairie and forest soils. Therefore, increasing the number of soil sample pools may lead to a positive species/area relationship, and insufficient sampling may result in incorrect diversity estimations (Grey et al., 2018). The

optimization of sample pooling size is a fundamental aspect for ecological studies as it may strongly affect results and their interpretations (Dickie et al., 2018). For example, insufficient number of samples may lead to higher stochasticity in sampled communities, increasing sampling error and unexplained variation, which should be reflected in beta diversity values. Therefore, it is important to explore whether it is possible to establish a minimum optimal sampling size to reduce stochasticity and infer diversity estimates.

In this study, we aim to identify the minimum number of pooled samples needed to reach diversity plateau, i.e. optimal sample pooling size, for a set of distinct forest types in Mediterranean area. This might help us to detect reliable diversity and compositional values for a given area in order to answer subsequent ecological questions in forest ecosystems using appropriate sampling effort. It is well known that fungal diversity and community structure in forests is influenced by dominant tree species (Urbanová et al., 2015; Nagati et al., 2018; Geml, 2019). Therefore, we performed our study over three contrasting forest types, dominated by i) a widely distributed evergreen pine species (*P. sylvestris*), ii) a common broadleaf oak (*Quercus robur*) and ii) a mixed pine-oak forest of both species (*P. sylvestris*-*Quercus robur*). Here, *Quercus* and *Pinus* species possess different root systems occupying different soil layers (Sardans and Peñuelas, 2013) and different leaf traits, i.e. broadleaf vs. evergreen (Ishida et al., 2007), thus harbouring different fungal communities (Ishida et al., 2007; Cavard et al., 2011; Suz et al., 2017). Therefore, we expect different optimal sample pools sizes for each forest type. In line with these premises, we hypothesized that:

i) Considering the species-area theory (MacArthur and Wilson, 1968; Hill, 1973; Whittaker and Fernández-Palacios, 2007) fungal diversity will increase in pools with more soil samples until an optimal pooling size when the asymptotic plateau is reached.
ii) When we increase the number of sample pools, we expect to characterize the most dominating communities at plot level, reducing β-diversity. Similarly, when pooling few samples, the probability to capture patchier communities increases, thus those species distributed in a patchier manner will cause an increase in soil fungal β-diversity in smaller sample pool sizes.
iii) Within each forest type, increasing the number of sample pools will produce a better characterization of the fungal community, because we will expect to sample the most abundant species as well as some species/communities distributed in a patchy manner. However, we hypothesize that these patchy distributed species will not have a great contribution to compositional differences but great effect over diversity.

2. Materials and Methods

2.1. Study sites and design

The study area was located in Northern-Eastern Spain (2º,4',18.61''E, 42º,15',46.42''N) at an altitude of 1149 m a.s.l., where three independent sites were selected. We choose three forest stands (100 m^2) in each site: a monospecific stand of *Pinus sylvestris*: named P, a monospecific stand of *Quercus robur*: named Q, and a mixed stand of *P. sylvestris* and *Q. robur* named M (total n=9). To avoid pseudo-replication, the forest stands at each site were randomly selected and the plots were more than 100 m distant from one another. Finally, to avoid tree proximity and represent under/out canopy, 20 samplings were considered in an area of 100 m^2, at least > 1 m from the nearby trees.

2.2. Soil sampling

In this study, 20 soil samples were randomly collected in November 2017 in each forest stand with a drillable cylinder corer (diameter: 5 cm; depth: 12 cm, 60 soil samples per forest type/site, 180 soil cores in total). In all cores, needles and oak leaves were eliminated, whereas humus and mineral soil were sampled together. Samples were sieved using 3 mm mesh and stored at 4 °C for less than 24 h until freeze-dried. Each sample was ground to fine powder using mortar and pestle to homogenize the soil core. The soil samples were manually pooled in order to obtain five composite independent samples representing an increasing gradient of mixing samples: pools of 3 samples, 6 samples, 10 samples, 15 samples and 20 samples. For this, the same volume (1 cm^3) from each soil sample that was used in the pooling was taken. This procedure was repeated for each plot in each site. From each of the 5 composite samples per stand we subsampled 500 mg of fine homogenized soil powder to extract the fungal DNA. The samples were coded with the corresponding forest type (P: *Pinus*, Q: *Quercus* and M: for mixed stands) followed by the number of soil samples pooled in each case, i.e. one sample pool: P1, Q1 and M1; for three sample pools: P3, Q3, M3; six sample pools: P6, Q6, M6; ten sample pools: P10, Q10, M10; fifteen sample pools: P15, Q15, M15; twenty sample pools: P20, Q20, M20. The resulting pooled samples were stored at −20 °C before DNA extraction.

2.3. Fungal community analyses

Fungal DNA was extracted from 500 mg aliquots using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol. Each sample was amplified using the gITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) primers to amplify the fungal ITS2 region, both fitted with unique 8-bp tags differing in at least three positions. The number of PCR cycles was optimised for each sample, with most of the samples amplifying at 23–26 cycles. The final concentrations in the PCRs were: 1× Buffer, 200 µM of each nucleotide, 2.75 mM $MgCl_2$, primers at 500 nM (gITS7) and 300 nM (ITS4) and 0.025 U μl^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA). PCR cycling conditions were as follows: 5 min at 95°C, followed by 23-26 cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C and final extension at 72°C for 7 min. Samples were amplified by triplicate together with negative extraction and PCR controls. Amplicons were purified using the NucleoMag® NGS Clean-up and Size Select (MACHEREY-NAGEL GmbH and Co) and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled. Samples were sequenced at Stab Vida, Caparica, Portugal on an Illumina MiSeq 2×300 bp.

2.4. Bioinformatic analysis

Sequences were quality filtered and clustered using the SCATA pipeline (https://scata.mykopat.slu.se/). We first removed DNA sequences with length <200 bp and were screened for sample tags and primers defining a primer match of at least 90%. Sequences were pair-wise compared using 'usearch' (Edgar, 2010) after collapsing homopolymers to 3 bp. Sequences were quality filtered removing data with amplicon quality score of <20 (averaged per sequence) and with a score of <10 at any position. Pairwise alignments were scored as follows: mismatch penalty of 1, gap open penalty of 0 and a gap extension penalty of 1. Putative chimera sequences were removed, and the quality-filtered sequences were clustered into species hypotheses (Kõljalg et al., 2013) using single linkage clustering, with a maximum distance of 1.5% to the closest neighbour required to enter clusters. Global singletons were excluded from further analyses. Switched tags were detected when the two primers from the same sequence were found to have two distinct DNA tags and therefore these sequences were further excluded from the data. Finally, the LULU (Frøsler et al., 2017) algorithm was applied (minimum_ratio_type = "min", minimum_match = 98.5, co_occ =0.8) to merge consistently co-occurring 'daughter' OTUs.Sequence data are archived at NCBI's Sequence Read Archive under accession number PRJNA613458. (www.ncbi.nlm.nih.gov/sra).

2.5. Taxonomic identification

We taxonomically identified the 1000 most abundant OTUs. We selected the most abundant sequence from each OTU for taxonomic identification, using PROTAX software (Somervuo et al. 2016) implemented in PlutoF, using a 50% probability of correct classification (called by Somervuo et al. (2017) as "plausible identifications"). These identifications were confirmed and some of them improved using massBLASTer in PlutoF against the UNITE (Abarenkov et al. 2010). Taxonomic identities at species level were assigned based on >98.5% similarity with database references, or to other lower levels using the next criteria: genus on >97%, family on >95%, order on >92% and phylum on >90% similarity.

2.6. Statistical analyses

Statistical analyses were implemented in R software environment (version 3.6.0, R Development Core Team 2019), using the iNEXT (Hiesh et al., 2016) package for fungal diversity analyses, the *vegan* package (Oksanen et al., 2019) for the multivariate analyses, and *adespatial* package (Dray et al., 2018) was used for beta diversity analyses.

We used Hill's diversity indices (Hills, 1973) to describe the differences in fungal diversity values between number of soil sample pools within each forest type. These analyses were performed on the overall fungal communities using the abundance-based matrices. Hill's diversity consists of three numbers: N0 is species richness; N1 is the antilogarithm of Shannon's diversity index; and N2 is the inverse of Simpson's diversity index. Therefore, to test the effect of sample pooling on fungal diversity, the iNEXT function was used to build rarefactions curves pooling together the individual samples. The extrapolated confidence intervals were used to visualize the differences between the number of sample pools. Moreover, the number of sequences also rarefied to 4000 to assess interpolated richness with increasing number of sequences. For all compositional analyses, the species abundance matrix was Hellinger transformed (square root of relative abundance data) to account for taxa with low counts numbers (Legendre and Gallagher 2001) and then the dissimilarity matrices were calculated based on Bray-Curtis index. Also, compositional matrix was transformed to presence-absence and Jaccard dissimilarity was evaluated to test qualitative compositional changes. Differences in fungal overall community composition between number of sample pools were tested using permutational multivariate analyses of variance (PERMANOVA, function *"adonis"*). Then, the variance of Bray-Curtis matrix between the number of sample pools for each forest type was compared through using the *betadisper* function which is analogue to a Levene's test.

Moreover, we expected species gains with increasing sample pools therefore, to assess ß-diversity patterns and whether the core of most abundant fungal species is maintained between sites*, we evaluated for each pool the* species (or abundances-per-species) losses (B) and species gains (C) using the *beta-indices* (tbi function, Legendre, 2019). Here, *we used the* one sample pool per each forest (sample 1) as a reference, and we compared pools with increasing number of samples (sample 3, 6, 10, 15 and 20) to identify species losses and gains. The statistical analyses' codes and some simulated data are freely accessible from the GitHub repository (Adamo et al. 2021, doi: 10.5281/zenodo.4434407).

3. Results

3.1. Sample pooling effect on fungal diversity

Species rarefaction curves showed significant differences in fungal richness across sample pools and between forest types. However, no clear differences in Shannon or Simpson fungal diversity indexes were detected across sample pools, since the extrapolated confidence intervals values overlapped. These two diversity variables ranged from 65.72-113.46/N1 and 52.11-125.26/N2 in *P. sylvestris*, from 52.11-136.21/N1 and 12.70-36.62/N2 in *Q. robur* and from 131.20-105.58/N1 and 52.11-125.26/N2 in mixed stands (Table S1). Considering species richness, there were significant differences between sample pools in *P. sylvestris* stands (Fig. 1a). The main difference was detected between P1, which had the lowest richness (= 428), and the other pools (> 650). The highest fungal richness was detected in P20 (= 916), followed by P15 (= 732), P10 (= 725) and P6 (= 704). In all cases, P3 observed richness values (= 657) were similar to observed values of higher number of sample pools (Fig.1a). Conversely, in *Q. robur* stands there were also significant differences in diversity across sample pools (Fig. 1b). Here, the extrapolated confidence intervals values of Q1 (714), Q6 and Q10 were significantly lower from Q15 (1019), and Q20 (868). On the other hand, no significant differences were detected between Q1, Q3, Q6 and Q10. Interestingly, Q3 richness values observed in *Q. robur* stands (857) were close to Q20 and Q15 (Fig.1b). Finally, in mixed pine-oak stands there were also significant differences in diversity across sample pools (Fig. 1c). The highest significant differences were detected between M1 or M3 (793) and the other sample pools. M6 showed the highest richness (1137) although it was not significantly different from M15 (1105) and M20 (1104). Moreover, no significant differences were detected between M3 and M6, therefore pooling from 3 to 6 samples will produce similar richness values (Fig.1c). Finally, when the number of sequences were rarefied to 4000, differences in interpolated richness increased with

increasing number of sequences (Fig. S1) similarly as previously described for interpolated and extrapolated Hill's N0 (Fig.1). The lower richness was detected in *P. sylvestris*, followed by *Q. robur* and mixed stands. For instance, 657 species were detected in P3, 857 in Q3, while 793 in M3. Conversely, mixed stands showed overall the highest richness values showing 30% more species than P and 10% more than Q stands (X^2 = 35.82, p <0.01).

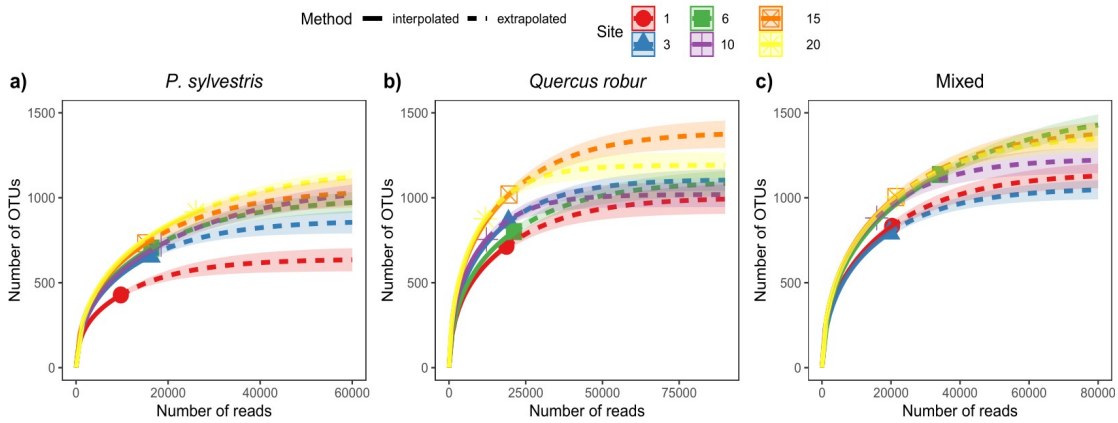

Fig. 1 Hill's N0 interpolated and extrapolated values across different sample pools in *P. sylvestris, Q. robur* and mixed pine-oak forest stand types. The values were obtained using the iNEXT fuction (iNEXT package, Hiesh et al., 2016). Hill's diversity consists of three numbers: N0 is species richness; N1 is the antilogarithm of Shannon's diversity index; and N2 is the inverse of Simpson's diversity index. Unbroken and dashed parts of the curve denote interpolated and extrapolated values respectively, and the shaded zone around each curve denotes the 95% confidence intervals. Significant differences appear where confidence interval do not overlap.

3.2. Sample pooling effect on fungal ß- diversity and species composition

ß-diversity values changed across sample pools in *P. sylvestris* and *Q. robur* stands ($F_{[5,12]}$=6.32, p-value < 0.01, $F_{[5,12]}$=13.12, p-value < 0.01) but not in mixed forest stands ($F_{[5,12]}$=0.67, p-value = 0.65; Fig.2). In contrast, no composition differences were observed across soil sample pools in any of the three forest stands ($F_{[5,12]}$=0.61, p-value = 0.98, $F_{[5,12]}$=0.63, p-value = 0.99, $F_{[5,12]}$=0.47, p-value = 0.98) since SD-ellipses of the six groups were clearly superposed in the centre of the ordination (a) NMDS stress=0.07, b) NMDS stress=0.09, c) NMDS stress=0.10 Fig. S2). ß-diversity was highest in sample pools P1 and P3 of *P. sylvestris* stands ($F_{[5,12]}$=6.32, p-value < 0.01), while the ß-diversity steadily decreased with increasing number of sample pools (>P6), with no significant differences (Fig. 2a). Conversely, in *Q. robur* stands, ß-diversity was significantly higher in Q1 pools ($F_{[5,12]}$=13.12,

p-value < 0.01) as compared to the other sample pools (>Q3, Fig. 2b). Here, ß-diversity values between larger pools other than Q1 were not significantly different (p>0.05). Finally, in mixed forest stands no significant differences in ß-diversity were detected between sample pools ($F_{[5,12]}$=0,67, p-value = 0.65, Fig. 2c), however, ß-diversity values increased but not significantly, from M1 to M20, with exception of M3. Similar results were obtained when the same analyses were performed over the presence-absence data using the dissimilarity matrices based on Jaccard index (data not shown).

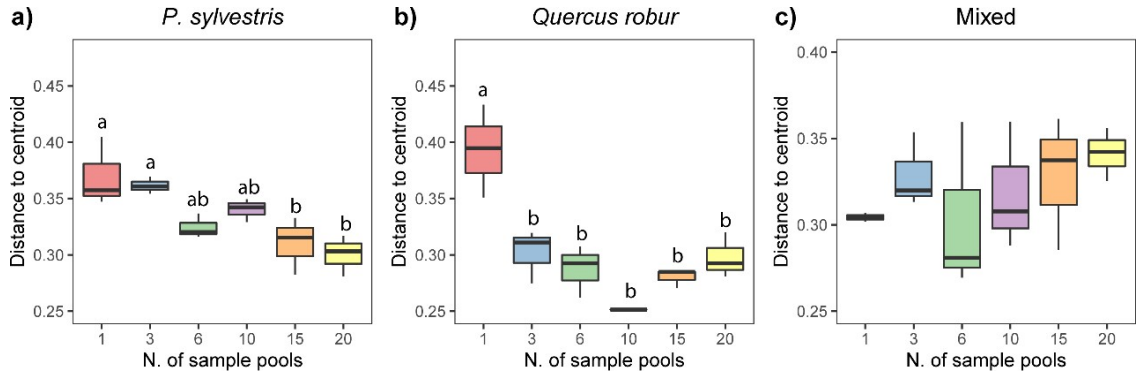

Fig. 2. Boxplots showing multivariate variance (Y-axis, ß-diversity values), sampled as distance to centroids, of each forest type in relation with the sample pools (X-axis). The species abundance matrix was Hellinger transformed and then the dissimilarity matrices were calculated based on Bray-Curtis index. Mean distance to centroids were compared with ANOVA and Tukey'HSD tests with letters denoting significant differences between number of sample pools.

There were differences in species loss and species gains between forest stands, however we did not find any significant p-values because of the low number of samples used in the permutations. In *P. sylvestris* stands, species loss values were not different between P3 and P10, while they slightly decreased between P15 (0.18) and P20 (0.16) (Table 1). Similarly, species gains values increased between P3 (0.27) and P20 (0.34). In *Q. robur* stands, species loss values were higher in Q3 (0.33) and Q10 (0.27), while they did not change across Q6, Q15 and Q20 (0.18). Conversely, no real changes in species gains were detected across Q3 and Q20, with the exception of Q6 (0.48) (Table 1). When mixed stands were analysed, species loss values decreased across M3 (0.39) and M6 (0.24) and did not change when they were compared with M1 and M20. On the other hand, species gains increased from M3 (0.36) to M6 (0.48), while there was a decrease in M10 (0.40). Yet, species gains values from M6 to M20 (0.44) decreased slightly (Table 1).

P. sylvestris	***Species loss***	***Species gains***	*p-value*
1-3	0.21 (± 0.05)	0.27 (± 0.05)	0.491
1-6	0.21 (± 0.05)	0.31 (± 0.06)	0.753
1-10	0.21 (± 0.01)	0.22 (± 0.03)	0.252
1-15	0.18 (± 0.04)	0.33 (± 0.02)	0.247
1-20	0.16 (± 0.02)	0.34 (± 0.02)	0.253
Q. robur	*Species loss*	*Species gains*	*p-value*
1-3	0.33 (± 0.11)	0.31 (± 0.12)	0.951
1-6	0.18 (± 0.07)	0.48 (± 0.04)	0.152
1-10	0.27 (± 0.11)	0.30 (± 0.08)	0.734
1-15	0.18 (± 0.03)	0.39 (± 0.04)	0.752
1-20	0.18 (± 0.04)	0.36 (± 0.04)	0.521
Mixed	*Species loss*	*Species gains*	*p-value*
1-3	0.39 (± 0.03)	0.36 (± 0.04)	0.953
1-6	0.24 (± 0.08)	0.48 (± 0.02)	0.502
1-10	0.37 (± 0.11)	0.40 (± 0.09)	0.814
1-15	0.31 (± 0.07)	0.45 (± 0.08)	0.712
1-20	0.32 (± 0.08)	0.44 (± 0.08)	0.758

Table 1. Mean (SE) ß-diversity components (loss and gain) across number of sample pools in *P. sylvestris, Q. robur* and mixed stand types. Temporal beta diversity was computed using the percentage difference index (Bray-Curtis) applied to the Hellinger transformed matrix. Total beta is the sum of 'species loss' and 'species gain' (Legendre, 2019). *P-values* were obtained using the *t.test.perm* option in the TBI function

4. Discussion

This study underlines the importance of sample pool size for accurate soil fungal diversity estimation in Mediterranean pure and mixed pine-oak forests, as increasing the number of soil sample pools, i.e. sampled volume, more reliable diversity predictions can be made with a positive species/area relationship (Whittaker and Fernández-Palacios, 2007). However, it seems not possible to standardise sampling pool protocols across distinct forest types, as our richness results showed that optimal soil sample pool size depended on forest type (e.g. pure or mixed forests). Moreover, increasing number of soil sample pools led to an increase in community similarity in pure forests, but not in mixed forests. Consequently, pools that represented less than three soil samples led to significant increases in ß-diversity values in pure forests, while values did not change in mixed forests. Finally, increasing the number of sample pools had no significant effect over species composition for any forest type, as we increased the sample pools while repeatedly sampling the same sites.

4.1. Sample pooling effect on fungal diversity

Our results demonstrate that increasing the number of soil sample pools leads to a positive species/area relationship regardless of the forest type investigated. Thus, the hypothesis 1 is accepted. These richness patterns are consistent with those reported in previous studies in agricultural fields and temperate forest sites, in which a positive relationship was detected between fungal diversity and increasing soil sample size (Ranjard et al., 2003; Song et al., 2015; Penton et al., 2016). Consequently, the number of samples pooled has important effects on the ecological interpretations also for fungal communities in soils, because insufficient sampling caused deviated richness values (Magurran, 2011). This implies that richness comparison between studies may be unreliable if distinct sampling strategies have been used, even comparing studies using the same lab protocols. These results are crucial for studies in which the total diversity is targeted (i.e. biodiversity monitoring), but also when rare species are targeted (Taberlet et al., 2018). The DNA extraction step also represents an important source of bias in community composition (Plassart et al. 2012), however, here DNA was carefully extracted following the same protocol for all the samples. In addition, PCR step is also known to be a source of bias and may affect final community composition. Nevertheless, we tried to keep biases as low as possible by reducing the number of PCR cycles and using an optimized protocol for fungal metabarcoding (Clemmensen et al., 2016). Finally, sequencing depth may also have an impact on the perceived diversity (Smith and Peay, 2014), however

based on the rarefaction curves (Fig.1) our sequencing depth was able to capture similar coverage of the fungal diversities of the community.

Surprisingly, neither Shannon nor Simpson fungal diversity indexes were affected by sampling pooling, although they slightly increased but not significantly. Thus, for Shannon and Simpson indexes the first hypothesis is not accepted. It is well known that diversity is dependent on richness and evenness, then it seems that richness increases are compensated in our case by evenness values (i.e. maintain or decrease slightly with sample pools). Finally, although not tested here we argue that future studies should consider both species-area and species-time relationship as it would lead to a deeper understanding of fungal diversity patterns (Ladau et al., 2019).

In forest ecosystems, differences in dominant tree species identity can lead to diversity and compositional changes (Ishida et al., 2007; Urbanová et al., 2015; Nagati et al., 2018). Simultaneously, mixed forests are expected to harbour higher taxonomical richness in all ecosystem compartments than pure stands (Ishida et al., 2007, Cavard et al., 2011). For instance, Suz et al. (2017) reported higher ectomycorrhizal richness in mixed pine-oak stands compared to pure pine stands. Our results follow these trends, with greater richness in mixed stands compared to pure ones (Fig 1). Consequently, the minimum number of sample pool size was different between pure and mixed stands. For example, pooling at least three soil samples already provide consistent richness estimations for *P. sylvestris and Q. robur* forests (same sampling effort), whereas for mixed stands pools should include almost six soil samples.

4.2. Sample pooling effect on fungal ß-diversity and species composition

In this study, we observed a steady decrease of ß-diversity values with increasing number of soil sample pools in both *P. sylvestris* and *Q. robur* stands, while there were no significant changes in mixed forest stands (Fig.2). Thus, hypothesis 2 is partially accepted. In pure *Pinus* and *Quercus* forest, the results followed the predicted trends, with a decrease of dispersion values when increasing the number of sample pools. This result indicates that pooling many samples reduces the ß-diversity estimation between sites, which means a higher compositional similarity between different sites. This is important, since by increasing the number of samples in each pool we may be able to reduce the type II error and therefore reduce the error variance or unexplained variation. The higher ß-diversity values observed in pools represented by low number of samples in *Pinus* or *Quercus* is likely attributed to insufficient sampling effort that failed in capturing the whole community in the site, with individual samples picking a different

subset of the community due to the patchiness distribution of each fungal species (Cairney, 2012). Thus, it seems that smaller sample pools, i.e. lower than three, will capture distinct subsets of the community, which would explain why there was much higher heterogeneous communities between sites with lower pools than with larger soil sample pools (Manter et al., 2010) since each new pool increased the species gains. Our results agree with Ranjard et al. (2003), who found higher replicate variation in small sample sizes. It seems that in pure pine or oak forest, soil sample pools lower than three are prone to profile the community in a more biased manner. Conversely, our results showed that second hypothesis was not applicable for mixed stands, since increasing the number of sample pools does not significantly affect soil fungal ß-diversity. It is possible that the higher taxonomical richness and greater species coexistence present in mixed forests (Cavard et al., 2011) could explain why ß-diversity is not higher when pooling low number of samples. Further studies of mixed forest are needed to identify if increasing the number of sample pools over more than 20 cores causes a reduction of ß-diversity values.

Interestingly, our ß-diversity findings were supported by species loss and gain values between sample pools (Table 1). In pure *Pinus* and *Quercus* forest, while species gains values slightly increased or decreased, we detected almost constant species loss values across sample pools. Thus, the core of most abundant fungal species is maintained between sites, with low increases of less abundant species causing a reduction of ß-diversity. In contrast, species loss and gain values did not change in mixed forest, thus there are different ß-diversity patterns between forest types, being more heterogeneous the communities found in mixed forest, since interquartile ranges were higher than in pure stands (Fig. 2). In any case, it seems that we are not collecting enough number of samples to pool to characterize ß-diversity patterns and species gains and loses properly in mixed forest.

Finally, increasing the number of sample pools had no significant effect on species composition for any forest type. These results are consistent with our last hypothesis, as we expected to not detect any influence of sample pools on community composition in each forest type. Since each low sample pool reflect a subset of the higher pools increasing the number of sample pools will not influence the species composition, qualitatively or quantitatively. Thus, it is possible that the main species are maintained, and the incorporation of new species is then reduced when increasing new sampling pools (see Fig. S1) (Magurran, 2011). Therefore, it seems that when profiling the core community (more abundant species) low sampling effort might be enough. However, an increase in the number of sampling cores may be desirable when targeting for

rare or less abundant species since many important processes may be driven by specific, low abundant species (Red list fungal species, Quarantine pathogens).

4.3. Conclusions

In this study, increasing number of sample pools had a significant effect on fungal richness in all the three forest types, indicating a positive positive species/area relationship. Moreover, our results indicate that the minimum number of sample pools to adequately estimate fungal richness and species composition will be lower in monospecific stands, three in our case, than for more diverse mixed forest where the optimal pooling will be almost six samples. Our results shed light on best soil sample monitoring implementations to be applied for characterizing pure and mixed forests ecosystems. However, further research is needed to test if these results can be extrapolated to different ecosystems in the area or in similar areas.

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Conflicts of interests

The authors declare they have no conflict of interest.

Authors' contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yasmin Piñuela, Carles Castaño, José Antonio Bonet, Irene Adamo and Josu G. Alday. The first draft of the manuscript was written by Irene Adamo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

References

Adamo I, Pinuela Y, Bonet JA, Castaño C, Martinez de Aragón J, Parladé J, Pera J, Alday JG. 2021.Statistical-analyses-optimal-sampling-size-in-Mediterranean-forest-ecosystems:first release GitHub repository http://github.com/ire1990/ Statistical-analyses-optimal-sampling-size-in-Mediterranean-forest-ecosystems. doi: 10.5281/zenodo.4434407)

Anslan S, Bahram M, Hiiesalu I, Tedersoo L, 2017. PipeCraft: Flexible open-source toolkit for bioinformatics analysis of custom high-throughput amplicon sequencing data. Mol. Ecol. Resour 17: e234–e240 https://doi.org/10.1111/1755-0998.12692

Arrhenius O, 1921. Species and Area. J. Ecol: 9: 95-99 . https://doi.org/10.2307/2255763

Bardgett RD, Wardle DA, 2010. Above-belowground linkages: biotic interaction, ecosystem processes and global chhange. Oxford University Press.

Bardgett RD, Van Der Putten WH, 2014. Belowground biodiversity and ecosystem functioning. Nature 515: 505–511. https://doi.org/10.1038/nature13855

Bjørnsgaard Aas A, Davey, ML, Kauserud H, 2017. ITS all right mama: investigating the formation of chimeric sequences in the ITS2 region by DNA metabarcoding analyses of fungal mock communities of different complexities. Mol. Ecol. Resour 17:4, 730-741. https://doi.org/10.1111/1755-0998.12622

Bonello P, Bruns T D, Gardes M, 1998. Genetic structure of a natural population of the ectomycorrhizal fungus Suillus pungens. New Phytol. 138: 533-543263–264. https://doi.org/10.1046/j.1469-8137.1998.00122.x

Cairney JWG, 2005. Basidiomycete mycelia in forest soils: Dimensions, dynamics and roles in nutrient distribution. Mycol. Res 109: 7-20. https://doi.org/10.1017/S0953756204001753

Cairney JWG, 2012. Extramatrical mycelia of ectomycorrhizal fungi as moderators of carbon dynamics in forest soil. Soil Biol. Biochem.48: 198-208 https://doi.org/10.1016/j.soilbio.2011.12.029

Cavard X, Macdonald SE, Bergeron Y, Chen HYH, 2011. Importance of mixedwoods for biodiversity conservation: Evidence for understory plants, songbirds, soil fauna, and ectomycorrhizae in northern forests. Environ. Rev. 19: 142-161 https://doi.org/10.1139/A11-004

Clemmensen KE, Ihrmark K, Durling MB, Lindahl BD, 2016. Sample preparation for fungal community analysis by high-throughput sequencing of barcode amplicons, in: Methods in

Environmental Genomics 1399: 61-88. https://doi.org/10.1007/978-1-4939-3369-3_4

De Filippis F, Laiola M, Blaiotta G, Ercolini D, 2017. Different amplicon targets for sequencing-based studies of fungal diversity. Appl. Environ. Microbiol 83: 1-9. https://doi.org/10.1128/AEM.00905-17

Dickie IA, Boyer S, Buckley HL, Duncan RP, Gardner PP, Hogg ID, Holdaway RJ, Lear G, Makiola A, Morales SE, Powell JR, Weaver L, 2018. Towards robust and repeatable sampling methods in eDNA-based studies. Mol. Ecol. Resour 18: 940-952. https://doi.org/10.1111/1755-0998.12907

Dopheide A, Xie D, Buckley TR, Drummond AJ, Newcomb RD, 2019. Impacts of DNA extraction and PCR on DNA metabarcoding estimates of soil biodiversity. Methods Ecol. Evol 10: 120-133. https://doi.org/10.1111/2041-210X.13086

Dray S, Bauman D, Guillaume B, Borcard D, Clappe S, Guenard G, Jombart T, Laroque G, Legendre P, Madi N, Wagner HH, 2018. Package "adespatial" 1–97.

Duarte S, Cássio F, Pascoal C, Bärlocher F, 2017. Taxa-area relationship of aquatic fungi on deciduous leaves. PLoS One 12: 1–19. https://doi.org/10.1371/journal.pone.0181545

Dunham SM, Kretzer A, Pfrender ME, 2003. Characterization of Pacific golden chanterelle (Cantharellus formosus) genet size using co-dominant microsatellite markers. Mol. Ecol. 12: 1607-1618. https://doi.org/10.1046/j.1365-294X.2003.01837.x

Edgar CRC, 2010. Search and clustering orders of magnitude faster then BLAST. Bionformatics 26:19, 2460-2461. https://doi.org/10.1093/bioinformatics/btq461

Frøslev TG, Kjøller R, Bruun HH, Ejrnæs R, Brunbjerg AK, Pietroni C, Hansen AJ, 2017. Algorithm for post-clustering curation of DNA amplicon data yields reliable biodiversity estimates. Nat. Commun 8: 1188. https://doi.org/10.1038/s41467-017-01312-x

Geml J, 2019. Soil fungal communities reflect aspect-driven environmental structuring and vegetation types in a Pannonian forest landscape. Fungal Ecol. 39: 63–79. https://doi.org/10.1016/j.funeco.2018.12.005

Grey EK, Bernatchez L, Cassey P, Deiner K, Deveney M, Howland KL, Lacoursière-Roussel A, Leong S CY, Li Y, Olds B, Pfrender ME, Prowse TAA, Renshaw MA, Lodge DM, 2018. Effects of sampling effort on biodiversity patterns estimated from environmental DNA metabarcoding surveys. Scientific Reports 8, 8843. https://doi.org/10.1038/s41598-018-27048-2

Grundmann GL, Debouzie DJ, 2000. Geostatistical analysis of the distribution of $NH4^-$ and $NO2^-$ oxidizing bacteria and serat the millimeter scale along a soil transect. FEMS Micro Ecol. 34: 57-62. https://doi.org/10.1111/j.1574-6941.2000.tb00754.x

Gweon HS, Oliver A, Taylor J, Booth T, Gibbs M, Read DS, Griffiths RI, Schonrogge K, 2015. PIPITS: An automated pipeline for analyses of fungal internal transcribed spacer sequences from the Illumina sequencing platform. Methods Ecol. Evol 6(8): 973-980. https://doi.org/10.1111/2041-210X.12399

Hibbett D, AbarenkovK, Kõljalg U, Öpik M, Chai B, Cole J, Wang Q, Crous P, Robert V, Helgason T, Herr JR, Kirk P, Lueschow S, O'Donnell K, Nilsson RH, Oono R, Schoch C, Smyth C, Walker DM, Porras-Alfaro A, Taylor JW, Geiser DM, 2016. Sequence-based classification and identification of Fungi. Mycologia 108: 1049-1068. https://doi.org/10.3852/16-130

Hibbett DS, Ohman A, Kirk PM, 2009. Fungal ecology catches fire. New Phytol 184: 277-279. https://doi.org/10.1111/j.1469-8137.2009.03042.x

Hill MO, 1973. Diversity and evenness: a unifying notation and its consequences. Ecology 54: 427-432.

Hsieh TC, Ma KH, Chao A, 2016. iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). Methods Ecol. Evol 7: 1451-1456. https://doi.org/10.1111/2041-210X.12613

Ihrmark K, Bödeker ITM, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, Strid Y, Stenlid J, Brandström-Durling M, Clemmensen KE, Lindahl BD, 2012. New primers to amplify the fungal ITS2 region - evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiol. Ecol. 82: 666–677. https://doi.org/10.1111/j.1574-6941.2012.01437.x

Ishida TA, Nara K, Hogetsu T, 2007. Host effects on ectomycorrhizal fungal communities: Insight from eight host species in mixed conifer-broadleaf forests. New Phytol 174: 430-440. https://doi.org/10.1111/j.1469-8137.2007.02016.x

Kang S, Mills AL, 2006. The effect of sample size in studies of soil microbial community structure. J. Microbiol. Methods 66: 242-250. https://doi.org/10.1016/j.mimet.2005.11.013

Koljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, 2013. Towards a unified paradigm for sequence-based identification of fungi. Mol. Ecol. 22: 5271–5277. https://doi.org/10.1111/mec.12481

Ladau J., Eloe-Fadrosh EA, 2019. Spatial, temporal, and phylogenetic scales of microbial ecology. *Trends Microbiol***27**:662–669. https://doi.org/10.1016/j.tim.2019.03.003

Legendre P, 2019. A temporal beta-diversity index to identify sites that have changed in

exceptional ways in space–time surveys. Ecol. Evol 9: 3500-3514. https://doi.org/10.1002/ece3.4984

Legendre P, Gallagher ED, 2001. Ecologically meaningful transformations for ordination of species data. Oecologia 129: 271-280. https://doi.org/10.1007/s004420100716

Lindahl BD, Nilsson RH, Tedersoo L, Abarenkov K, Carlsen T, Kjøller R, Kõljalg U, Pennanen T, Rosendahl S, Stenlid J, Kauserud H, 2013. Fungal community analysis by high-throughput sequencing of amplified markers - a user's guide. New Phytol. 199: 288-299. https://doi.org/10.1111/nph.12243

MacArthur RH, 1965. Patterns of biological diversity. Biol. Rev 40: 510-533. https://doi.org/10.1111/j.1469-185x.1965.tb00815.x

MacArthur RH, Wilson EO, 1968. The Theory of Island Biogeography. Geogr. J 134: 592. https://doi.org/10.2307/1796430

Magurran AE, McGill BJ, 2011. Biological diversity: frontiers in measurement and assessment. Oxford University Press.

Manter DK, Weir TL, Vivanco JM, 2010. Negative effects of sample pooling on PCR-Based estimates of soil microbial richness and community structure. Appl. Environ. Microbiol. 76: 2086–2090. https://doi.org/10.1128/AEM.03017-09

Mueller GM, Schmit JP, 2007. Fungal biodiversity: What do we know? What can we predict? Biodivers. Conserv. 16: 1–5. https://doi.org/10.1007/s10531-006-9117-7

Murata H, Ohta A, Yamada A, Narimatsu M, Futamura N, 2005. Genetic mosaics in the massive persisting rhizosphere colony "shiro" of the ectomycorrhizal basidiomycete *Tricholoma matsutake*. Mycorrhiza 15: 505-512. https://doi.org/10.1007/s00572-005-0358-1

Nagati M, Roy M, Manzi S, Richard F, Desrochers A, Gardes M, Bergeron Y, 2018. Impact of local forest composition on soil fungal communities in a mixed boreal forest. Plant Soil 432: 345–357. https://doi.org/10.1007/s11104-018-3806-3

Nilsson RH, Wurzbacher C, Bahram M, Coimbra VRM, Larsson E, Tedersoo L, Eriksson J, Ritter CD, Svantesson, S, Sánchez-García M, Ryberg M, Kristiansson E, Abarenkov K, 2016. Top 50 most wanted fungi. MycoKeys 12: 29-40. https://doi.org/10.3897/mycokeys.12.7553

Nguyen NH, Song Z, Bates ST, Branco S, Tedersoo L, Menkel J, Schilling JS, Kennedy PJ, 2016. FUNGuild: an open annotation tool for parsing fungal communty datasetes by ecological guild. Fungal Ecol 20: 241-248

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H, 2019. vegan: Community Ecology Package. R package version 2,5-2. Cran R.

Pauvert C, Buèe M, Laval V, Edel-Hermann V, Fauchery L, Gautier A, Lesur I, Valance J, Vacher C, 2019. Bionformatics matters: The accurancy of plant and soil fungal communities data is highly dependent on the metabarcoding pipeline. Fungal ecol. 41: 23-33. https://doi.org/10.1016/j.funeco.2019.03.005

Penton CR, Gupta VVSR, Yu J, Tiedje JM, 2016. Size matters: Assessing optimum soil sample size for fungal and bacterial community structure analyses using high throughput sequencing of rRNA gene amplicons. Front. Microbiol 7,824. https://doi.org/10.3389/fmicb.2016.00824

Pérez-Izquierdo L, Zabal-Aguirre M , González-Martínez SC, Buée M, Verdú M, Rincón A, Goberna M, 2019. Plant intraspecific variation modulates nutrient cycling through its below ground rhizospheric microbiome. Journal of Ecology 107(4): 1594-1605. doi:10.1111/1365-2745.13202

Plassart P, Terrat S, Thomson B, Griffiths R, Dequiedt S, Lelievre M, Regnier T,Nowak V, Bailey M, Lemanceau P, Bispo A, Chabbi A, Maron PA, Mougel C, Ranjard L, 2012. Evaluation of the ISO Standard 11063 DNA extraction for assessing soil microbial abundance and community structure. Plos One 7 1-8. https://doi.org/:10.1371/journal.pone.0044279

Ranjard L, Lejon DPH, Mougel C, Schehrer L, Merdinoglu D, Chaussod R, 2003. Sampling strategy in molecular microbial ecology: Influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial communities. Environ. Microbiol 5: 1111-1120. https://doi.org/10.1046/j.1462-2920.2003.00521.x

Rognes T, Flouri T, Nichols B, Quince C, Mahé F, 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4,: e2584. https://doi.org/10.7717/peerj.2584

Sardans J, Peñuelas J, 2013. Plant-soil interactions in Mediterranean forest and shrublands: Impacts of climatic change. Plant Soil 365: 1-33. https://doi.org/10.1007/s11104-013-1591-6

Sawyer NA, Chambers SM, Cairney JWG, 1999. Molecular investigation of genet distribution and genetic variation of Corrinarius rotundisporus in eastern Australian sclerophyll forests. New Phytol. 42: 561-568. https://doi.org/10.1046/j.1469-8137.1999.00417.x

Smith ML, Bruhn JN, Anderson JB. 1992. The fungus Armillaria bulbosa is among the largest and oldest living organisms. Nature 356: 428–431

Smith S, Read D, 2008. Mycorrhizal Symbiosis, Mycorrhizal Symbiosis 637-768. https://doi.org/10.1016/B978-0-12-370526-6.X5001-6

Somervuo P, Koskela S, Pennanen J, Nilsson RH, Ovaskainen O, 2016. Unbiased probabilistic taxonomic classification for DNA barcoding. Bioinformatics 32:2920-2927. https://doi.org/10.1093/bioinformatics/btw346

Song Z, Schlatter D, Kennedy P, Kinkel LL, Kistler HC, Nguyen N, Bates ST, 2015. Effort versus reward Preparing samples for fungal community characterization in high-throughput sequencing surveys of soils. PLoS One 1-13. https://doi.org/10.1371/journal.pone.0127234

Suz LM, Kallow S, Reed K, Bidartondo MI, Barsoum N, 2017. Pine mycorrhizal communities in pure and mixed pine-oak forests: Abiotic environment trumps neighboring oak host effects. For. Ecol. Manage. 40: 370–380. https://doi.org/10.1016/j.foreco.2017.09.030

Taberlet P, Bonin A, Zinger L, Coissac E, 2018. Environmental DNA: for biodiversity research and monitoring, https://doi.org/10.1093/oso/9780198767220.001.0001

Tedersoo L, Bahram M, Põlme S, Kõljalg U, Yorou NS, Wijesundera R, Abarenkov K, 2014. Global diversity and geography of soil fungi. Science 346: 6213. https://doi.org/10.1126/science.1256688

Urbanová M, Šnajdr J, Baldrian P, 2015. Composition of fungal and bacterial communities in forest litter and soil is largely determined by dominant trees. Soil Biol. Biochem 84: 53-64. https://doi.org/10.1016/j.soilbio.2015.02.011

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics in PCR protocols. 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Whittaker RJ, Fernández-Palacios JM, 2007. Island biogeography: ecology, evolution and conservation. 2nd edition, Oxford University Press.

Supplementary material

Table S1. Hill's N1 and Hill's N2 observed values across different sample pools in *P. sylvestris, Q. robur* and mixed pine-oak forest stand types. The values were obtained using the iNEXT fuction (iNEXT package, Hiesh et al., 2016). Hill's diversity consists of three numbers: N0 is species richness; N1 is the antilogarithm of Shannon's diversity index; and N2 is the inverse of Simpson's diversity index.

P. sylvestris	*Hill's N1*	*Hill's N2*
P1	65.72 (±1.29)	26.10 (±0.51)
P3	80.83 (±1.32)	25.88 (±0.46)
P6	84.80 (±1.27)	27.39 (±0.50)
P10	97.58 (±1.44)	39.83 (±0.59)
P15	113.47 (±1.83)	44.46 (±0.73)
P20	107.79 (±1.17)	38.03 (±0.54)
Q. robur	*Hill's N1*	*Hill's N2*
Q1	52.11 (±0.89)	12.70 (±0.24)
Q3	97.72 (±1.43)	30.74 (±0.51)
Q6	65.38 (±0.96)	16.18 (±0.25)
Q10	90.50 (±1.92)	22.40 (±0.52)
Q15	136.21 (±2.45)	36.62 (±0.64)
Q20	125.26 (±2.52)	27.52 (±0.76)
Mixed	*Hill's N1*	*Hill's N2*
M1	109.26 (±1.46)	46.41 (±0.60)
M3	105.57 (±1.36)	46.40 (±0.59)
M6	126.57 (±1.20)	44.23 (±0.55)
M10	131.12 (±1.90)	48.38 (±0.88)
M15	120.74 (±1.70)	33.74 (±0.6)
M20	126.77 (±1.62)	41.48 (±0.68)

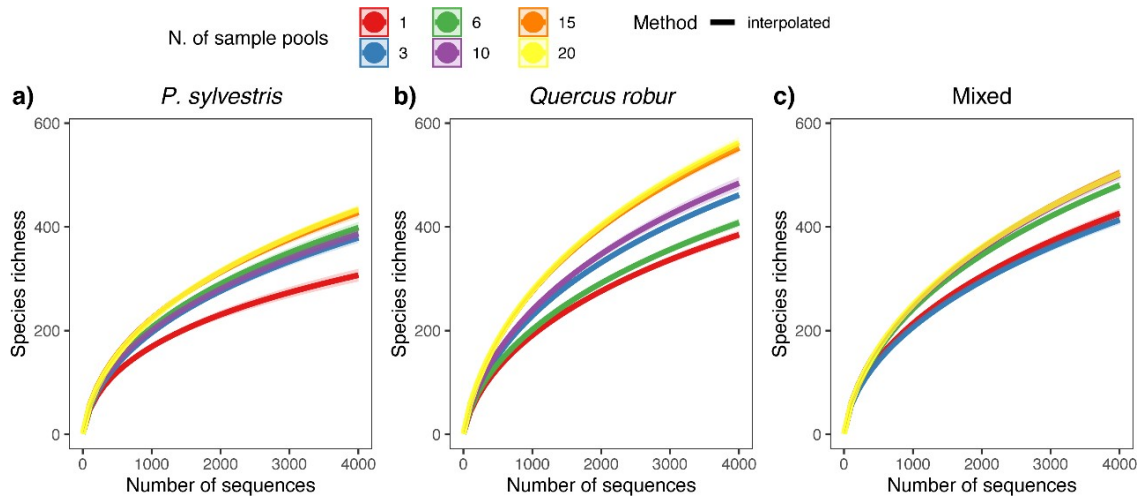

Fig. S1. Hill's N0 interpolated values across different sample pools in *P. sylvestris, Q. robur* and mixed pine-oak forest stand types. The values were obtained using the iNEXT function (iNEXT package, Hiesh et al., 2016). Hill's diversity consists of three numbers: N0 is species richness; N1 is the antilogarithm of Shannon's diversity index; and N2 is the inverse of Simpson's diversity index.

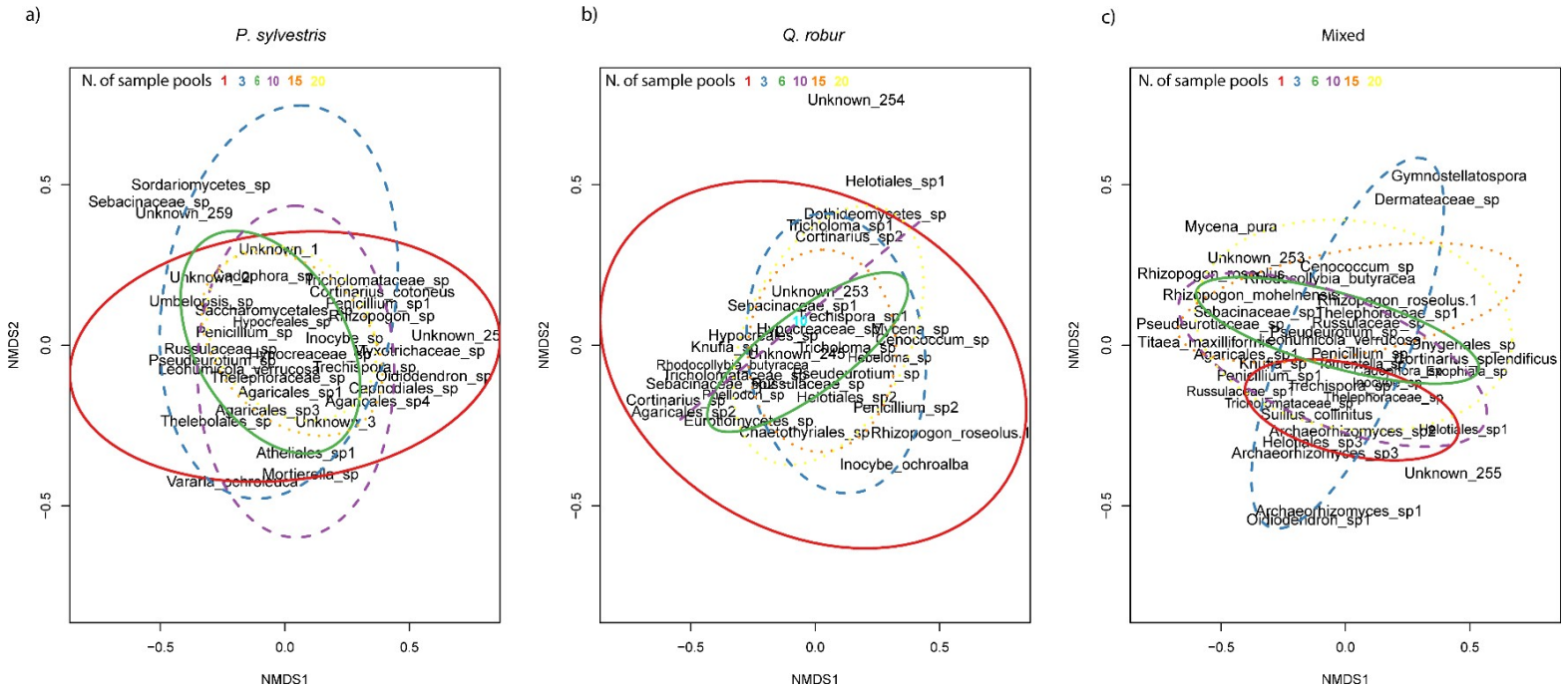

Fig. S2. Non-metric multidimensional scaling (NMDS) representing compositional differences in the overall communities between number of soil sample pools (1, 3, 6, 10, 15, 20) in a) *P. sylvestris* b) *Q. robur* c) mixed forests.

CHAPTER II

"Soil physico-chemical properties have a greater effect on soil fungi than host species in Mediterranean pure and mixed pine forests"

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Soil physico-chemical properties have a greater effect on soil fungi than host species in Mediterranean pure and mixed pine forests

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Abstract

Soil fungi are fundamental drivers of forest ecosystem processes. Soil physico-chemical parameters and vegetation features such as host type or stand structure can affect soil fungal communities. However, there is a lack of comprehensive studies describing the relative importance of niche processes (soil physico-chemistry and forest structural drivers) versus neutral processes (geographical distance) driving soil fungal community assemblages, especially in less-studied drought-prone ecosystems such as Mediterranean forests. In this study, we performed Pacific Biosciences sequencing of internal transcribed spacer 2 amplicons to characterize the soil fungal community composition and diversity of 42 forests dominated by either pure *Pinus nigra*, *Pinus halepensis* or *Pinus sylvestris* or a *P. nigra–P. halepensis* or *P. nigra–P. sylvestris* mixture. Our specific aims were to identify and disentangle the relative importance of the main soil characteristics and the spatial and forest structural factors that accounted for the greatest proportion of fungal community variation along a regional gradient in the Mediterranean Pre-Pyrenees. Soil parameters accounted for the greatest significant proportion of the total variance in the overall fungal community (25%), and in the mycorrhizal (23%) and saprotrophic (22%) communities, while geographical distance accounted for 14% of the variance in the overall fungal community, 7% in the mycorrhizal and 22% in the saprotrophic communities. Conversely, forest structure did not significantly affect the soil fungal community, as fungal composition and diversity did not differ significantly among the pine hosts. Moreover, pH, followed by P and the C:N ratio explained the largest differences in the composition of the overall fungal community and in the mycorrhizal fungal community. By contrast, the largest proportion of differences in saprotrophic composition were explained by geographical distance, closely followed by the C:N ratio and N. Our results show that, in these Mediterranean pine forests, soil parameters are the most important driving forces shaping soil fungal communities at the regional scale given that ectomycorrhizal and saprotrophic fungi were more influenced by soil physico-chemical parameters or geographical distance than by *Pinus* species or forest structural variables. Finally, P content in soils also emerged as a significant factor driving differences in mycorrhizal communities.

Highlights

- Soil fungal communities were profiled in 42 pure and mixed pine forests.
- Soil chemistry significantly influenced variation in soil fungal communities.
- Pine species and stand structure had no effect on the soil fungal communities.

- pH, P and the C:N ratio were the strongest predictors shaping fungal communities.

Keywords: DNA metabarcoding, community composition, ectomycorrhizal fungi, saprotrophic fungi, fungal diversity, pH, N, P.

1. Introduction

Soil fungi are fundamental drivers of ecosystem processes (Bardgett and van der Putten, 2014), such as organic matter decomposition, soil nutrient release and plant nutrient uptake (Bardgett and Wardle, 2010). Given that these communities are able to determine plant communities at multiple spatial scales, understanding the main processes shaping fungal assemblages is, therefore, a central goal of the microbial ecology research field. Soil fungal communities are highly influenced by differences in soil physico-chemical properties and vegetation features such as host type or stand structure (i.e., niche processes; Větrovský et al., 2019). However, previous studies have found that geographical distance (i.e., neutral processes; Green and Bohannan, 2006) can have a primary role in shaping fungal community structure (Peay et al., 2012; Bahram et al., 2013; Peay and Bruns, 2014). Nevertheless, both niche and neutral processes have been described as extremes of a continuum, whereas biological communities are usually located somewhere between these two theoretical extremes (Gravel et al., 2006), raising the need to determine the relative importance of each process on soil fungal community assembly in different ecosystems (Cao et al., 2019). For example, we still lack comprehensive studies describing simultaneously the relative importance of niche processes, such as environmental filtering (e.g., soil parameters and forest stand drivers), and neutral processes, such as distance decay similarity (e.g., geographical distance, Bahram et al., 2013), in driving soil fungal community assemblages, especially in less-studied drought-prone ecosystems such as Mediterranean forests.

Previous research from boreal and temperate ecosystems have demonstrated that soil physico-chemical properties and nutrient availability can have a strong influence in shaping soil fungal composition and diversity (Read and Perez-Moreno, 2003; Kyaschenko et al., 2017). In this regard, pH seems to have a strong role in regulating fungal communities worldwide (Tedersoo et al., 2014; Goldmann et al., 2015; Zhang et al., 2016; Glassman et al., 2017; Tedersoo et al., 2020), and often affects nutrient cycling (Adamczyk et al., 2016), determining the availability of soil nutrients such as Nitrogen, Phosphorus and Potassium

(Awad et al., 2019; Guo et al., 2020). Nitrogen is often the main limiting nutrient in soils, especially in colder terrestrial ecosystems where decomposition is limited (Read and Perez-Moreno, 2003; Kyaschenko et al., 2017). However, the primary productivity of Mediterranean terrestrial plants is generally limited by P and not by N (Du et al., 2020), suggesting that P could be a more important nutrient trader than N in these ecosystems in fungal–tree interactions. Therefore, based on the biological market theory (Konvalinková et al., 2017), P trading in these ecosystems might show similar patterns to the plant–ectomycorrhizal N trading model (Hortal et al., 2017). For example, Pérez-Izquierdo et al. (2020) found that enzymatic activity in root tips was significantly influenced by low P availability in Mediterranean *P. pinaster* and *P. halepensis* forests. In addition to the soil physico-chemistry, Mediterranean forests are highly influenced by water availability (Sardans and Peñuelas 2013; Castaño et al., 2018b), and the long-lasting dry summer periods that are typical of these ecosystems alter biologically controlled soil elements such as C and N (Jarvis et al., 2007; Delgado-Baquerizo et al., 2017). Thus, given that soil conditions and water limitation during the summer months are factors that determine plant communities (Thullier et al., 2008), soil parameters may shape fungal communities in Mediterranean soils differently than in other forest ecosystems. However, this issue remains to be explored. The tree host has also been observed to influence soil fungi, either directly via intraspecific (Pérez-Izquierdo et al., 2019) or interspecific variability that can affect tree–mycorrhizal associations (Kernaghan and Patriquin 2011; Arfi et al., 2012; Hagenbo et al., 2020), or indirectly via changes in soil chemistry that can affect saprotrophic community structure (i.e., litter chemistry; Lladó et al., 2017). Mixed forests are of interest because they are more adaptable to climate change or disturbances than monocultures (Bravo-Oviedo et al., 2014). The coexistence of tree species may be supported by complementary niches for tree growth and nutrient uptake, increasing forest resistance to disturbances (Bello et al., 2019). Thus, due to these complementary niches, mixed forests are expected to harbour higher levels of taxonomical richness in ecosystem niches (Ishida et al., 2007, Cavard et al., 2011). In this regard, previous studies comparing soil fungal communities under distinct tree hosts in boreal and temperate ecosystems have reported higher levels of soil fungal richness in mixed stands than in pure stands (Ishida et al., 2007; Nagati et al., 2018). Although some mycorrhizal fungi are known to have relatively broad host ranges and, therefore, are rarely specific to a tree host genus (Molina et al., 1992), clear differences in soil fungal communities between pure and mixed forests have been found in studies comparing host trees with contrasting traits (i.e., deciduous vs conifers) (Ishida et al., 2007). However, it

is unclear whether these differences also occur when host trees have similar traits or belong to the same genus (i.e., *Pinus*).

Forest stand variables can also influence soil fungal community composition (Santos Silva et al., 2011). For instance, in Mediterranean ecosystems, Tomao et al. (2017) found that the basal area of trees in a stand significantly affected mushroom yield production. Therefore, forest silviculture not only affects soil fungal communities directly by disrupting symbiotic associations with the host (Jones et al., 2003) but also indirectly by changing soil microclimate and biochemistry (Varenius et al., 2016; Kyaschenko et al., 2017; Sterkenburg et al., 2019). However, the extent to which forest stand variables shape soil fungal communities and diversity with regard to interspecific changes in tree host, geographical distance and soil physico-chemistry have not been analysed in Mediterranean ecosystems (Tedersoo et al., 2013).

In this study, we collected soil samples from 42 different forests in the Mediterranean Spanish Pre-Pyrenees mountain range. The overall aim of this study was to characterize the soil fungal community composition and diversity of these forests, which were dominated by either pure *Pinus nigra*, *Pinus halepensis* or *Pinus sylvestris* or a *P. nigra–P. halepensis* or *P. nigra–P. sylvestris* mixture. Given that these forests have different soil properties and forest structural characteristics (e.g., trees per hectare and basal area), we also tried to identify the main soil type and spatial and forest structural factors that accounted for the highest proportion of fungal community variation. More specifically, we had four aims. Our first aim was to identify to what extent niche processes (i.e., soil physico-chemical parameters and forest structural factors) *vs* a neutral process (i.e., distance decay similarity measured as spatial distance) shape the overall, mycorrhizal and saprotrophic soil community assemblages given that they may respond differently to changes in soil physico-chemical parameters (Averill & Hawkes, 2016). Our second aim was to determine whether there is fungal specificity across across habitats with distinct pine hosts. We expected host trees to have little or no significant effect on fungi given that closely related tree species tend to share more similar fungal communities than distantly related tree species (Losos, 2008; Tedersoo et al., 2008). Our third aim was to determine whether mixed species forests have distinct or more diverse soil fungal communities than pure pine forests, which could potentially explain why mixed forests are better adapted to disturbances (Bello et al., 2019). Our fourth aim was to disentangle the main soil physico-chemical and forest structural drivers of community composition.

2. Materials and Methods

2.1. Site selection

We conducted this study in the mountainous pre-Pyrenees region of Catalonia in north-eastern Spain (see map in Fig. 1). We analysed a set of long-term monitoring plots in which fungal fruiting has been recorded for ~20 years (Martínez de Aragón et al., 2007). The climate is Mediterranean, with an intense period of drought occurring in the summer from June until August, mean annual temperatures ranging from 6° to 9°C (Alday et al., 2017), and most of the precipitation occurring in spring and autumn. We randomly selected 42 pine forest plots from the 579 sites included in the 1992 Forest Ecological Inventory of Catalonia carried out by the Centre de Recerca Ecològica i Aplicacions Forestals (CREAF, 1992) (Bonet et al., 2010). The plots were randomly distributed throughout Catalonia in numbers proportional to the area occupied by each tree species, with eleven plots of *P. nigra*, six plots of *P. sylvestris*, and four plots of *P. halepensis*. Stand age of these plots ranges from 23 to 88 years and elevation ranges from 500 to 1500 m. Of these 42 plots, 32 comprised pure pine forest: 14 plots of *P. nigra*, 14 plots of *P. sylvestris* and 4 plots of *P. halepensis*. Ten of the plots comprised a mix of *Pinus* species: 7 plots of *P. sylvestris* and *P. nigra* and 3 plots dominated by *P. nigra* and *P. halepensis*. The main features of the study plots are summarized in Table 1.

Fig. 1. Map of Catalonia showing the location of the 42 plots sampled in this study: the different types of pine tree stand are indicated by different coloured symbols.

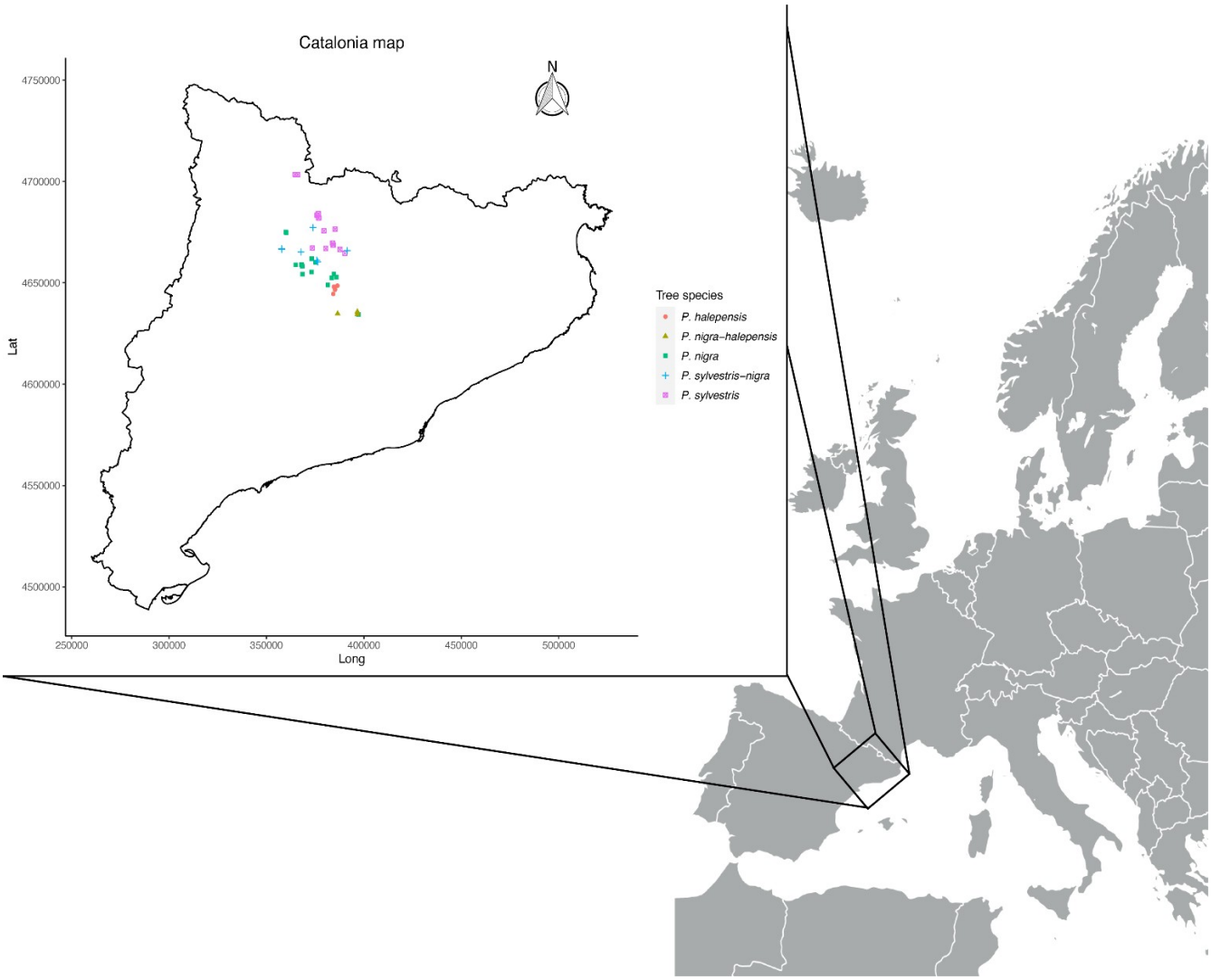

Table 1. Characteristics of the study plots

Forest type (no. of plots)	Range	BA, m^2 ha^{-1}	No. of trees per hectare	Altitude, m a.s.l	Slope, %	pH*	C:N ratio	P mg/kg
Ps	Min.	18.0	681	854	4	4.8	6.9	2.0
(14)	Mean	29.8	1362	1197	22	7.2	12.4	5.8
	Max.	41.5	1517	1615	37	8.3	19.5	9.0
Pn	Min.	16.1	638	397	5	8.0	4.0	3.0
(14)	Mean	27.7	1692	763	16	8.2	14.4	5.0
	Max.	39.1	2838	1040	32	8.4	21.3	9.0
Ph	Min.	24.0	1006	520	10	8.2	12.5	3.0
(4)	Mean	28.8	2093	612	16	8.3	13.6	4.8
	Max.	33.6	3088	661	34	8.4	14.8	6.0
Ps–Pn	Min.	11.5	477	1030	8	6.6	12.1	2.0
(7)	Mean	23.5	1161	1085	24	7.7	14.5	3.3
	Max.	31.8	2870	1148	31	8.3	19.8	5.2
Pn–Ph	Min.	17.6	1229	390	9	8.2	11.1	2.0
(3)	Mean	19.7	1806	469	12	8.3	13.2	4.0
	Max.	20.9	2761	577	13	8.4	15.4	5.0

Abbreviations: BA, basal area; P, phosphorus; Ps, *Pinus sylvestris*; Pn, *Pinus nigra*; Ph, *Pinus halepensis*; Ps–Pn, *P. sylvestris–P. nigra*; Pn–Ph, *P. nigra–P. halepensis.* * *significant differences were found in pH between tree host, with P.sylvestris plots showing similar pH values that P. sylvestris-P. nigra but significant lower pH values compared to the other tree hosts.*

2.2. Soil sampling

Soils were sampled during the autumn season (October and November). Prior to this study, a 10 × 10 m plot had already been established in the centre of each of the selected forest stands for the long-term monitoring of fungal fruiting (Martínez de Aragón et al., 2007). We extracted four soil subsamples, one from the centre of each of the four sides of these plots. The upper litter layer was discarded from all soil cores to reduce the sampling of needle-associated saprotrophs (Voříšková et al., 2014). We used a rectangular steel drill to extract a soil core with a depth of 30 cm and a width of 6 ξ 4.5 cm. The four soil subsamples were pooled in the field and approximately 1 kg of the mixed sample was stored at 4°C for < 24 h before being sieved through a 3-mm mesh sieve and then stored at –20°C. A subset of the sieved sample was used to determine soil physico-chemical parameters and the remainder was freeze-dried and homogenized, using a pestle and mortar to form a fine powder and then stored at –20°C.

2.3. *Soil analyses*

The soil samples were analysed in the laboratory using the methodology described by Alday et al. (2012). Each sample was air-dried and then sieved (≤2-mm mesh). Soil texture was analysed (i.e., clay, sand and lime proportions) using the Bouyoucos-method (Day, 1965). We determined the soil characteristics using the following techniques: soil pH and electrical conductivity (EC) using a conductivity meter in a 1:2.5 soil:deionized water slurry (Allen, 1989); total N concentration using the Kjeldahl method (Bremner and Mulvaney, 1982); available P concentration using the Olsen method (Olsen and Sommers, 1982); total organic matter and total carbon concentration using the Walkley–Black method (Walkley, 1947); and, finally, exchangeable cations as sodium (Na^+), potassium (K^+) and magnesium (Mg^{2+}) using atomic absorption spectroscopy after extraction with 1 N ammonium acetate (pH 7; Allen, 1989; Anderson and Ingram,1993).

2.3. Fungal community analyses

Fungal DNA was extracted from 500 mg of homogenized soil using a NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol. We amplified the fungal internal transcribed spacer 2 (ITS2) region in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) using the primers gITS7 (Ihrmark et al., 2012), ITS4 (White et al., 1990) and ITS4arch (Sterkenburg et al., 2018). Each primer was fitted with 8-bp tags differing in at least three positions to individually identify each sample during *a posteriori* bioinformatics analyses. We optimized the number of PCR cycles in each sample with the aim of obtaining PCR products that formed weak to medium PCR bands on agarose gels to reduce size length biases (Castaño et al., 2020), which was achieved in most of the samples by using 21–26 cycles. The final concentrations in the PCR reactions were: 25 ng template, 200 μM of each nucleotide, 2.75 mM $MgCl_2$, gITS7 primer at 500 nM, ITS4 and ITS4A primers at 300 nM and 0.025 U μL^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA) in 1X buffer in 50 μL reactions. PCR cycling conditions were as follows: 5 min at 95°C, followed by 21–30 cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C and a final extension step at 72°C for 7 min. Samples were amplified in triplicates together with negative controls obtained during the DNA extraction and PCR. Amplified products were purified using an AMPure kit (Beckman Coulter Inc. Brea, CA, USA) and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled, and the mix was purified using an EZNA Cycle Pure kit (Omega Bio-Tek) following the protocol. Amplicons were quantified and visualized using a 7500 DNA chip in a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Samples were sequenced at

SciLifeLab NGI, Uppsala, Sweden on a PacBio RS II system (Pacific Biosciences, Menlo Park, CA, USA) using four SMRT cells. The PacBio RS II system was chosen because although significantly lower sequencing depths are obtained with this system compared with those obtained with other sequencing platforms, recent studies have shown that PacBio sequencing results are less distorted than those obtained using other sequencing platforms, even at low levels of sequence output (Castaño et al., 2020).

2.4. Bioinformatics analyses

Sequences were quality filtered and clustered using the SCATA pipeline (https://scata.mykopat.slu.se/). We first removed DNA sequences with lengths of <200 bp before screening for sample tags and primers with at least a 90% primer match. Sequences were pair-wise compared using 'usearch' (Edgar, 2011) after collapsing homopolymers to 3 bp. Pairwise alignments were scored as follows: mismatch penalty of 1, gap open penalty of 0 and a gap extension penalty of 1. We clustered the sequences into operational taxonomic units (OTUs) using single linkage clustering, with a maximum distance of 1.5% to the closest neighbour required to enter clusters. Global singletons were excluded from further analyses. Sequence data are archived at NCBI's Sequence Read Archive under accession number PRJNA641823(www.ncbi.nlm.nih.gov/sra). In total, we obtained 31,642 ITS2 sequences after quality control.

2.5. Taxonomic and functional identification

We taxonomically identified the 600 most abundant OTUs, which represented 93% of the total sequences. We selected the most abundant sequence from each OTU for taxonomic identification using PROTAX software (Somervuo et al., 2016) implemented in PlutoF, using a 50% probability of correct classification (considered by Somervuo et al. (2016) to be "plausible identifications"). These identifications were confirmed and some of them improved using massBLASTer in PlutoF against the UNITE database (Abarenkov et al., 2010). Taxonomic identities at species level were assigned based on >98.5% similarity to database reference sequences, or to other lower levels using the following criteria: genus based on >97% similarity, family based on >95% similarity, order based on >92% similarity and phylum based on >90% similarity. OTUs were assigned to the following functional guilds: (a) root-associated basidiomycetes, (b) root-associated ascomycetes, (c) moulds, (d) yeasts, (e) litter-associated basidiomycetes, (f) litter-associated ascomycetes, (g) pathogens, (h) moss-associated fungi, (i) soil saprotrophs (saprotrophic taxa commonly found in N-rich mineral soils) or (j) unknown function, based on the UNITE database, DEEMY (www.deemy.de) or FUNGuild (Nguyen et

al., 2016). However, for specific analyses, we used mycorrhizal community (which included root-associated basidiomycetes and root-associated ascomycetes) and saprotrophic fungal community (which included moss-associated fungi and soil saprotrophs).

2.6. Statistical analyses

Statistical analyses were implemented in the R software environment (version 3.6.0, R Development Core Team 2019). The *vegan* package was used for multivariate analyses (Oksanen et al., 2018), the *iNEXT* package for fungal diversity analyses (Hsieh et al., 2016), and the *ecodist* package for multiple distance matrix regressions (Goslee and Urban, 2007). For all compositional analyses, the species abundance matrix was first transformed, keeping only the OTUs that were present in more than 10% of the samples. Then, a Hellinger transformation was performed to account for taxa with low counts (Legendre and Gallagher, 2001).

First, variation partitioning (function "*varpart"*) was used to determine the relative contribution of soil parameters (i.e., sand content, pH, EC, N, P, C:N ratio, organic matter, K, Mg and Na), geographical distances and stand structure (i.e., host tree species, altitude, slope, number of trees per hectare and basal area) to the overall, mycorrhizal and saprotrophic community composition. To avoid multicollinearity, highly correlated soil variables were removed ($r > 0.7$, i.e., EC) before variation partitioning analysis was performed. Prior to analysis, the geographical distances were evaluated using principal coordinates of neighbours' matrices spatial eigenvectors (PCNM, *pcnm* function) based on UTM coordinates of the sampled stands with Euclidean distances. Moreover, in a second matrix we included mean annual temperature and annual precipitation to account for climatic regional and compare differences that may account for the geographic distance effect on the fungal community with and without mesoclimatic variables. Climatic data for the sampling locations were downloaded from the WorldClim database (www.worldclim.org). Thus, significant spatial eigenvectors were forward selected to be used as explanatory variables in the variation partitioning, together with soil and stand structural variables. The significance of each partition was tested using multivariate ANOVAs.

Second, differences in the overall fungal community composition between pure and mixed pine forests were assessed using permutational multivariate analyses of variance (PMAV, function "*adonis*") of a Bray–Curtis dissimilarity matrix. After that, the overall community matrix was split by main functional guilds (i.e., mycorrhizal and saprotrophs) into two matrices and analysed individually in the same way. Then, non-metric multidimensional scaling (NMDS, function "*metaMDS*") was implemented in order to visualize compositional differences in the

overall, mycorrhizal and saprotrophic guilds between pine hosts. Standard deviational ellipses were used to visualize the dispersion of each forest in the ordination space. Then, the variance of the Bray–Curtis dissimilarity matrix between pine hosts for each forest type was compared using the *betadisper* function, which is an analogue of a Levene's test.

Third, we used Hill's diversity indices (Hill, 1973) to describe differences in fungal diversity between pure and mixed pine forests. The overall, ectomycorrhizal and saprotrophic communities were analysed separately using linear models. Hill's diversity consists of three numbers: N0 is species richness; N1 is the anti-logarithm of Shannon's diversity index; and N2 is the inverse of Simpson's diversity index.

Fourth, for the description of the main environmental and geographical drivers of fungal species composition, we used multiple regression on distance matrices (MRM, function "*MRM*"; Goslee and Urban, 2007). For the overall, mycorrhizal and saprotrophic soil communities, distance-based regressions using Bray–Curtis dissimilarity as the response to environmental and geographical distances were fitted with 10,000 permutations to test statistical significance. Euclidean pair-wise distances between plots were calculated using matrices of geographical distances, soil physico-chemistry and forest structure. These models were repeated including all soil and forest variables to describe the main dissimilarity drivers. Coefficients from these models were used to predict Bray–Curtis scores resulting from the maximum sampled distance for each variable in isolation to compare their relative influence on fungal assemblages across sites (Guerin et al., 2014). Finally, the main mycorrhizal and saprotrophic species were correlated with the most influential environmental variables described.

3. Results

Overall, Basidiomycota was the most abundant phylum (57.8 ± 2.6% sequences) followed by Zygomycota (22.2 ± 2.5% sequences) and Ascomycota (19.8 ± 1.5% sequences). The most abundant guilds were moulds and mycorrhizal fungi, representing 39.8 ± 3.7% and 41.6 ± 3.7% of the sequences, respectively, followed by yeasts (7.5 ± 0.9%), saprotrophs (6.6 ± 1.7%) and. Finally, most of the root-associated fungi were mycorrhizal (41.6± 3.7%, ectomycorrhizal, ericoid mycorrhizal and arbuscular mycorrhizal), particularly ectomycorrhizal (39.6 ± 4.1 %).

3.1. Main drivers of fungal communities

When determining the relative importance of soil parameters (soil), geographical distance (distance) and forest structure (structure) to the overall fungal community composition, soil accounted for the greatest significant proportion of the total variance (25%, p-value <0.010), followed by geographical distance (14%, p-value <0.010). Forest structure accounted for only 7% of the variance, which was not significant (p-value = 0.149), and with only a slight shared variance with soil and geographic variables (Fig. 2a). When mycorrhizal and saprotrophic communities were analysed separately, soil still accounted for a significant proportion of the total variance (p-value <0.05): 23% and 22%, respectively. However, when considering the mycorrhizal guild, geographical distance accounted for 7% of the variance (p-value <0.05) and forest structure accounted for 5% of the variance; however, this effect was not significant (p-value = 0.488). Moreover, the shared variation between soil, distance and forest structure accounted for 6% of the total variance (Fig. 2b). Although soil and geographical distance accounted for a similar amount of variation in the saprotrophic community (22%, p-value <0.05), saprotrophs were not influenced by forest structure (<5% of variance, p-value = 0.368, Fig. 2c). Finally, similar results were found when the relative importance of soil parameters (soil), geographical distance (distance) and forest structure (structure) was assessed including mean annual temperature and mean annual precipitation (Fig.S4).

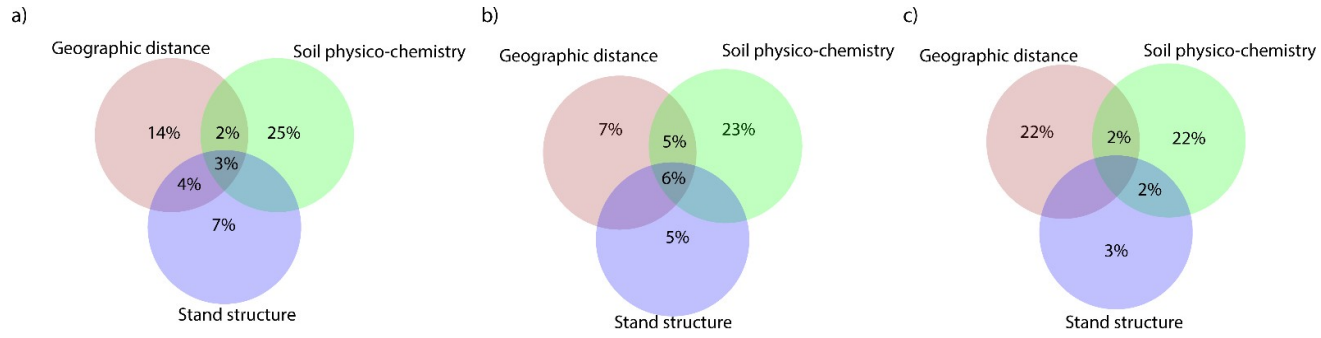

Fig. 2. Variance partitioning analyses of (a) overall, (b) mycorrhizal and (c) saprotrophic communities showing the effects of (i) geographical distance, (ii) soil physico-chemistry and (iii) stand structure. Values show the fraction of variation explained by each group of parameters as well as the shared contribution of each combination of them.

3.2. Similar fungal communities are found in pure and mixed forests and in all the pine species hosts

Overall, there were no significant differences in soil fungal composition between pure and mixed pine forests (PMAV: $r^2 = 0.03$, $F_{[1,41]} = 1.01$, p-value = 0.398) given that the standard deviational ellipses of both groups were clearly superposed in the centre of the ordination (NMDS stress = 0.16, Fig. 3). Similarly, when *P. halepensis*, *P. nigra*, *P. sylvestris*, *P. nigra–P. halepensis* and *P. sylvestris–P. nigra* forests were compared, there were no significant differences in fungal community composition among them ($F_{[4,41]} = 0.51$, p-value = 0.116, Fig. 4a). The lack of differences between pure and mixed forests was maintained independently of whether mycorrhizal ($F_{[4,41]} = 0.60$, p-value = 0.178, Fig. 4b) or saprotrophic communities ($F_{[4,41]} = 1.01$, p-value = 0.476, Fig. 4c) were analysed. Moreover, analysis of the community composition of the overall fungal community revealed a significant difference in the variance of the Bray–Curtis dissimilarity matrix between pine hosts ($F_{[4,41]} = 2.94$, p-value = 0.033); however, these differences were marginally significant for the mycorrhizal community and non-significant for the saprotrophic community ($F_{[4,41]} = 2.54$, p-value = 0.055 and $F_{[4,41]} = 1.82$, p-value >0.05, respectively). Finally, *Suillus spp*, *Rhizopogon mohelnensis, Phellodon niger* were abundant in all the pines hosts, while *Boletus edulis* was abundant only in *P. halepensis-P. nigra* forests. Conversely, *Cortinarius vernus* was abundant only in *P. halepensis* and *P. halepensis-P. nigra* forests, while *Inocybe ochroalba* was abundant only in *P. sylvestris* and *P. sylvestris-P. nigra* forests (Table S1).

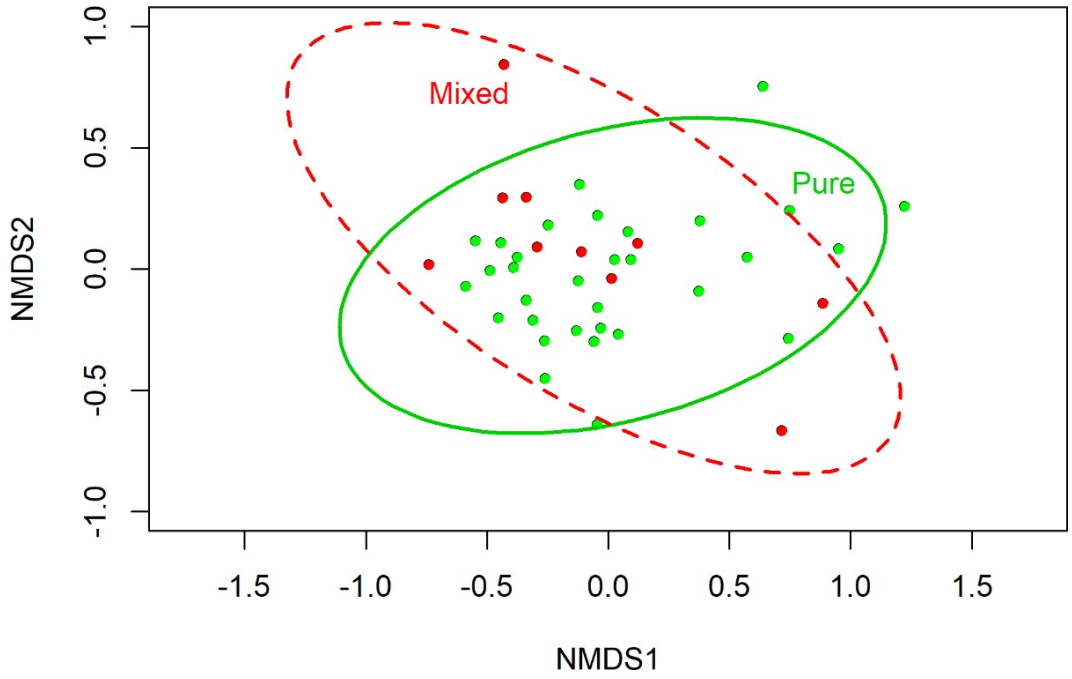

Fig. 3. Non-metric multidimensional scaling (NMDS) showing the overall fungal community similarity between pure (green) and mixed stands (red).

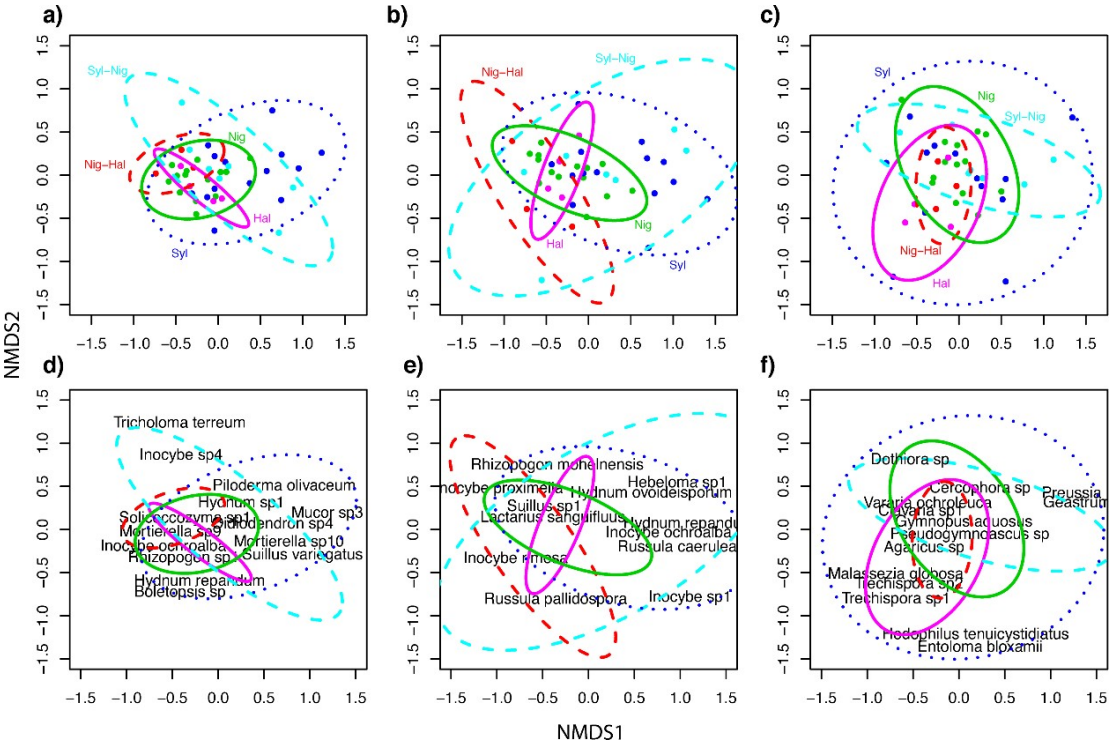

Fig. 4. Non-metric multidimensional scaling (NMDS) showing (a) overall, (b) mycorrhizal and (c) saprotrophic compositional differences between forest stands: *P. nigra–P. halepensis* (Nig–

Hal, red); *P. nigra* (Nig, green); *P. halepensis* (Hal, magenta); *P. sylvestris* (Syl, blue); *P. sylvestris–P. nigra* (Syl–Nig, cyan). (d) The most abundant species detected in the overall fungal community: *Inocybe* spp., *Piloderma olivaceum*, *Solicoccozyma* spp., *Hydnum* spp., *Oidiodendron* sp., *Knufia* spp., *Mortierella* spp., *Mucor* spp., *Suillus variegatus*, *Hydnum repandum*, *Boletopsis* spp., *Rhizopogon* spp., *Inocybe ochroalba*, *Knufia peltigerea* and *Tricholoma terreum*. (e) The most abundant species detected in the mycorrhizal community: *Rhizopogon mohelnensis*, *Inocybe proximella*, *Suillus* spp., *Lactarius sanguifluus*, *Inocybe rimosa*, *Russula pallidospora*, *Hebeloma* spp., *Hydnum ovoideisporum*, *Hydnum repandum*, *Inocybe ochroalba*, *Tricholoma* spp., *Russula caerulea* and *Inocybe* spp. (f) The most abundant species detected in the saprotrophic community: *Entoloma bloxamii*, *Hodophilus tenuicystidiatus*, *Trechispora* spp., *Trechispora invisitata*, *Agaricus* spp., *Pseudogymnoascus* spp., *Clavaria* spp., *Dothiora* spp., *Malassezia globosa*, *Gymnopus aquosus*, *Cercophora* spp., *Preussia* spp. and *Geastrum pectinatum*.

3.3. Fungal diversity between forest types

Shannon diversity values showed that the diversity of the overall and mycorrhizal fungal communities differed significantly among pine forests ($p < 0.05$), with values for the overall fungal community ranging from N1 = 28 to 73 and for the mycorrhizal community ranging from N1 = 6 to 20, but this was not the case for saprotrophic fungi (N1 = 4–7). Shannon diversity values of mycorrhizal fungi were higher in *P. sylvestris* and *P. sylvestris–P. nigra* forests (N1 = 54.2 and 73.4, respectively) than in *P. halepensis* and *P. nigra–P. halepensis* forests (N1 = 29.0 and 34.6, respectively). By contrast, no significant differences in Simpson diversity values were detected between pine forests for the overall (N2 = 11–23), mycorrhizal (N2 = 4–12) and saprotrophic fungal communities (N2 = 2–4).

Moreover, significant differences in fungal richness were detected among pine forests since the extrapolated confidence intervals did not overlap. Overall, the fungal richness of *P. sylvestris* forests (N0 = 448) was greater than that of *P. nigra–P. halepensis* (N0 = 193) or *P. halepensis* forests (N0 = 171). In the case of mycorrhizal richness, we found significant differences between *P. nigra–P. halepensis* or *P. halepensis* forests (observed richness <60) and *P. sylvestris* or *P. nigra* forests (observed richness >84; Fig. S1a). Conversely, we found significant differences in saprotrophic richness values, which were mainly due to the low richness values of the *P. nigra–P. halepensis* stands (observed richness = 16) compared with those of the *P. sylvestris* and *P. nigra* stands (observed richness >25; Fig. S1b). Finally, we

found no significant effect of the environmental variables on neither fungal richness nor diversity (data not shown).

3.4. Disentangling environmental drivers of fungal communities

The general distance-based regressions showed that overall, mycorrhizal and saprotrophic community compositions were shaped primarily by soil parameters and geographical distances (Table 2). When the relative contributions of geographic, soil and stand drivers of fungal community composition were analysed, we observed that the largest proportion of overall fungal dissimilarities between forests were explained by geographical distance (28% of dissimilarities, $R^2 = 0.15$), closely followed by pH (27% of dissimilarities, $R^2 = 0.47$; Fig. 5a). In both cases, increases in pH (ranging from 4.8 to 8.5) and geographical distance between forests were associated with significantly increased compositional dissimilarity between forest stands. In addition, there were significant changes in community composition as P (ranging from 2 to 9 mg/Kg) and the C:N ratio (ranging from 4 to 21.33) changed, but both variables explained smaller proportions of dissimilarity (19% of dissimilarities, $R^2 = 0.09$ and 16% of dissimilarities, $R^2 = 0.07$, respectively) than that explained by pH or geographical distance. When considering structural variables, only altitude had a significant effect on soil fungal composition (16% of dissimilarity, $R^2 = 0.10$), although this effect was also lower than that explained by pH or geographical distance. The mycorrhizal community showed similar general trends to that of the overall community, with geographical distance being the most important driver of community dissimilarities (29%, $R^2 = 0.15$), followed by pH (26%, $R^2 = 0.33$), P and the C:N ratio (17%, $R^2 = 0.07$, 17%, $R^2 = 0.06$ respectively), and altitude (16%, $R^2 = 0.08$, Fig. 5b). In both cases, pH was the only significant factor that was positively correlated with overall richness and mycorrhizal richness ($F_{[1,41]} = 23.41$, p-value <0.001, $R^2 = 0.33$). Finally, the largest proportion of saprotrophic community composition variation was explained by geographical distance (25%, $R^2 = 0.08$), followed by the C:N ratio, N (23% and 21% with $R^2 = 0.08$, respectively) and pH (17% $R^2 = 0.07$; Fig. 5c). Thus, when the values of these variables between forest stands increased saprotrophic compositional dissimilarity significantly increased. In addition, N was positively associated with saprotrophic richness ($F_{[1,41]} = 4.06$, p-value = 0.050, $R^2 = 0.05$). Moreover, we found no significant effect of the environmental variables on neither fungal richness nor diversity (data not shown).

Finally, the main mycorrhizal and saprotrophic species associated with these soil variables are described in Figure S3.

Table 2 Multiple regression analyses of distance matrices on overall, mycorrhizal and saprotrophic community composition

	Variables	Estimates	t-values	p-values
Overall	*Intercept*	0.39 ± 0.02	25.51	<0.001
$R^2 = 0.25$	Dist (geo)	0.23 ± 0.02	9.65	<0.001
	Dist (soil)	0.02 ± 0.001	9.77	<0.001
	Dist (forest)	0.003 ± 0.004	0.53	0.594
Mycorrhizae	*Intercept*	0.39 ± 0.02	21.70	<0.001
$R^2 = 0.23$	Dist (geo)	0.31 ± 0.03	10.87	<0.001
	Dist (soil)	0.02 ± 0.001	9.39	<0.001
	Dist (forest)	0.003 ± 0.004	1.03	0.305
Saprotrophs	*Intercept*	0.32 ± 0.02	13.95	<0.001
$R^2 = 0.13$	Dist (geo)	0.21 ± 0.04	5.79	<0.001
	Dist (soil)	0.02 ± 0.002	8.22	<0.001
	Dist (forest)	–0.005 ± 0.006	0.55	0.581

In all regressions, the Bray–Curtis community dissimilarity was used to measure fungal community distances and Euclidean distances for explanatory distance matrices, i.e., geographical distance (geo), soil, and forest variables (forest).

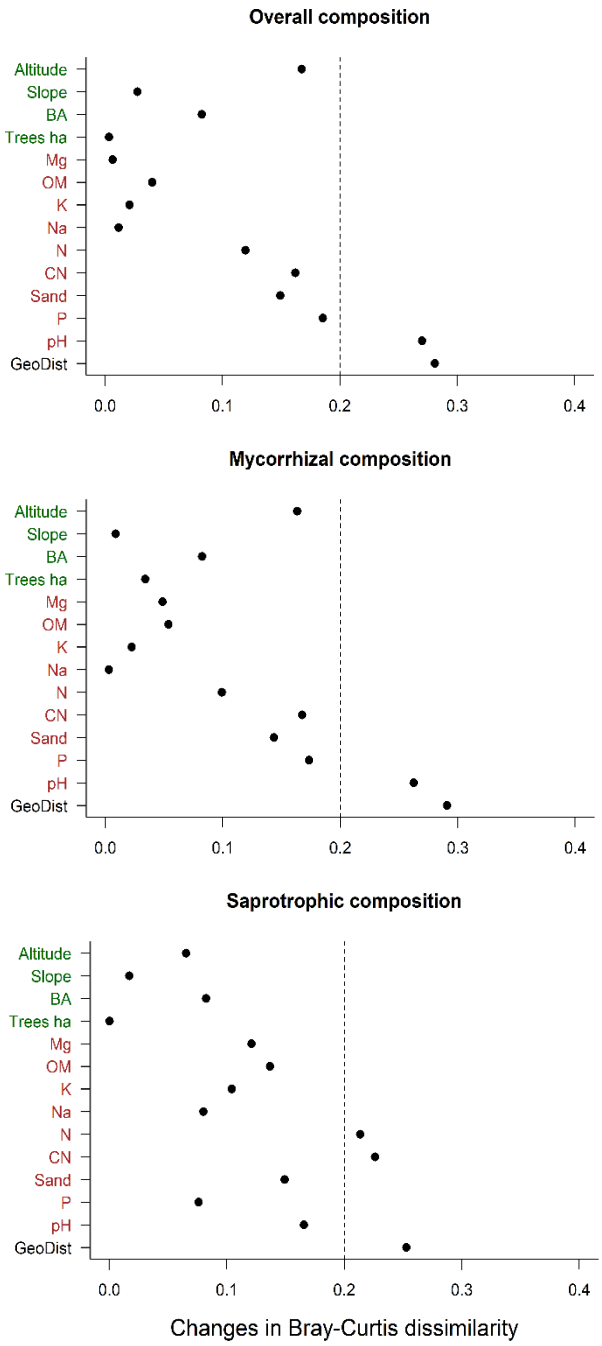

Fig. 5. Multiple regressions on distance matrices predicted changes in Bray–Curtis dissimilarity (horizontal axis) with environmental, i.e., soil physico-chemistry (brown) and stand variables (green), and geographical distance for (a) overall, (b) mycorrhizal and (c) saprotrophic community composition. The horizontal axis represents the maximum distance differences between plots of Bray–Curtis dissimilarity for each variable in isolation (y-axis). The vertical line marks the middle of the figure. Abbreviations: BA, basal area; Tree ha, number of trees per hectare; CN, C:N ratio; GeoDist, geographical distance; OM, organic matter. Considering that soil physico-chemistry and stand structure matrices effects are decomposed in the dissimilarity explained by their vectorial parts, the overall effect for both should include all their parts. In contrast, the geographical distance effect is plotted as the overall effect including all its vectorial variables.

4. Discussion

At regional spatial scales, niche processes, such as environmental filtering, and neutral processes, such as dispersal limitation driven by spatial structure, can be important determinants in structuring fungal communities (Cao et al., 2019). Our analyses revealed that niche processes dominated over neutral processes, and among them niche processes related with soil parameters largely determined the fungal community assemblages rather than other niche processes such as interspecific differences between *Pinus* species (i.e. host effects). However, the relative importance of soil variables on fungal community assembly varied between mycorrhizal and saprotrophic guilds because the mycorrhizal communities were primarily shaped by pH and P effects, whereas the saprotrophic communities were shaped mainly by the C:N ratio and N. Thus, these results suggest that different assembly mechanisms are involved in the structuring of mycorrhizal and saprotrophic communities. Nevertheless, our models indicate that pH, geographical distance, P and the C:N ratio were the strongest drivers shaping fungal communities at regional scales in these Mediterranean pine forests.

4.1. Fungal communities determined by geographical distance and soil rather than forest structure

There is a consensus that changes in soil physico-chemical properties, particularly the availability of nutrients such as N and P (Read and Perez-Moreno, 2003), shape soil fungal communities at global (Tedersoo et al., 2014), regional (Kivlin et al., 2014) and fine spatial scales (Glassman et al., 2015). Our results at regional scale agree with this given that mycorrhizal and saprophytic communities were primarily influenced by site-specific soil properties but also by geographical distance (i.e., dispersal limitation; Peay et al., 2012; Peay and Bruns, 2014). However, only a small proportion of compositional variation in this study was explained by the tree host (i.e., *Pinus* species) and stand structural variables. The lack of specificity between fungal and pine species may be related to the close phylogenetic relationships of the host trees considered here (Tedersoo et al., 2013). Moreover, the lack of stand effects on the soil fungal communities could be because fungal networks were sufficiently preserved and all stands had enough live roots to harbour similar mycorrhizal communities (Castaño et al., 2018a; Sterkenburg et al., 2019). Our results resemble those reported by Castaño et al. (2018b) in smaller-scale Mediterranean *Pinus pinaster* stands, where fungal communities were strongly affected by soil biochemistry and geographical distance. Thus, our analyses indicate that processes such as environmental filtering produced by abiotic

soil physico-chemical parameters play a dominant role in soil fungal compositional patterns in these Mediterranean pine forest ecosystems. Other processes like dispersal limitation or stochastic processes such as niche pre-emption (i.e. priority effects, Kennedy et al., 2009) might have a secondary role, potentially due to continuous forest cover promoting inoculum arrival from nearby similar forests (Redondo et al., 2020), but still mainly shaping ectomycorrhizal compositional patterns at regional spatial scales. The geographical distance effect on the soil fungal communities observed here is in accordance with distance decay similarity patterns observed in different ecosystems at local and regional scales (e.g. Bahram et al., 2013). Here, we attempted also to explore whether several other drivers could be confounded with the geographical distance effects on the soil fungal communities, such as altitude, stand structure, climate (mean annual temperature (MAT) and mean annual precipitation (MAP) and soil parameters (Fig.S4). However, only soil parameters and geographical distance emerged as significant.

4.2. Fungal community composition does not differ between pine species

As predicted, there was a lack of soil fungal compositional differences between pure and mixed forest, as well as when pure and mixed groups were split to consider the main *Pinus* host identities. Fungal community dissimilarity is tightly related to the phylogenetic distance between host tree species present in pure and mixed forests (Smith et al., 2009; Tedersoo et al., 2013; Glassman et al., 2017). In our study, the phylogenetic distance between studied pines was low because all hosts were congeneric and closely related within the *Pinus* genus (Gernandt et al., 2005). Although mycorrhizal fungi are known to show host specificity (Hausmann and Hawkes, 2010), in these pine forests tree–mycorrhizal fungal interactions between congeneric hosts are not significantly different (Tedersoo et al., 2013). Thus, there seems to be a lack of a host filtering effect on soil fungal community composition in areas where forests are dominated by phylogenetically related congeneric species, which has also been observed in North American pine forests, Mediterranean ecosystems (Glassman et al., 2015, Pérez-Izquierdo et al 2020) and for distinct *Salix* species (Erlandson et al., 2016).

The high level of heterogeneity between the selected forests in this study (high beta-diversity measured as deviational area) may explain the lack of compositional differences in the fungal communities among hosts (Fig 3). Previous studies have also reported higher community compositional dissimilarity when highly diverse and distant forests are grouped in compositional analyses (Alday et al., 2013). Therefore, recent studies have mainly focused on close homogeneous forests comprising hosts of different families or genera to detect

compositional differences in soil fungal communities between pure and mixed forests (Suz et al., 2017; Nagati et al., 2018) or in common garden experiments (Pérez-Izquierdo et al., 2019). Although these considerations should not bias the conclusions from our study, care should be taken in the forest-site selection process when aiming to test the drivers of community assembly.

As observed in previous smaller-scale studies of *P. pinaster* forests (Castaño et al., 2018b), similar compositional patterns were found in the overall and ectomycorrhizal communities (Fig. 4), therefore ectomycorrhizal taxa appeared to be the main source of the overall fungal community changes in these ecosystems. In addition, saprophytic community composition was similar in all pine forest types. Previous studies of saprophytic fungal communities have reported that these communities are mainly influenced by litter origin and chemistry (Štursová et al., 2020), which can vary with forest host and stands (Li et al., 2019). Most of the soil litter in our study plots originates from closely related species belonging to the *Pinus* genus, thus, the litter chemistry should not differ significantly among the examined pine stands (Otsing et al., 2018). Moreover, saprotrophic communities tend to be species-specific but converge compositionally with forest age (Štursová et al., 2020). Therefore, the lack of a host effect on the soil saprophytic community composition may be partially explained by the age of the forests under study. Nevertheless, further studies of saprophytic community composition should be undertaken to formally test this hypothesis in Mediterranean climates.

4.3. Fungal diversity across pure and mixed forest types

Mixed forests were expected to harbour higher levels of diversity than pure stands (Ishida et al., 2007; Cavard et al., 2011). Although the diversity of the overall and mycorrhizal fungal communities significantly differed between pure and mixed stands, the highest diversity values were detected in soils extracted from pure *P. sylvestris* stands. Pure and mixed forests shared a great number of OTUs (i.e., more than 80; Fig. S2), reducing the probability of finding compositional differences, which is likely to be related to the close phylogenetic relationships of the pine species in the study stands (Tedersoo et al., 2008). Nevertheless, there were significant differences in the OTU richness of the overall, mycorrhizal and saprophytic fungal communities, mainly because the richness values of *P. nigra–P. halepensis* stands were very low in comparison to those obtained for the rest of the stands, which is the opposite of the situation we had expected for a mixed forest. Unfortunately, the process behind these patterns in richness values is unknown but could relate to differences between habitats (i.e., mixed plots were located in the poorest and driest sites). Similar results have been described by

Erlandson et al. (2016), who concluded that ectomycorrhizal species richness differences were produced by soil properties and not by hosts that belonged to the same genus.

4.4. Disentangling environmental drivers of fungal communities

Our analyses indicate that niche processes are the most important driving forces shaping soil fungal communities in Mediterranean pine forests at the regional scale, with niche soil properties explaining greater compositional variation than geographical distance or other niche processes, as stand structure variation. This was consistent with previous studies that highlighted the importance of soil as an abiotic filter shaping arbuscular and ectomycorrhizal fungal composition (Lekberg et al., 2007; Glassman et al., 2017). Soil fertility has been reported to be a primary factor in determining the dominance of mycorrhizal communities, which is related to tree nutritional modes (Read and Perez-Moreno, 2003; Clemmensen et al., 2015). In addition, in our study, pH was a stronger driver of soil fungal composition than soil fertility. Previous studies have reported that pH shapes soil fungal and bacterial communities (Lladó et al., 2018; Goldmann et al., 2015) and influences soil processes (Härdtle et al., 2004) and the availability of nutrients (Adamczyk et al., 2016), such as N, which in turn determine the presence of specific mycorrhizal fungi (Read and Perez-Moreno, 2003; Kjøller et al., 2012; Morrison et al., 2016; de Witte et al., 2017). Thus, it seems that the relative importance of niche processes is maintained among mycorrhizal and saprotrophic guilds, however soil properties differently affect the two functional communities (Fig.5).

Although N has been described as an important soil element that determines fungal assemblages in boreal and temperate forests (Kyaschenko et al., 2017; Read and Perez-Moreno, 2003), in our study mycorrhizal fungal species composition was primarily driven by soil pH and P. In contrast, saprotrophic species composition was primarily associated with the C:N ratio and N. Free-living saprotrophs can have a strong influence on C:N ratios by assimilating C and N but then releasing the C into the atmosphere during respiration (Boddy et al., 2007). Conversely, some mycorrhizal fungi can affect the C:N ratio by taking N, which can then be transferred to the host trees, therefore increasing soil C:N ratios (e.g., Averill et al., 2014; Smith and Read, 2008; Clemmensen et al., 2015). P is a limiting element for primary productivity in Mediterranean ecosystems (Du et al., 2020), thus, it seems that P may be a more relevant trading element than N during the plant-fungal interactions in Mediterranean forests (Smith and Read, 2008), similar to the importance of N in other ecosystems (Hortal et al., 2017). The P gradient in our study ranged from 2 to 9 mg kg^{-1}, which are considered low values for Mediterranean ecosystems (Recena et al., 2016). Moreover, in these forests, soils are

characterized by high pH values where P is mainly present in bound forms and not freely available (Antoniadis et al., 2016). Previous studies already described mycorrhizal compositional and diversity changes across P gradients in Mediterranean and temperate forests (Zavišić et al., 2016; Pérez-Izquierdo et al., 2017, Almeida et al., 2019). Therefore, in these pine forests mycorrhizal compositional dissimilarities might be explained by the intraspecific differences in the absorption of P.

4.5. Conclusions

Our analyses indicate that niche processes (soil physico-chemistry) dominate over neutral processes (geographical distance), being the main drivers of fungal community composition and are more influential than tree hosts or forest stand structure in our set of pure and mixed *Pinus* forests along the Pre-Pyrenees. Fungal communities are not influenced by closely related congeneric host species but are primarily affected by soil properties, with pH, P and the C:N ratio the strongest predictors shaping fungal communities in these forest ecosystems. Importantly, mycorrhizal communities are significantly affected by P but not N, therefore a fundamental nutrient trader in these ecosystems. Conversely, saprotrophic communities are significantly influenced by the C:N ratio and N. Further research should focus on a better mechanistic understanding of how variations in soil P and C:N ratios affect soil fungal communities and, consequently, Mediterranean ecosystem functioning, especially in the current climate change context.

Acknowledgements

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References

Abarenkov, K., Nilsson, R.H., Larsson, K.H., Alexander, I.J., Eberhardt, U., Erland, S., ... & Koljalg, U., 2010. The UNITE database for molecular identification of fungi - recent updates and future perspectives. New Phytologist 186(2), 281-285. doi:10.1111/j.1469-8137.2009.03160.x

Adamczyk, B., Ahvenainen, A., Sietio, O. M., Kanerva, S., Kieloaho, A.-J., Smolander, A., & … Heinonsalo, J., 2016. The contribution of ericoid plants to soil nitrogen chemistry and

organic matter decomposition in boreal forest soil. Soil Biology and Biochemistry 104, 394-404. doi:10.1016/j.soilbio.2016.09.016

Allen S.E., 1989. Chemical Analysis of Ecological Materials. Blackwell's, Oxford.

Alday, J.G., Marrs, R.H., Martínez-Ruiz, C., 2012. Soil and vegetation development during early succession on restored coal wastes: A six-year permanent plot study. Plant and Soil 353, 305-320. doi:10.1007/s11104-011-1033-2

Alday, J.G., Cox, E.S., Pakeman, R.J., Harris M.P.K., Le Duc, M.G., Marrs, R.H., 2013. Overcoming resistance and resilience of an invaded community is necessary for effective restoration: a multi-site bracken control study. *Journal of Applied Ecology* 50, 156-167. doi:10.1111/1365-2664.12015

Alday, J.G., Martìnez de Aragòn, J., de-Miguel, S., Bonet, J.A., 2017. Mushroom biomass and diversity are driven by different spatio-temporal scales along Mediterranean elevation gradients. Scientific Reports 7, 45824. doi:10.1038/srep45824

Anderson, J.M., Ingram, J.S.I., 1993. Tropical Soil Biology and Fertility: A Handbook of Methods, second ed. C.A.B. International, Wallingford.

Antoniadis, V., Koliniati, R., Efstratiou, E., Golia, E., Petropoulos, S., 2016. Effect of soils with varying degree of weathering and pH values on phosphorus sorption. Catena 139, 214–219. doi:10.1016/j.catena.2016.01.008

Arfi, Y., Buée, M., Marchand, C., Levasseur, A., Record, E., 2012. Multiple markers pyrosequencing reveals highly diverse and host-specific fungal communities on the mangrove trees Avicennia marina and Rhizophora stylosa. FEMS Microbiology Ecology 79(2), 433-444. doi:10.1111/j.1574-6941.2011.01236.x

Averill, C., Turner B., Finzi A., 2014. Mycorrhiza-mediated competition between plants and decomposers drive soil carbon storage. Nature 505, 543-545. doi:10.1038/nature12901

Averill, C., Hawkes, C., 2016. Ectomycorrhizal fungi slow soil carbon cycling. Ecology Letters 19(8), 937-947. doi:10.1111/ele.12631

Awad, A., Majcherczyk, A., Schall, P., Schröter, K., Schöning, I., Schrumpf, M., and Seidel, D., 2019. Ectomycorrhizal and saprotrophic soil fungal biomass are driven by different factors and vary among broadleaf and coniferous temperate forests. Soil Biology and Biochemistry 131, 9-18. doi:10.1016/j.soilbio.2018.12.014.

Bahram, M., Kõljalg, U.; Courty, P.E.; Diédhiou , A.G., Kjølle, R., Põlme, S., Ryberg, M., Veldre, B., Tedersoo, L. 2013. The distance devay similarity in communities of ectomycorrhizal fungi in different ecosystems and scales. Journal of Ecology 101(5),

1335-1344. doi:10.1111/1365-2745.10120

Bardgett, R.D., van der Putten, W.H., 2014. Belowground biodiversity and ecosystem functioning. Nature 515, 505-511. doi:10.1038/nature13855

Bardgett R.D., Wardle D.A., 2010. Above-belowground Linkages: Biotic Interaction, Ecosystem Processes and Global Change. Oxford University Press.

Bello, J., Hasselquist, N.J., Vallet, P., Kahmen, A., Perot, T., Korboulewsky, N., 2019. Complementary water uptake depth of *Quercus petraea* and *Pinus sylvestris* in mixed stands during an extreme drought. Plant and Soil 437, 93-111. doi:10.1007/s11104-019-03951-z

Boddy, E., Hill, P.W., Farrar, J., David, L.J., 2007. Fast turnover of low molecular weight components of the dissolved organic carbon pool of temperate grassland field soils. Soil Biology and Biochemistry 39(4), 827-835. doi:10.1016/j.soilbio.2006.09.030

Bonet, JA., Palahí, M., Colinas, C., Pukkala, T., Fisher, CR., Miina, J., Martinez de Aragón, J. 2010. Modelling the production and species richness of wild mushrooms in pine forests of the Central Pyrenees in northeastern Spain. Canadian Journal of Forest Research 40(2), 347-356. doi:10.1139/X09-19

Bravo-Oviedo, A., Pretzsch, H., Ammer, C., Andenmatten, E., Barbati, A., ... & Zlatanov, T., 2014. European mixed forests: Definition and research perspectives. Forest Systems 23(3), 518-533. doi:10.5424/fs/2014233-06256

Bremner, J.M., Mulvaney, C.S., 1982. Nitrogen total. In: Miller, A.L., Keeney, D.R. (Eds.) Methods of Soil Analysis, second ed. American Society of Agronomy, Madison, 595–624.

Cao, M., Jia, T., Mi, J., Jing, J., Chai, B., 2019. Relative roles of niche and neutral processes on turnover of plant, fungal and bacterial communities in arid and semi-arid areas at the regional scale. Basic and Applied Ecology 40, 43-54. doi:10.1016/j.baae.2019.08.005

Castaño, C., Alday, J.G., Lindahl, B.D., Martínez de Aragón, J., de-Miguel, S., Colinas, C., Parladé, J., Pera, J., Bonet, J.A., 2018a. Lack of thinning effects over inter-annual changes in soil fungal community and diversity in a Mediterranean pine forest. Forest Ecology and Management 424, 420–427. doi:10.1016/j.foreco.2018.05.004

Castaño, C., Lindahl, B.D., Alday, J.G., Hagenbo, A., Martínez de Aragón, J., Parladé, J., Pera, J., Bonet, J.A., 2018b. Soil microclimate changes affect soil fungal communities in a Mediterranean pine forest. New Phytologist 220, 1211–1221. doi:10.1111/nph.15205

Castaño, C., Berlin, A., Brandström Durling, M., Ihrmark, K., Lindahl, B.D., Stenlid, J., Clemmensen K.E., Olson, Å. 2020. Optimized metabarcoding with Pacific biosciences

enables semi-quantitative analysis of fungal communities. New Phytologist 228(3). doi:10.1111/nph.16731

Cavard, X., Macdonald, S.E., Bergeron, Y., Chen, H.Y.H., 2011. Importance of mixedwoods for biodiversity conservation: Evidence for understory plants, songbirds, soil fauna, and ectomycorrhizae in northern forests. Environmental Reviews 19(NA), 142-161. doi:10.1139/A11-004

Clemmensen, K.E., Finlay, R.D., Dahlberg, A., Stenlid, J Wardle, D.A., 2015. Carbon sequestration is related to mycorrhizal fungal community shifts during longterm succession in boreal forests. New Phytologist 205, 1525-1536. doi:10.1111/nph.13208

Day, P.R., 1965. Particle fractionation and particle-size analysis. In: Black, C.A. (Ed.), Methods of Soil Analysis Part 1. Agronomy No. 9. American Society of Agronomy, Madison, WI.

de Witte, L.C., Rosenstock, N.P., van der Linde, S., Braun, S., 2017. Nitrogen deposition changes ectomycorrhizal communities in Swiss beech forests. Science of the Total Environment 605-606, 1083-1096. doi:10.1016/j.scitotenv.2017.06.142

Delgado-Baquerizo, M., Eldridge, D.J., Ochoa, V., Gozalo, B., Singh, B.K., Maestre, F.T., 2017. Soil microbial communities drive the resistance of ecosystem multifunctionality to global change in drylands across the globe. Ecology Letters 20(10), 1295-1305. doi:10.1111/ele.12826

Du, E., Terrer, C., Pellegrini, A.F.A., Ahlström, A., van Lissa, C.J., Zhao, X., Xia, N., Wu, X., Jackson, R.B., 2020. Global patterns of terrestrial nitrogen and phosphorus limitation. Nature Geoscience 13, 221-226. doi:10.1038/s41561-019-0530-4

Edgar, R.C., 2011. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19), 2460-2461. doi:10.1093/bioinformatics/btq461

Erlandson, S.R., Savage, J.A., Cavender-Bares, J.M., Peay, K.G., 2016. Soil moisture and chemistry influence diversity of ectomycorrhizal fungal communities associating with willow along an hydrologic gradient. FEMS Microbiology Ecology 92(1), fiv148. doi:10.1093/femsec/fiv148

Gernandt, D.S., Geada López, G., Ortiz García, S., Liston, A., 2005. Phylogeny and classification of Pinus. Taxon 54(1), 29-42. doi:10.2307/25065300

Glassman, S.I., Peay, K.G., Talbot, J.M., Smith, D.P., Chung, J.A., Taylor, J.W., Vilgalys, R., Bruns, T.D., 2015. A continental view of pine-associated ectomycorrhizal fungal spore banks: A quiescent functional guild with a strong biogeographic pattern. New Phytologist 205(4), 1619-1631. doi:10.1111/nph.13240

Glassman, S.I., Wang, I.J., Bruns, T.D., 2017. Environmental filtering by pH and soil nutrients drives community assembly in fungi at fine spatial scales. Molecular Ecology 26(24), 6960-6973. doi:10.1111/mec.14414

Goldmann, K., Schöning, I., Buscot, F., Wubet, T., 2015. Forest management type influences diversity and community composition of soil fungi across temperate forest ecosystems. Frontiers in Microbiology 6, 1300. doi:10.3389/fmicb.2015.01300

Goslee, S.C., Urban, D.L., 2007. The ecodist package for dissimilarity-based analysis of ecological data. Journal of Statistical Software 22(7), 1-19. doi:10.18637/jss.v022.i07

Gravel, D., Canham, C.D., Beaudet, M., Messier, C., 2006. Reconciling niche and neutrality: The continuum hypothesis. Ecology Letters 9(4), 399-409. doi:10.1111/j.1461-0248.2006.00884.x

Green, J., Bohannan, B.J.M., 2006. Spatial scaling of microbial biodiversity. Trends in Ecology and Evolution 21(9), 501-507. doi:10.1016/j.tree.2006.06.012

Guerin, G.R., Biffin, E., Jardine, D.I., Cross, H.B., Lowe, A.J., 2014. A spatially predictive baseline for monitoring multivariate species occurrences and phylogenetic shifts in mediterranean southern Australia. Journal of Vegetation Science 25(2): 338-348. doi:10.1111/jvs.12111

Guo, J., Ling, N., Chen, Z., Xue, C., Li, L., Liu, L., ... & Vandenkoornhuyse, P., 2020. Soil fungal assemblage complexity is dependent on soil fertility and dominated by deterministic processes. New Phytologist 226(1), 232-243. doi:10.1111/nph.16345

Hagenbo, A., Piñuela, Y., Castaño, C., Martínez de Aragón, J., de-Miguel, S., Alday, J.G., Bonet, J.A. 2020. Production and turnover of mycorrhizal soil mycelium relate to variation in drought conditions in Mediterranean *Pinus pinaste*r, *Pinus sylvestris* and *Quercus ilex* forests. New Phytologist 230, 1609-1622.

Hausmann, N.T., Hawkes, C.V., 2010. Orders of plant host establishment alters the composition of arbuscular mycorrhizal communities. Ecology 91, 2333-2343. doi:10.1078/0367-2530-00142

Härdtle, W., Von Oheimb, G., Friedel, A., Meyer, H., Westphal, C., 2004. Relationship between pH-values and nutrient availability in forest soils - The consequences for the use of ecograms in forest ecology. Flora 199, 134-142. doi:10.1078/0367-2530-00142

Hill, M.O., 1973. Diversity and evenness: a unifying notation and its consequences. Ecology 54, 427-432.

Hortal, S., Plett, K.L., Plett, J.M., Cresswell, T., Johansen, M., Pendall, E., Anderson, I.C., 2017. Role of plant-fungal nutrient trading and host control in determining the competitive

success of ectomycorrhizal fungi. ISME Journal 11(12), 2666-2676. doi:10.1038/ismej.2017.116

Hsieh, T.C., Ma, K.H., Chao, A., 2016. iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). Methods in Ecology and Evolution 7(12), 1451-1456. doi:10.1111/2041-210X.12613

Ihrmark, K., Bödeker, I.T.M., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., Strid, Y., Stenlid, J., Brandström-Durling, M., Clemmensen, K.E., Lindahl, B.D., 2012. New primers to amplify the fungal ITS2 region - evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiology Ecology 82, 666–677. doi:10.1111/j.1574-6941.2012.01437.x

Ishida, T.A., Nara, K., Hogetsu, T., 2007. Host effects on ectomycorrhizal fungal communities: Insight from eight host species in mixed conifer-broadleaf forests. New Phytologist 174(2), 430-440. doi:10.1111/j.1469-8137.2007.02016.x

Jarvis, P., Rey, A., Petsikos, C., Wingate, L., Rayment, M., Pereira, J., ... & Manca, G., 2007. Drying and wetting of Mediterranean soils stimulates decomposition and carbon dioxide emission: The "Birch effect,". Tree Physiology 27(7), 929-940. doi:10.1093/treephys/27.7.929

Jones, M.D., Durall, D.M., Cairney, J.W.G., 2003. Ectomycorrhizal fungal communities in young forest stands regenerating after clearcut logging. New Phytologist 57(3), 399-422. doi:10.1046/j.1469-8137.2003.00698.x

Kennedy, PG., Peay, KG., Bruns, TD. 2009. Root tip competition among ectomycorrhizal fungi. Are priority effects a rule or an exception? Ecology 90(8), 2098-2107. doi:10.1111/mec.15493

Kernaghan, G., Patriquin, G., 2011. Host Associations Between Fungal Root Endophytes and Boreal Trees. Microbial Ecology 62, 460-473. doi:10.1007/s00248-011-9851-6

Kivlin, S.N., Winston, G.C., Goulden, M.L., Treseder, K.K., 2014. Environmental filtering affects soil fungal community composition more than dispersal limitation at regional scales. Fungal Ecology 12, 14-25. doi:10.1016/j.funeco.2014.04.004

Kjøller, R., Nilsson, L.O., Hansen, K., Schmidt, I.K., Vesterdal, L., Gundersen, P., 2012. Dramatic changes in ectomycorrhizal community composition, root tip abundance and mycelial production along a stand-scale nitrogen deposition gradient. New Phytologist 194(1), 278-286. doi:10.1111/j.1469-8137.2011.04041.x

Konvalinková, T., Püschel, D., Řezáčová, V., Gryndlerová, H., Jansa, J., 2017. Carbon flow from plant to arbuscular mycorrhizal fungi is reduced under phosphorus fertilization. Plant

and Soil 419, 319-333. doi:10.1007/s11104-017-3350-6

Kyaschenko, J., Clemmensen, K.E., Karltun, E., Lindahl, B.D., 2017. Below-ground organic matter accumulation along a boreal forest fertility gradient relates to guild interaction within fungal communities. Ecology Letters 20, 1546–1555. doi:10.1111/ele.12862

Legendre, P., Gallagher, E.D., 2001. Ecologically meaningful transformations for ordination of species data. Oecologia 129, 271-280. doi:10.1007/s004420100716

Lekberg, Y., Koide, R.T., Rohr, J.R., Aldrich-Wolfe, L., Morton, J.B., 2007. Role of niche restrictions and dispersal in the composition of arbuscular mycorrhizal fungal communities. Journal of Ecology 95(1), 95-105. doi:10.1111/j.1365-2745.2006.01193.x

Li, Y., Bezemer, M., Yang, J., Xiaotao, L., Xinyu, L., Wenju, L., Xingguo, H., Li, Q., 2019. Changes in litter quality induced by N deposition alter soil microbial communities. Soil Biology and Biochemistry 130, 33-42. https://doi.org/10.1016/j.soilbio.2018.11.025

Lladó, S., López-Mondéjar, R., Baldrian, P., 2017. Forest Soil Bacteria: Diversity, Involvement in Ecosystem Processes, and Response to Global Change. Microbiology and Molecular Biology Reviews 81(2), e0063-16. doi:10.1128/mmbr.00063-16

Lladó, S., López-Mondéjar, R., Baldrian, P., 2018. Drivers of microbial community structure in forest soils. Applied Microbiology and Biotechnology 102, 4331-4338. doi:10.1007/s00253-018-8950-4

Losos, J.B., 2008. Phylogenetic niche conservatism, phylogenetic signal and the relationship between phylogenetic relatedness and ecological similarity among species. Ecology Letters 11(10), 995-1003. doi:10.1111/j.1461-0248.2008.01229.x

Martìnez de Aragòn, J., Bonet J.A., Fisher C.R., Colinas C., 2007. Productivity of ectomycorrhizal and edible saprotrophic fungi in pine forests of the pre-Pyrenees mountains, Spain: Predictive equations for forest management of mycological resources. Forest Ecology and Management 252, 239-256. doi:10.1016/j.foreco.2007.06.040

Molina, R., Massicotte, H.B., Trappe, J.M. 1992. Specificity phenomena in mycorrhizal symbioses: community-ecological consequences and practical implications. In: Allen, M.F., (Ed.), Mycorrhizal Functioning. An Integral Plant–Fungal Process. Chapman & Hall, New York, 357-423.

Morrison, E.W., Frey, S.D., Sadowsky, J.J., van Diepen, L.T.A., Thomas, W.K., Pringle, A., 2016. Chronic nitrogen additions fundamentally restructure the soil fungal community in a temperate forest. Fungal Ecology 23, 48-57. doi:10.1016/j.funeco.2016.05.011

Nagati, M., Roy, M., Manzi, S., Richard, F., Desrochers, A., Gardes, M., Bergeron, Y., 2018.

Impact of local forest composition on soil fungal communities in a mixed boreal forest. Plant and Soil 432, 345–357. doi:10.1007/s11104-018-3806-3

Nguyen, N.H., Song, Z., Bates, S.T., Branco, S., Tedersoo, L., Menke, J., Schilling, J.S., Kennedy, P.G., 2016. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecology 20, 241–248. doi:10.1016/j.funeco.2015.06.006

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H., Szoecs, E., Wagner, H., Oksanen, M.J., 2018. Package "vegan."

Olsen, S.R., Sommers, L.E., 1982. Phosphorus. In: Miller, A.L., Keeney, D.R. (Eds.), Methods of Soil Analysis. American Society of Agronomy, Madison, pp. 403–427

Otsing, E., Barantal, S., Anslan, S., Koricheva, J., Tedersoo, L. Litter species richness and composition effects on fungal richness and community structure in decomposing foliar and root litter, Soil Biology and Biochemistry, 2018 125, 328-339. doi:10.1016/j.soilbio.2018.08.006

Peay, K.G., Bruns, T.D., 2014. Spore dispersal of basidiomycete fungi at the landscape scale is driven by stochastic and deterministic processes and generates variability in plant-fungal interactions. New Phytologist 204(1), 180-191. doi:10.1111/nph.12906

Peay, K.G., Schubert, M.G., Nguyen, N.H., Bruns, T.D., 2012. Measuring ectomycorrhizal fungal dispersal: Macroecological patterns driven by microscopic propagules. Molecular Ecology 21(16), 4122-4136. doi:10.1111/j.1365-294X.2012.05666.x

Pérez-Izquierdo, L., Zabal-Aguirre, M., Flores-Rentería D., González-Martínez, S.C., Buée, M., Rincón, A., 2017. Functional outcomes of fungal communitiy shifts driven by tree genotype and spatial-temporal factors in Mediterranean pine forests Environmental Microbiology 19(4), 1639-1652. doi:10.1111/1462-2920.13690

Pérez-Izquierdo, L., Zabal-Aguirre, M., González-Martínez, S.C., Buée, M., Verdú, M., Rincón, A., Goberna, M., 2019. Plant intraspecific variation modulates nutrient cycling through its below ground rhizospheric microbiome. Journal of Ecology 107(4), 1594-1605. doi:10.1111/1365-2745.13202

Pérez-Izquierdo, L., Zabal-Aguirre, M., Verdú, M., Rincón, A., 2020. Ectomycorrhizal fungal diversity decreases in Mediterranean pine forests adapted to recurrent fires. Molecular Ecology 29(13), 2463-2476. doi:10.1111/1365-2745.13202

Read, D., Perez-Moreno J., 2003. Mycorrhizal nutrient cycling in ecosystems-A journey towards relevance? New Phytologist 157(3), 475-492. doi: 10.1046/j.1469-

8137.2003.00704.x

Recena, R., Díaz, I., del Campillo, M.C., Torrent, J., Delgado, A., 2016. Calculation of threshold Olsen P values for fertilizer response from soil properties. Agronomy for Sustainable Development 36, 54. doi:10.1007/s13593-016-0387-5

Redondo, M.A., Berlin, A., Boberg, J., Oliva J., 2020. Vegetation type determines spore deposition within a forest–agricultural mosaic landscape. *FEMS Microbiology Ecology*, 96(6), fiaa082. doi:10.1093/femsec/fiaa082

Santos-Silva, C., Gonçalves, A., Louro, R., 2011. Canopy cover influnce on macrofungal richness and sporocarp production in montado ecosystems. Agroforestry systems 82, 149-159. doi:10.1007/s10457-011-9374-7.

Sardans, J., Peñuelas, J., 2013. Plant-soil interactions in Mediterranean forest and shrublands: Impacts of climatic change. Plant and Soil 373, 1-15. doi:10.1007/s11104-013-1591-6

Smith, M.E., Douhan, G.W., Fremier, A.K., Rizzo, D.M., 2009. Are true multihost fungi the exception or the rule? Dominant ectomycorrhizal fungi on Pinus sabiniana differ from those on co-occurring Quercus species. New Phytologist 182(2), 295-299. doi:10.1111/j.1469-8137.2009.02801.x

Smith, S.E., Read, D.J., 2008. Mycorrhizal Symbiosis, third ed. Academic Press, San Diego.

Somervuo, P., Koskela, S., Pennanen, J., Henrik Nilsson, R., Ovaskainen, O., 2016. Unbiased probabilistic taxonomic classification for DNA barcoding. Bioinformatics 23(19), 2920-2927. doi:10.1093/bioinformatics/btw346

Sterkenburg, E., Clemmensen, K.E., Ekblad, A. Finlay, R.D., Lindahl, B.D., 2018. Contrasting effects of ectomycorrhizal fungi on early and late stage decomposition in a boreal forest. *ISME J* 12, 2187–2197 2018. https://doi.org/10.1038/s41396-018-0181-2

Sterkenburg, E., Clemmensen, K.E., Lindahl, B.D., Dahlberg, A., 2019. The significance of retention trees for survival of ectomycorrhizal fungi in clear-cut Scots pine forests. Journal of Applied Ecology 56(6), 1367-1378. doi:10.1111/1365-2664.13363

Štursová, M., Šnajdr, J., Koukol, O., Tláskal, V., Cajthaml, T., Baldrian, P., 2020. Long-term decomposition of litter in the montane forest and the definition of fungal traits in the successional space. Fungal Ecology 46, 100193. doi:10.1016/j.funeco.2020.100913

Suz, L.M., Kallow, S., Reed, K., Bidartondo, M.I., Barsoum, N., 2017. Pine mycorrhizal communities in pure and mixed pine-oak forests: Abiotic environment trumps neighboring oak host effects. Forest Ecology and Management 406, 370-380. doi:10.1016/j.foreco.2017.09.030

Tedersoo, L., Jairus, T., Horton, B.M., Abarenkov, K., Suvi, T., Saar, I., Kõljalg, U., 2008. Strong host preference of ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest as revealed by DNA barcoding and taxon-specific primers. New Phytologist 180(2), 479-490. doi:10.1111/j.1469-8137.2008.02561.x

Tedersoo, L., Mett, M., Ishida, T.A., Bahram, M., 2013. Phylogenetic relationships among host plants explain differences in fungal species richness and community composition in ectomycorrhizal symbiosis. New Phytologist 11(3), 822-831. doi:10.1111/nph.12328

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N.S., Wijesundera, R., ... & Abarenkov, K., 2014. Global diversity and geography of soil fungi. Science 346(6213): 1256688. doi:10.1126/science.1256688

Tedersoo, L., Anslan, S., Bahram, M., Drenkhan, R., Pritsch, C., S., Buegger, F., Padari, A., Hagh-Doust, N., Mikryukov, V., , Gohar, D., ... & Abarenkov, K., 2020. Regional scale in-depth analysys of soil fungal diversity reveals strong pH and plant speciea effect in Northern Europe. Frontiers in Microbiology 11, 1953. doi:10.3389/fmicb.2020.01953

Tomao, A., Bonet, J.A., Martínez de Aragón, J., de-Miguel, S., 2017. Is silviculture able to enhance wild forest mushroom resources? Current knowledge and future perspectives. Forest Ecology and Management 402, 102-114. doi:10.1016/j.foreco.2017.07.039

Thuiller, W., Albert, C., Araújo, M.B., Berry, P.M., Cabeza, M., Guisan, A., Hickler, T., Midgley, G.F., Paterson, J., Schurr, F.M., Sykes, M.T., Zimmermann, N.E., 2008. Predicting global change impacts on plant species' distributions: Future challenges. Perspectives in Plant Ecology, Evolution and Systematics 9(3-4), 137-152. doi:10.1016/j.ppees.2007.09.004

Varenius, K., Kårén, O., Lindahl, B., Dahlberg, A., 2016. Long-term effects of tree harvesting on ectomycorrhizal fungal communities in boreal Scots pine forests. Forest Ecology and Management 380, 41-49. doi:10.1016/j.foreco.2016.08.006

Větrovský, T., Kohout, P., Kopecký, M., Machac, A., Man, M., Bahnmann, B.D., ... & Baldrina, P., 2019. A meta-analysis of global fungal distribution reveals climate-driven patterns. Nature Communications 5142. doi:10.1038/s41467-019-13164-8

Voříšková J., , Brabcová V., Cajthaml, T., Baldrian P., 2014. Seasonal dynamics of fungal communities in a temperate oak forest soil. New Phytologist 201(1), 269-278. doi:10.1111/nph.12481

Walkley, A., 1947. A critical examination of rapid method for determining organic carbon in soils. Soil Science 63, 251–254.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal

ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, pp. 315–322. doi:10.1016/B978-0-12-372180-8.50042-1

Zavišić, A, Nassal, P., Yang, N., Heuck, C., Spohn, M., Marhan, S., Pena, R., Kandeler, E., Polle, A., 2016. Phosphorus availabilities in beech (Fagus selvatica L.) forests impose habitat filtering on ectomycorrhizal communities and impact tree nutrition. Soil Biology and Biochemistry 98, 127-137. doi:10.1016/j.soilbio.2016.04.006

Zhang, T., Wang, N.F., Liu, H.Y., Zhang, Y.Q., Yu, L.Y., 2016. Soil pH is a key determinant of soil fungal community composition in the Ny-Ålesund Region, Svalbard (High Arctic). Frontiers in Microbiology 17:227. doi:10.3389/fmicb.2016.00227

Supplementary material

Table S1. Most abundant mycorrhizal species detected in pure and mixed stands of *Pinus sp.*

P. halepensis	***P. nigra*–*P. halepensis***	***P. nigra***	***P. sylvestris*–*P. nigra***	***P. sylvestris***
Suillus sp.	*Suillus* sp.	*Suillus* sp.	*Suillus* sp.	*Hydnum repandum*
Phellodon niger	*Phellodon niger*	*Phellodon niger*	*Phellodon niger*	*Phellodon niger*
Rhizopogon mohelnensis	*Inocybe* sp.	*Tricholoma* sp.	*Tricholoma* sp.	*Inocybe ochroalba*
Hydnum sp.	*Lactarius sanguifluus*	*Hydnum repandum*	*Hydnum repandum*	*Tricholoma terreum*
Russula pallidospora	*Cortinarius hydrobivelus*	*Russula* sp.	*Russula pallidospora*	*Russula caerulea*
Inocybe ochroalba	*Russula pesudoaeruginea*	*Inocybe* sp.	*Inocybe ochroalba*	*Suillus* sp.
Cortinarius vernus	*Craterellus lutescens*	*Craterellus cornucopioides*	*Rhizopogon molhensis*	*Rhizopogon molhensis*
Tomentella subclavigera	*Boletopsis* sp.	*Boletus edulis*	*Inocybe asterospora*	*Russula* sp.
Lactarius sanguifluus	*Lactarius deliciosus*	*Lactarius sanguifluus*	*Sebacina flagelliformis*	*Lactarius sanguifluus*
Craterellus lutescens	*Tricholoma* sp.	*Tomentella* sp.	*Tricholoma terreum*	*Craterellus lutescens*
Russula delica	*Rhizopogon mohelnensis*	*Lactarius deliciosus*	*Craterellus lutescens*	*Lactarius deliciosus*
Hydnum repandum	*Boletus edulis*	*Hydnellum* sp.	*Boletopsis* sp.	*Sebacina* sp.
Lactarius deliciosus	*Cortinarius vernus*	*Sebacina* sp.	*Sebacina cystidiata*	*Boletus edulis*

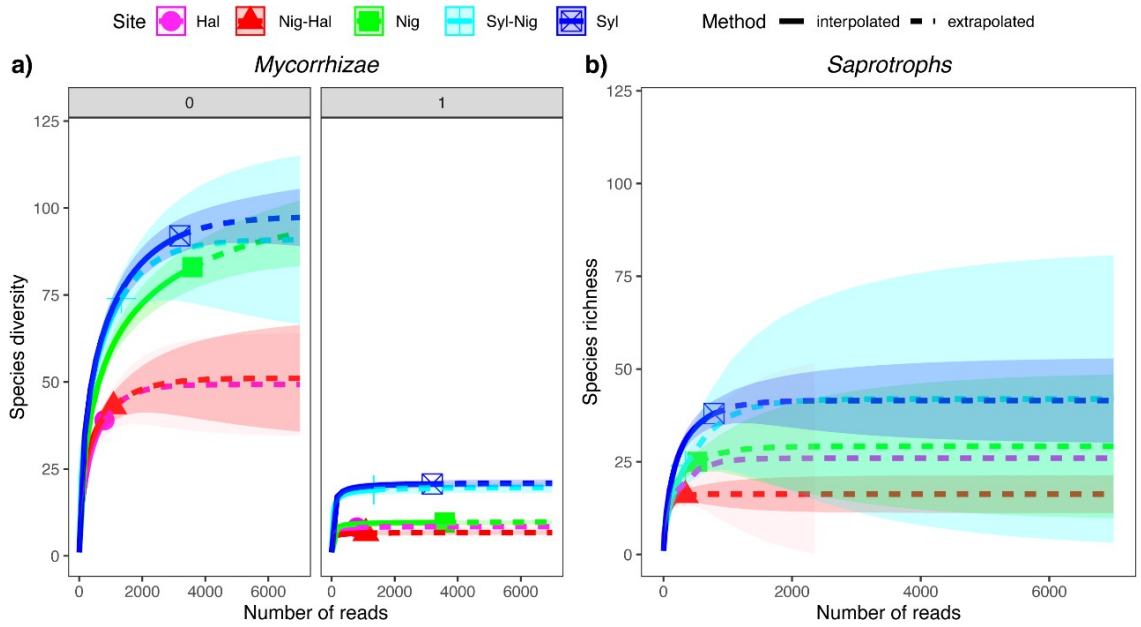


Fig. S1. Hill's N0 interpolated and extrapolated values for (a) mycorrhizal and (b) saprotrophic guilds between forest stands. Stands *P. halepensis* (Hal, magenta); *Pinus nigra–Pinus halepensis* (Nig–Hal, red); *P. nigra* (Nig, green); *P. sylvestris–P. nigra* (Syl–Nig, cyan); *Pinus sylvestris* (Syl, blue). N0 is species richness, N1 is the anti-logarithm of Shannon diversity. Unbroken and dashed parts of the curve denote interpolated and extrapolated values, respectively, and the shaded zone around each curve denotes the 95% confidence interval.

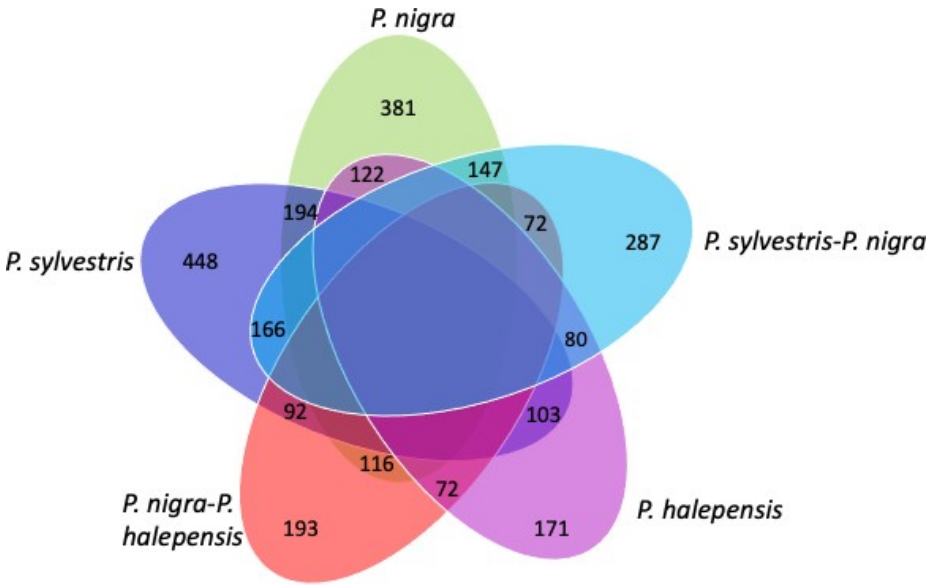
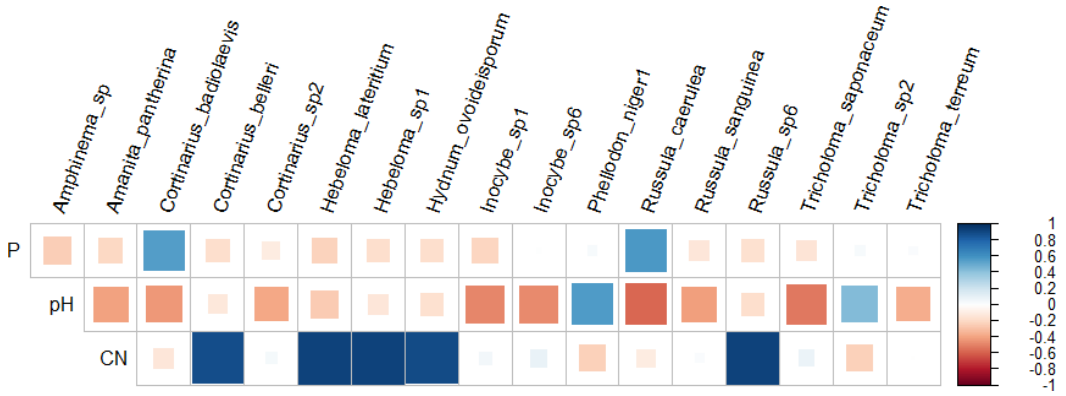


Fig. S2. Venn diagram showing the number of shared species in the overall fungal communities of the different pine host stands.

a)



b)

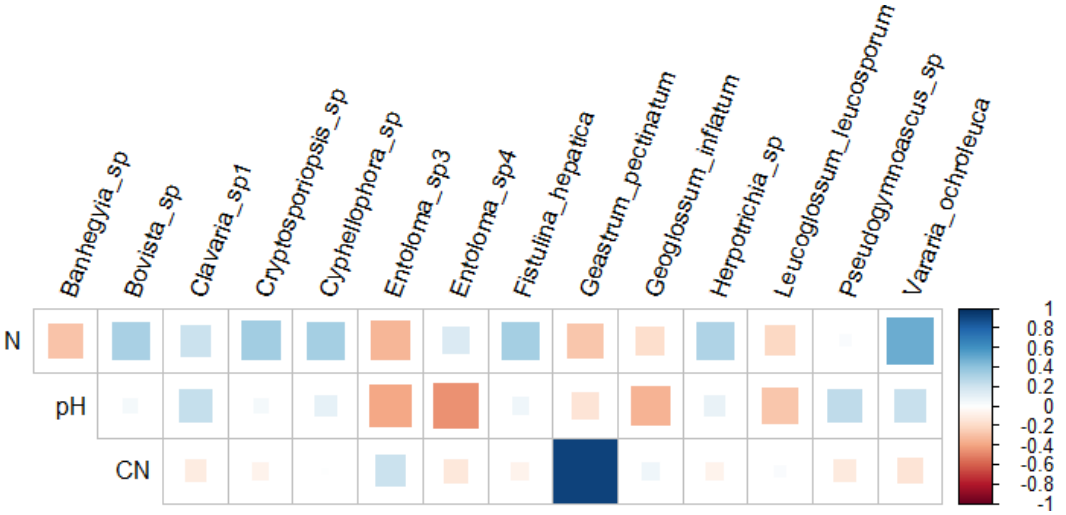


Fig. S3. Pearson correlations between soil variables (i.e., N, pH and the C:N ratio) associated with the main fungal compositional dissimilarities and the most abundant and frequent (a) mycorrhizal and (b) saprotrophic fungi. Square colours indicate positive and negative correlations and the size the correlation level.

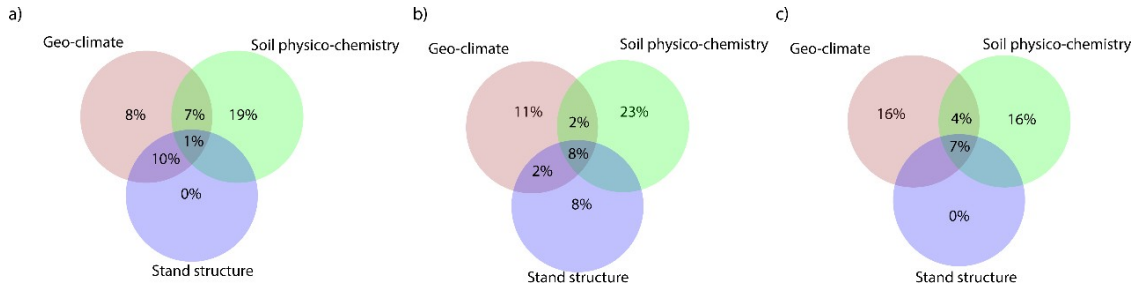


Fig. S4. Variance partitioning analyses of (a) overall, (b) mycorrhizal and (c) saprotrophic communities showing the effects of (i) geo- climate (matrix including geographic distance, mean annual temperature (MAT) and mean annual precipitation (MAP), (ii) soil physico-chemistry and (iii) stand structure. Values show the fraction of variation explained by each group of parameters as well as the shared contribution of each combination of them.

CHAPTER III

"Lack of phylogenetic differences in ectomycorrhizal fungi among distinct Mediterranean Pine forest habitats"

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Lack of phylogenetic differences in ectomycorrhizal fungi among distinct Mediterranean pine forest habitats

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Abstract: Understanding whether the occurrences of ectomycorrhizal species in a given tree host are phylogenetically determined can help assessing different conservational needs for each fungal species. In this study, we characterized ectomycorrhizal phylogenetic composition and phylogenetic structure in 42 plots with five different Mediterranean pine forests: i.e. pure forests dominated by *P.nigra, P.halepensis* and *P.sylvestris,* and mixed forests of *P.nigra-P.halepensis* and *P.nigra-P.sylvestris,* and tested whether phylogenetic structure of ectomycorrhizal communities differs among these. We found that ectomycorrhizal communities were not different among pine tree hosts neither in phylogenetic composition nor in structure and phylogenetic diversity. Moreover, we detected a weak abiotic filtering effect (4%), with pH being the only significant variable influencing phylogenetic ectomycorrhizal community, while phylogenetic structure was slightly influenced by the shared effect of stand structure, soil and geographic distance. However, phylogenetic community similarity increased at lower pH values, supporting that fewer, closely related species were found at lower pH values. Also, no phylogenetic signal was detected among exploration types, although short and contact were the most abundant types in these forest ecosystems. Our results demonstrate that pH but not tree host, acts as a strong abiotic filter on ectomycorrhizal phylogenetic communities in Mediterranean pine forests at local scale. Finally, our study shed light on dominant ectomycorrhizal foraging strategies in drought-prone ecosystems such as Mediterranean forests.

Keywords: DNA metabarcoding 1, phylogenetic structure 2, habitat filtering 3.

1. Introduction

Ectomycorrhizal fungi are essential organisms in forests, as they form symbiotic relations with trees providing them nutrients in exchange for photosynthetic carbon (Allen, 2007; Van Der Heijden, 2008; Smith & Read, 2008). Some ectomycorrhizal fungi are host specific (Allen 2007, Van Der Linde et al., 2018), and are influenced by tree species as well as by soil abiotic factors such as pH and nutrient availability (Kyaschenko et al., 2017; Suz et al., 2017). Therefore, host effect and abiotic soil parameters are often fundamental drivers of ectomycorrhizal community assembly (Dumbrell et al., 2010; Perez-Izquierdo et al., 2019). Moreover, previous studies showed that ectomycorrhizal taxonomic community composition does not significantly change between Mediterranean congeneric pine species (Adamo et al., 2021). Nevertheless, how ectomycorrhizal fungi are phylogenetically structured among Mediterranean pine host species and whether at both taxonomic and phylogenetic level respond to similar abiotic factors has not been assessed yet. Previous studies showed that ectomycorrhizal responses to climate warming are modulated by host plant performance and nutrient availability (Fernandez et al., 2016; Mohan et al., 2014). Therefore, it is crucial to disentangle whether these drivers influence ectomycorrhizal phylogenetic composition and structure, to better understand forest ecosystem functioning (Sardans & Peñuelaz, 2013).

Phylogenetic analyses are useful tools to estimate the relative importance of evolutionary and ecological forces structuring communities (Webb et al., 2002; Maherali & Klironomos, 2007; Peay et al., 2010). In this regard, phylogenetic indices have been implemented to calculate phylogenetic relatedness of an observed community and compare the value to expectations of community assembly under neutral processes from a regional species pool (Webb, 2000). Therefore, these indices enable us to characterize whether communities are more phylogenetically related (phylogenetic clustering) or less phylogenetically related (phylogenetic over dispersion) than expected by chance (Webb 2000, Webb 2002). In general, habitat filtering is the dominant assembly process when closely related species that share similar traits are selected to coexist within the community (i.e. phylogenetic clustering). In contrast, competition processes occur when distantly related species with dissimilar traits are selected to co-occur within a community (i.e. phylogenetic over dispersion, (Webb 2002), while random phylogenetic

structure is detected when none of the above processes are inferred (Webb 2002, Vamos et al., 2009). For example, Edwards et al. (2009), observed phylogenetic clustering of Agaricomycotina communities (including mycorrhizal and saprotrophs) and observed that xeric oak-dominated forests acted as a filter for these communities. Likewise, Egan et al. (2017), found phylogenetically clustered arbuscular mycorrhizal communities along an altitudinal gradient and observed that environment was the primary ecological factor structuring these communities, either via changes in host plant or fungal niches. Although the ecological processes filtering communities have recently received criticism (Kraft et al., 2015), investigating the communities' phylogenetic responses to environment in different ecosystems is fundamental to understand the mechanisms that structure communities (Emerson & Gillespie, 2008, Tucker et al., 2017). However, how ectomycorrhizal communities are phylogenetic structured in Mediterranean pine forests has not been studied yet.

The description of phylogenetic relations between ectomycorrhizal fungi might help to understand the evolutionary ecology of traits, species, and entire communities (Weber & Agrawal, 2012). In this regard, exploration types of ectomycorrhizal fungi represent an important group of functional traits, which are defined according to the hyphal morphology, i.e. long distance, medium distance, medium distance fringe, short distance, or contact exploration types (Agerer, 2001). The hyphal morphology determines access to distinct nutrient sources, for example nitrogen (N) (Hobbie & Agerer, 2010, Tedersoo & Smith 2013). Ectomycorrhizal species with short, contact and medium smooth distance exploration types may preferentially use soluble inorganic forms of N close to the host roots due to the lack the enzymes to access organic N forms (Lilleskov et al., 2002; Hobbie & Agerer, 2010). Conversely, some fungi have enzymes (i.e. fenton peroxidase) to access insoluble N substrates such as organic substrates and they usually show medium mat and long-distance exploration types (Tedersoo & Smith 2013, Koide et al., 2014). However, long and medium fringe exploration types might demand higher carbon cost on the host than shorter distance exploration types (Agerer et al. 2001; Fernandez et al., 2016), therefore species with shorter exploration types may be favored under stressful conditions (Fernandez et al., 2016, Castaño et al., 2018). In this regard, several studies have addressed ectomycorrhizal exploration types' responses to environmental drivers (Lilleskov et al., 2011; Defrenne et al., 2019), however, the phylogenetic pattern of the trait in Mediterranean ecosystems has rarely been assessed. Thus, understanding the phylogenetic relationships between ectomycorrhizal species and evolution of hyphal morphologies in the current climate change context might shed light on the future impacts on Mediterranean ecosystem functioning.

In this study, we aim to characterize ectomycorrhizal phylogenetic composition and phylogenetic structure in 42 plots of five different Mediterranean pine forests: i.e. pure forests dominated by *P. nigra, P. halepensis* and *P. sylvestris,* and mixed forests of *P. nigra-P. halepensis* and *P. nigra-P. sylvestris*. In line with the above premises, we hypothesized that:

Considering that *P. halepensis, P. nigra* and *P. sylvestris* are phylogenetically closely related (Liston et al., 2001; Gernandt et al., 2005; Saladin et al., 2017), we expect that ectomycorrhizal phylogenetic composition, structure and diversity will not be different among them due to co-evolutionary processes (Cavender-Bares et al., 2009).

Previous studies have identified that ectomycorrhizal taxonomic composition is influenced by soil parameters followed by geographical distance (Narwani et al., 2015; Egan et al., 2017). Thus, we hypothesized that soil physico-chemistry will act as the main habitat filter on ectomycorrhizal phylogenetic composition (Pérez-Valera et al., 2018). Finally, among abiotic filters pH, P and CN ratio strongly influenced ectomycorrhizal taxonomic community composition (Adamo et al., 2021). Here, we tested if these filters would act similarly over ectomycorrhizal phylogenetic composition.

In Mediterranean ecosystems, soil N might not be limiting due to warmer temperatures which may enhance N mineralization by increasing decomposition of the organic matter (Pérez-Valera et al., 2018, Adamo et al., 2021). Therefore, short exploration types could uptake nutrients close to the host roots. Here, we expected that short and contact exploration types will be dominant, thus both traits will be overrepresented and dispersed across the ectomycorrhizal phylogenetic tree in comparison with medium and long-term exploration types.

2. Materials and Methods

2.1. Sites selection

The study was conducted in the mountainous pre-Pyrenees region of Catalonia, North-eastern Spain (Fig. S1) in a set of long-term monitoring plots in which fungal fruiting has been recorded for ~20 years (Martinez de Aragon, 2007). The region is under the influence of Mediterranean climate, with a summer drought period from June to August and mean annual temperatures from 9° to 6 °C with most of the precipitation occurring in spring and autumn (Alday et al., 2017). The 42 pine forest were randomly selected from the 579 sites included in the Forest Ecological Inventory of Catalonia carried out by Centre de Recerca Ecològica i Aplicacions Forestals (CREAF, 1992), trying to preserve even-aged forest. From the total 42 forest, 32 correspond to pure pine forests, with 14 plots corresponding to *P. nigra* and *P. sylvestris*

species and 4 to *P. halepensis*, whilst 10 plots were mixed plots (7 mixed plots of *P. sylvestris* and *P. nigra* species and 3 plots dominated by *P. nigra* and *P. halepensis*). The main features of the study plots are summarized in the Table 1, Table S1.

2.2. Soil sampling

Soils were sampled during the Autumn season (October and November) in 2009. In each of the selected forest stands, a 10 × 10 m plot was stablished in the center for long-term monitoring of fungal fruiting (Alday et al., 2017). In each plot, we took four soil subsamples, i.e. one per plot-side (Adamo et al., 2021a), with a rectangular steel drill (30 cm depth and 6 x 4.5 width). The four soil subsamples were pooled in the field and around 1 kg of the mixed sample was placed on ice and taken to the laboratory for fungal DNA extraction. Similar procedure was followed for soil samples to determine soil physico-chemical parameters.

2.3. Soil analysis

Soil samples were analysed using the methodology described in Alday et al. (2012). Each sample was air-dried, sieved (≤2 mm mesh) and soil texture (clay, sand, and lime proportions) was analysed using the Bouyoucos--method (Day, 1965). Soil pH and electrical conductivity (EC) using a conductivity meter in a 1:2.5 soil:deionized water slurry (Allen, 1989). Total nitrogen concentration using the Kjeldahl-method (Bremner, 1952). Moreover, available phosphorus concentration using the Olsen method (Olsen, 1982); total organic matter and total carbon concentration using the Walkley-Black method (Walkley, 1947). Finally, exchangeable cations such as sodium (Na), potassium (K+) and magnesium (Mg2+) with atomic absorption spectroscopy after extraction with 1 N ammonium acetate (pH 7; (Allen, 1989; Anderson & Ingram, 1993).

Table 1. Table summarizing the main features of the study plots: BA (Basal area), Number of trees per hectare, Altitude, Slope, pH, CN ratio and P (Phosphorus). Ps: *P. sylvestris*, Pn: *P. nigra*, Ph: *P. halepensis*, Ps-Pn: *P.sylvestris-nigra*, Pn-Ph: *P. nigra-halepensis.*

Forest type	Range	BA, m^2 ha^{-1}	N. of tree per hectare	Altitude, m a.s.l	Slope, %	pH	CN ratio	P
Ps	Min.	18.0	681	854	4	4.8	6.9	2
(14)	Mean	29.8	1362	1197	22	7.2	12.4	5.8
	Max.	41.5	1517	1615	37	8.3	19.5	9
Pn	Min.	16.1	638	397	5	8.0	4.0	3
(14)	Mean	27.7	1692	763	16	8.2	14.4	5.0
	Max.	39.1	2838	1040	32	8.4	21.3	9
Ph	Min.	24.0	1006	520	10	8.2	12.5	3
(4)	Mean	28.8	2093	612	16	8.3	13.6	4.8
	Max.	33.6	3088	661	34	8.4	14.8	6
Ps–Pn	Min.	11.5	477	1030	8	6.6	12.1	2
(7)	Mean	23.5	1161	1085	24	7.7	14.5	3.3
	Max.	31.8	2870	1148	31	8.3	19.8	5
Pn–Ph	Min.	17.6	1229	390	9	8.2	11.1	2
(3)	Mean	19.7	1806	469	12	8.3	13.2	4.0
	Max.	20.9	2761	577	13	8.4	15.4	5

2.4. Fungal community and bioinformatic analysis

Fungal DNA was extracted from 0.5 g of homogenized soil using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol. Fungal internal transcribed spacer 2 (ITS2) region was amplified the in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) using the primers gITS7 (Ihrmark et al., 2012; ITS4 and ITS4A (White et al., 1990; Sterkenburg et al., 2018). We optimised the number of PCR cycles in each sample aiming for weak to medium PCR bands at the agarose gels, which was achieved in most of the samples by using 21-26 cycles. The final concentrations in the PCR reactions, PCR conditions, DNA purification and sequencing and bioinformatics analyses were as in explained Adamo et al. (2021). Sequence data are archived at NCBI's Sequence Read Archive under accession number PRJNA641823 (www.ncbi.nlm.nih.gov/sra).

2.5. Taxonomic and functional identification

We taxonomically identified the 600 most abundant OTUs, which represented 93% of the total sequences. We selected the most abundant sequence from each OTU for taxonomic identification, using PROTAX software (Somervuo et al., 2016) implemented in PlutoF, using a 50% probability of correct classification (called "plausible identifications") (Somervuo et al., 2016). These identifications were confirmed and some of them improved using massBLASTer in PlutoF against the UNITE (Abarenkov et al., 2010). Taxonomic identities at species level were assigned based on > 98.5% similarity with database references, or to other lower levels using the next criteria: genus on > 97%, family on > 95%, order on > 92% and phylum on >90% similarity. OTUs were assigned to the following functional guilds: a) root-associated basidiomycetes, b) root-associated ascomycetes, c) moulds, d) yeasts, e) litter-associated basidiomycetes, f) litter-associated ascomycetes, g) pathogens, h) moss-associated fungi, i) soil saprotrophs (saprotrophic taxa commonly found in N-rich mineral soils), j) unknown function, based on the UNITE database, DEEMY (www.deemy.de) or FUNGuild (Nguyen et al., 2016) . ECM species were assigned to exploration types according to the DEEMY database (Agerer & Rambold, 2004-2016).

2.6. Phylogenetic and statistical analyses

The ghost-tree approach (Fouquier et al., 2016), which allows sequence data to be integrated into a single tree, was used to reconstruct the fungal phylogenetic tree. Foundation phylogeny at the family level was derived by (Treebase ID S20837) (Zhao et al., 2017), following methodology and was based on the sequences of six genes 18sS rRNA, 28SrRNA, 5.8S rRNA, translation elongation factor 1-α(tef1α) and RNA polymerase II (two subunits: RPB1 and RPB2) (Mikryukov et al., 2020).

Statistical analyses were implemented in R software environment (version 3.6.1, R Development Core Team 2019). The *ape* package was used to load and manipulate the phylogenetic tree in newick format (Paradis et al., 2004), while the *phyloseq* package was used to import and handle OTU counts (McMurdie & Holmes, 2013), taxonomic assignments and associated phylogenetic tree. The philR package was used to analyse compositional data using the phylogenetic tree information (Silverman et al., 2017). The *picante* package was used to calculate the ectomycorrhizal phylogenetic structure indices (NRI and NTI) and Faith's phylogenetic

diversity (Faith, 1992; Kembel et al., 2010. The *vegan* package was used for the multivariate analyses (Oksanen et al., 2018).

For all compositional analyses, the ectomycorrhizal species abundance matrix was previously filtered to exclude the taxa that were not seen in at least 10% of samples to eliminate random noise. We analysed ectomycorrhizal phylogenetic community composition using philR which enables to transform compositional data into an orthogonal unconstrained space with phylogenetic and evolutionary interpretation (Silverman et al., 2017). First, PhilR Isometric Log Ratio transformations were built from the phylogenetic tree utilizing a weighted reference (Silverman et al., 2017), then a Euclidean distance matrix was built from the philR transformed data. After that, Redundancy analysis, (RDA function *"rda"*) was used to visualize ectomycorrhizal phylogenetic compositional differences between tree host species. Moreover, ectomycorrhizal phylogenetic differences between tree host species were tested using permutational multivariate analyses of variance (PMAV, function *"adonis"*) on the Euclidean dissimilarity matrix based on the philR transformed data. To test the phylogenetic structure of ectomycorrhizal communities between tree host species were calculated the standardized effect size of mean pairwise distances and mean nearest taxon distances using *ses.mpd* (Standardised effect of mean pairwise distances in communities) and *ses.mntd* (Standardised effect of nearest taxon index in communities) functions from *picante*. In each stand type, we compared the MPD and MNTD values with the MPD and MNTD distributions of random communities in order to identify whether communities were more over-dispersed or under-dispersed than expected by chance. We used the *independentswap* null model, which randomizes community data matrix with the independent swap algorithm maintaining species occurrence frequency and sample species richness, to construct from 9999 randomly assembled communities (Gotelli, 2000). After calculating SES.MPD and SES.MNTD, the values were multiplied by -1 as these values are equivalent to -1 times NRI (net relatdness index) and NTI (nearest taxon index), respectively. Importantly, an increase in the NRI value indicates increasing phylogenetic clustering (or decreasing overall relatedness) of a set of species relative to the source pool (Vamosi et al., 2017). On the other hand, the nearest taxon index (NTI) is a standardized measure of the mean phylogenetic distance to the nearest taxon in each sample/community (Vamosi et al., 2017). The NRI measures the standardized effect size of the mean phylogenetic distance (MPD), which estimates the average phylogenetic relatedness between all possible pairs of taxa in a community. The NTI calculates the mean nearest phylogenetic neighbor among the individuals in a community. The ectomycorrhizal phylogenetic diversity comparisons between tree host

species were done using Faith's PD phylogenetic diversity index with the function *pd*. Moreover, to assess the phylogenetic relationships among species change across space, we computed multiple-site phylogenetic turnover, nestedness and phylo-beta diversity (Sorensen similarity index) per tree host species using "*phylo.beta.multi"* function in the betapart package (Baselga & Olmes, 2012).

Second, variation partitioning (function "*varpart"*) was used to test the relative importance as variation sources of geographical distances, soil parameters (i.e. Sand content, K, Mg, organic matter, Na, N, P, water pH and CN ratio), and stand structure (i.e. Tree species, Altitude, Slope, Trees per hectare and Basal Area) in ectomycorrhizal phylogenetic composition (philR transformed data) and structure (NRI, NTI). To avoid multicollinearity before variation partitioning analysis highly correlated environmental variables were removed (r>0.7). The geographical distances included were previously evaluated using principal coordinates of neighbours matrices spatial eigenvectors (PCNM, *pcnm* function) based on UTM coordinates of the sampled stands with Euclidean distances. Thus, significant spatial eigenvectors were forward selected to be used as explanatory variables in the variation partitioning, together with soil and stand structural variables. The significance of each partition was tested using multivariate ANOVAs. Moreover, to evaluate the effect of pH, CN and P on the ectomycorrhizal phylogenetic composition we conducted a redundancy analysis (*rda* function). In addition, the *sm.density.compare (*n. of permutations = 999) from the *sm* package was used to randomly assign pH values between the five tree hosts and estimate how different the densities were using a permutational test of density equality (Bowman & Azzanini, 2018). Lastly, to visualize if the ectomycorrhizal phylogenetic communities were clustering across the pH gradient, we performed a hierarchical cluster analysis on the ectomycorrhizal phylogenetic compositional data based on the Euclidian distance matrix using the function *hclust* in the *stats* package.

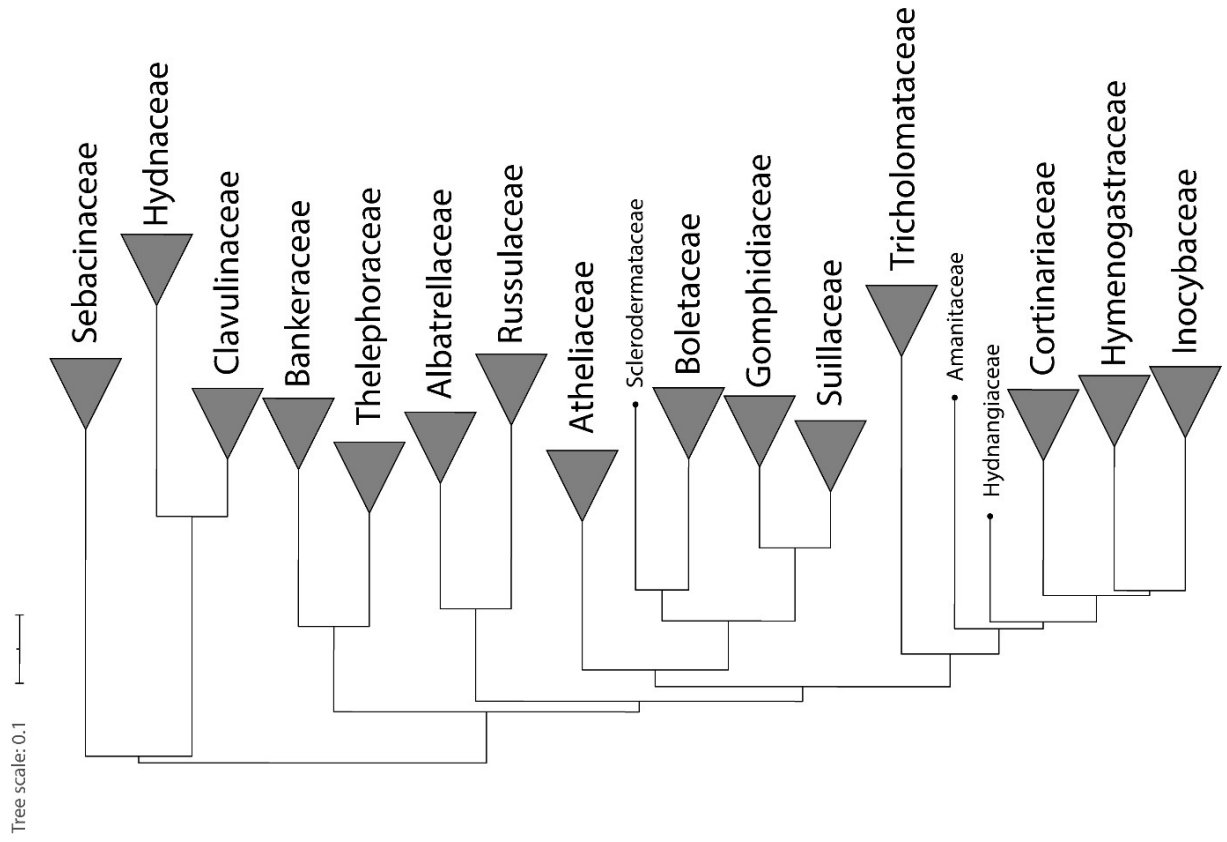
Finally, a binary data matrix was compiled with ectomycorrhizal exploration traits (contact, short, medium smooth, medium mat, medium-fringe and long). Then, we calculated the trait *ses.mpd* and *ses.mntd* using the *independentswap* null model to assess trait structure following the same methodology for communities. Finally, to test for a phylogenetic signal to exploration types, K' Blomberg statistics was calculated for the presence of the traits using the function *MultiPhylosignal* in the *picante* package (Blomberg et al., 2010; Kembel et al., 2010) . Moreover, the traits were visualized on the phylogenetic tree by plotting the exploration types at the tips of the phylogenetic tree following (Kembel et al., 2010).

3. Results

3.1. Ectomycorrhizal phylogenetic description

The hybrid phylogenetic tree of ectomycorrhizal fungi was consistent with Mikryukov et al. (2020) (Fig.1). The families Sebacinaceae, Clavulinaceae and Hydnaceae clearly formed a monophyletic group, while Bankeraceae Thelephoraceae, Russulaceae and Albatrellaceae formed two distinct clades (Fig.1). Moreover, two other family groups were identified, one including Atheliaceae, Sclerodermataceae, Boletaceae, Gomphidiaceae and Suillaceae and the other including Tricholomataceae, Amanitaceae, Hydnangiaceae, Cortinariaceae, Hymenogastraceae and Inocybaceae (Fig.1). Finally, the most abundant species in each tree host were indicated in table S2.

a)



b)

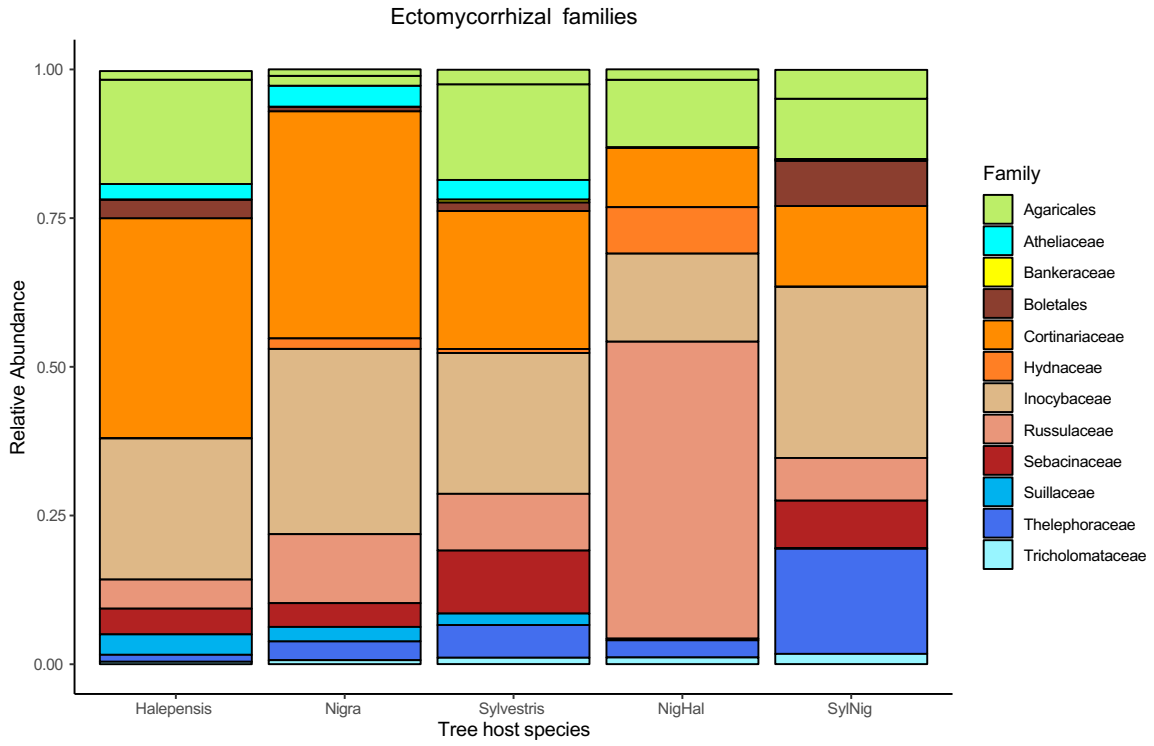


Fig.1 a) The hybrid phylogenetic tree of ectomycorrhizal families based on the foundation phylogeny derived by Zhao et al., (2017), based on the sequences of six genes 18sS rRNA, 28SrRNA, 5.8S rRNA, translation elongation factor 1-α(tef1α) and RNA polymerase II. b) Relative abundance of the most abundant ectomycorrhizal families.

3.2. Ectomycorrhizal phylogenetic composition, structure, and diversity

There were no significant differences in the ectomycorrhizal phylogenetic composition among tree host species (r^2= 0.10, $F_{[4,41]}$ = 1.12, p = 0.281). The RDA and the sd-ellipses based on the philR Euclidean distance matrix clearly showed that all forest types were overlapping at the ordination center (Fig. 2). Redundancy analyses resulted in two main axes that explained together 22% of the variance. However, *P. halepensis-nigra*, *P.halepensis* and *P. nigra* communities were less spread (homogeneous), while, *P.sylvestris* and *P.sylvestris-nigra* communities were more over dispersed in the ordination space (heterogeneous). Regarding ectomycorrhizal phylogenetic structure, no significant difference was detected for NRI ($F_{[4,41]}$ = 0.26, p = 0.901) between tree host species. Positive mean values of NRI were detected in *P. halepensis* (0.51±0.09), indicating ectomycorrhizal higher phylogenetic clustering. *P. nigra-halepensis* (0.14±0.83), *P. sylvestris-nigra* (0.06±0.25) and *P. sylvestris* (0.05±0.27), and *P. nigra* (-0.02±0.26) showed a dispersion of NRI values positive and negative around 0 (Fig. 3a). However, we detected significant differences in NTI values ($F_{[4,41]}$ = 2.96, p = 0.031) between tree

host species. Mean positive NTI values were detected across all tree host species, except in *P. sylvestris-nigra* (-0.46±0.37), indicating ectomycorrhizal phylogenetic clustering in *P. halepensis*, *P. nigra-halepenesis*, while *P. nigra*, *P. sylvestris* were not clearly defined, with values around 0, and a marginal phylogenetic over dispersion was detected in *P. sylvestris-nigra* (Fig. 3b).

The ectomycorrhizal phylogenetic diversity analysis showed no significant differences between tree host species ($F_{[4,41]} = 0.92$, $p = 0.458$, Fig 3c), with PD mean values ranging from 6.6 of *P. nigra* and 8.3 *P. sylvestris* (Fig 3c). In addition, analysing of multiple-site phylogenetic similarities showed that total beta diversity values were similar across host tree species (Table S1), although, species turnover resulted strongly higher than species nestedness across the tree host species and with similar values, except for *P. nigra-halepensis* (Phylo beta.sim: 0.40; Phylo beta.sne: 0.14; Table S1).

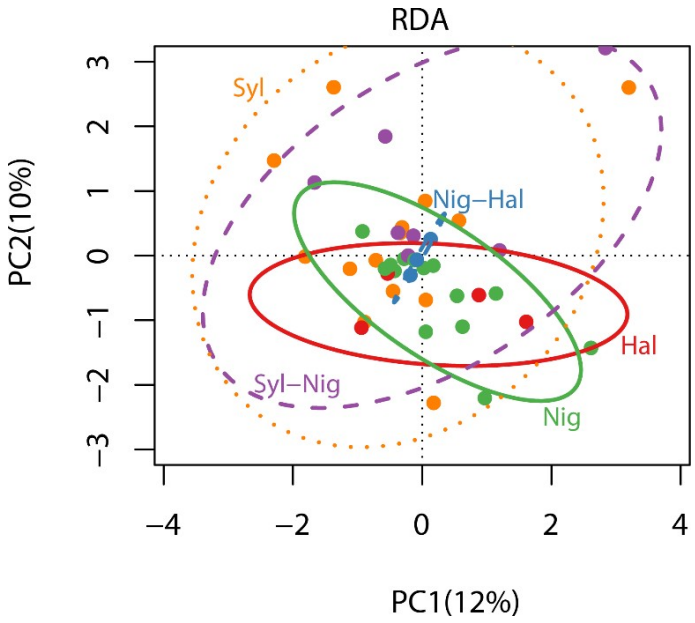


Fig. 2. philR RDA ordination based on Euclidean distance matrix displaying ectomycorrhizal phylogenetic community composition of *P. halepensis, P. nigra-halepensis, P. nigra, P. sylvestris-nigra* and *P. sylvestris* forest and the sd ellipses of each forest.

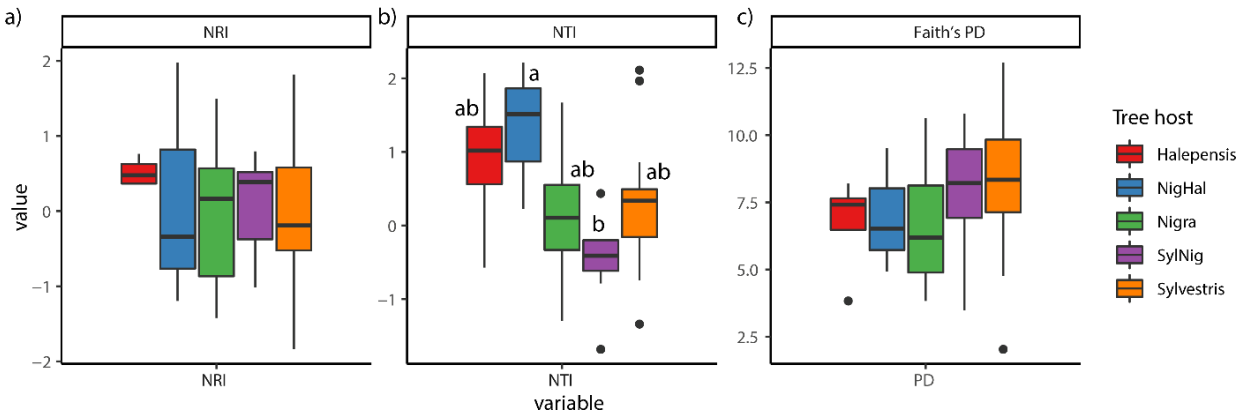


Fig. 3. Boxplots displaying a) Net Relatedness Index (NRI) values b) Nearest Taxon Index (NTI) c) Faith's PD values between tree host species (Halepensis: *P. halepensis,* NigHal*: P. nigra-halepensis,* Nigra*: P. nigra,* SylNig*: P. sylvestris-nigra* and Sylvestris: *P. sylvestris*). Means were compared using ANOVA and Tukey's HSD tests, with letters denoting significant differences between host species.

3.3. Main drivers of ectomycorrhizal phylogenetic composition and structure

When testing the relative importance of geographic distance, soil parameters and stand structure on ectomycorrhizal phylogenetic composition, soil accounted for the greatest proportion the total variance (4%) followed by geographic distance, however, these fractions were not significant ($p > 0.05$, Fig. 4a). Moreover, stand structure, soil and geographic distance shared 4% of the total variance. Conversely, when phylogenetic structure was analysed, stand structure, soil and geographical distance shared 8% proportion of variation, while stand structure accounted for 4% of the total variance ($p > 0.05$) (Fig 4b). Finally, phylogenetic structure was marginally influenced by soil (2%) and not by geographic distance.

pH was the only significant soil predictor influencing phylogenetic composition (Variance = 0.55, $F = 2.76$, $p = 0.002$). Thus, the distribution of pH values was significantly different across tree hosts ($p = 0.041$). Moreover, when pH densities were compared only P. sylvestris and P. nigra differed significantly ($p = 0.009$) showing a larger left tail towards lower pH values (Fig. S2). The hierarchical clustering of the ectomycorrhizal phylogenetic composition showed that communities were clustered into two main groups (Fig. S3), Here, the group composed by *P. halepensis, P. sylvestris* and *P. sylvestris-nigra* communities clustered at lower pH values (<7), while *P. nigra* and *P. nigra-halepensis* communities only occurred at higher pH values (>7) (Fig. S3).

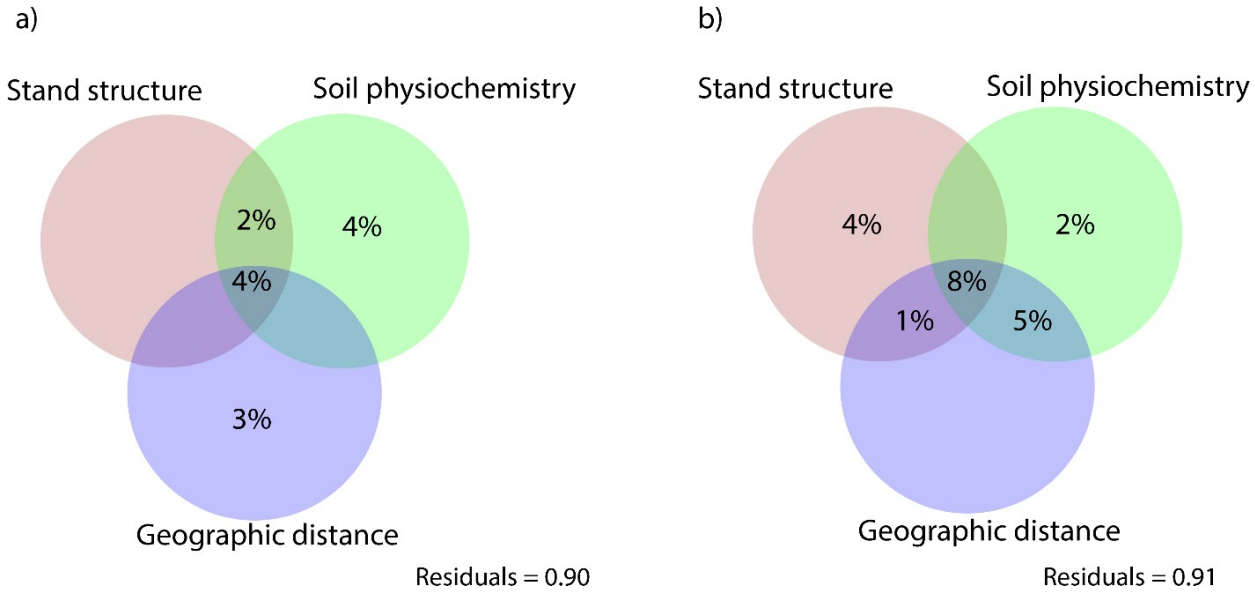


Fig. 4. Variance partitioning analyses for a) ectomycorrhizal phylogenetic composition and b) ectomycorrhizal phylogenetic structure (NRI, NTI indices) in response to stand structure, soil physiochemistry and geographic distance. Values show the fraction of variation explained by each group of parameters, as well as the shared contribution of each combination of them.

3.4. Trait evolution of the exploration types

When the exploration traits were visualized on the phylogenetic tree, 59 OTUs out of 184 had short exploration types up to, 53 had contact exploration types, while 39 OTUs and 25 OTUs had medium fringe and medium smooth exploration types. Conversely, medium mat and long exploration types were the least abundant with 9 and 8 OTUs, respectively. Finally, we did not find any phylogenetic signal for any exploration type ($0.25 < K < 0.77$, $p > 0.05$), as exploration types were dispersed across the phylogenetic tree (Fig. 5).

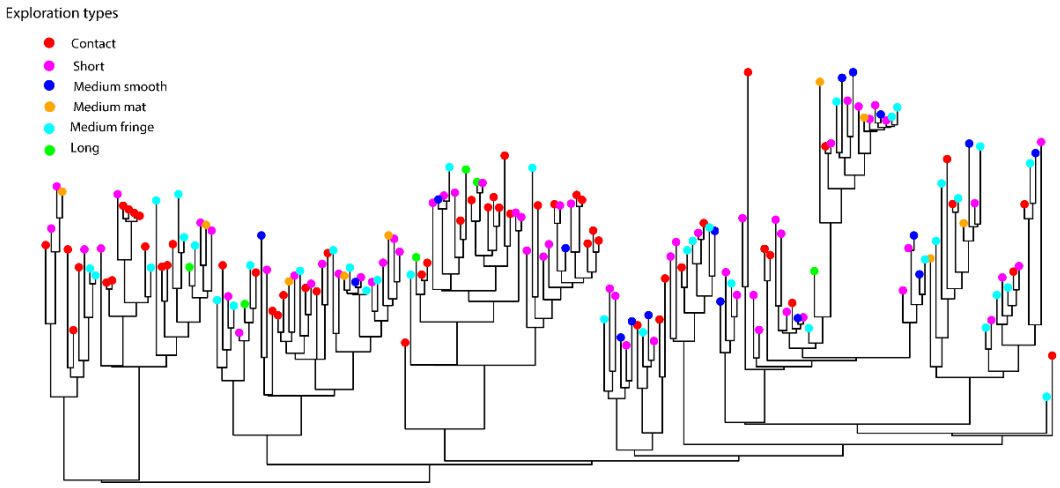


Fig. 5. The hybrid phylogenetic tree displaying the distribution of the exploration types (Contact, short, medium smooth, medium fringe and long).

4. Discussion

The results of our phylogenetic study on ectomycorrhizal communities in Mediterranean pine forests, showed that phylogenetic composition, structure and diversity were similar among habitats with distinct pine tree hosts. However, significant differences were found in nearest taxon index values between *P. nigra-halepensis* and *P. sylvestris-nigra*, probably not directly caused by differences in tree hosts but due to higher differences in the local abiotic conditions in *P. sylvestris-nigra* than in *P.halepensis-nigra* sites. Moreover, we detected a weak abiotic filtering effect on ectomycorrhizal phylogenetic compositional variation, being pH the only variable among soil variables that significantly influence ectomycorrhizal phylogenetic community. This finding suggests that pH acts as a strong abiotic filter on ectomycorrhizal community at both phylogenetic and taxonomic level (Adamo et al., 2021). In contrast, ectomycorrhizal phylogenetic structure variation was marginally influenced only by the shared effect of stand structure, soil and geographic distance. Therefore, phylogenetic structure may be indirectly influenced by other processes (i.e. competition; Tucker et al., 2017) not directly tested in this study. Finally, we identified that short and contact exploration types were the most abundant in these

forest ecosystems. Conversely, long exploration types were the least abundant, although, there was no phylogenetic signal since exploration types were dispersed across the phylogenetic tree.

4.1. Ectomycorrhizal phylogenetic description

Our study allowed us to investigate the phylogenetic relationships between 256 OTUs using a multiple gene tree at family level as a foundation tree which allows to build a better supported tree (Fig. 1a) (Mikryukov et al., 2020). Also, we were able to identify monophyletic groups of families, such as Sebacinaceae, Clavulinaceae and Hydnaceae, and Atheliaceae, Sclerodermataceae, Boletaceae, Gomphidiaceae and Suillaceae, however, this last clade formed a paraphyletic group with Russulaceae and Albatrellaceae. Moreover, two other family groups were identified, one including Bankeraceae, Thelephoraceae, Sclerodermataceae, Boletaceae, Gomphidiaceae and Suillaceae and the other including Tricholomataceae, Amanitaceae, Hydnangiaceae, Cortinariaceae, Hymenogastraceae and Inocybaceae. Therefore, the resolved phylogenetic tree resulted in a strong backbone for the downstream analyses as the level of resolution allows to perform reliable phylogenetic diversity analyses (Tomao et al., 2020). Finally, disentangling the ectomycorrhizal phylogenetic community structure in our study region, where the current climate change may lead to changes in ecosystems functioning, is crucial to predict the impacts on ectomycorrhizal taxonomic and phylogenetic community composition and diversity (Winter et al., 2013).

4.2 Ectomycorrhizal phylogenetic composition, structure, and diversity

Our results demonstrated that ectomycorrhizal phylogenetic community and diversity were not significantly different among pine tree host species or in NRI values, although there were differences in NTI values between *P. sylvestris-nigra* and *P.nigra-halepensis*. These results are in accordance with previous taxonomical studies on ectomycorrhizal communities between congeneric tree hosts (Erlandson et al., 2017; Arraiano-Castilho et al., 2020), in which a lack of phylogenetic differences was observed. Similarly, ectomycorrhizal community composition was not different between phylogenetically related pines in China (Ning et al. 2019). In contrast, several studies reported taxonomical differences between ectomycorrhizal communities between hosts of different families or genera (Suz et al., 2017; Nagati et al., 2018). Thus, it seems that at both taxonomic and phylogenetic level, ectomycorrhizal communities are not varying significantly among phylogenetically close related tree hosts (Tedersoo et al., 2014).

Similar phylogenetic studies detected phylogenetic clustering of Agaromycotina communities in xeric oak dominated forests and concluded that oak acted as a main habitat filter (Edwards et al., 2010). Here, our results showed an opposite trend, with no significant differences in ectomycorrhizal phylogenetic structure and diversity between pine tree hosts. However, we observed significant differences in phylogenetic dispersion among habitats with distinct pines hosts. For example, ectomycorrhizal species in P. *halepensis-nigra* forest resulted phylogenetic clustered, while in *P. sylvestris-nigra* were slightly more over dispersed at the tip of the phylogeny (Fig.3a), probably due to the low number of *P. nigra-halepensis* sites which may have caused underestimation of the differences between phylogenetic taxa. In this regard, the three *P. nigra-halepensis* sites showed similar soil properties (i.e. values range from pH: 8.18-8.38, CN: 15-11, P: 5-2, N: 0.16-0.12), which may have resulted in the occurrence of closely related species that are adapted to these similar abiotic conditions. These results may imply that Mediterranean pine host tree species are weak habitat filters for ectomycorrhizal fungi probably due to a lack of host specificity among congeneric hosts. Thus, our results are in agreement with the hypothesis that the lack of phylogenetic composition, structure and diversity between pine host species may be partially explained by possible conserved symbiosis between *Pinus* and ectomycorrhizal fungi (Tedersoo et al., 2017).

Finally, we found high and similar turnover values in all the tree host species forest, while nestedness was significantly lower, except in the case of *P. nigra-halepensis* forest. It seems that both environmental filtering by soil and dispersal limitation may, to a certain extent, promote species replacement among sites (Glassman et al., 2017). However, in *P. nigra-halepensis* forest higher nestedness might indicate local species loss probably due to its soil site conditions that resulted in the occurrence of locally adapted a subset of species

4.3 Main drivers of ectomycorrhizal phylogenetic composition and structure

In this study, we observed that soil parameters influenced ectomycorrhizal phylogenetic composition, while phylogenetic structure variation was primarily influenced by the shared effect of the three environmental filters. However, these three fractions were not significant and explained a residual amount of variation, thus, the second hypothesis is not accepted. Although previous studies have identified that soil parameters are main drivers of taxonomic ectomycorrhizal community variation in Mediterranean pine forest (Adamo et al., 2021), here, soil

parameters were marginally important in driving ectomycorrhizal phylogenetic composition. This may imply that at phylogenetic level, the lack of strong abiotic gradients results in the occurrence of non-closely related species which are adapted to heterogeneous but not specific environmental conditions (Pescador et al., 2021).

Soil properties have been widely described as a strong abiotic filter on taxonomic fungal communities at different spatial scales (Tedersoo et al., 2014; Sterkenburg et al., 2018; Lladó et al., 2017). In contrast, we observed weak abiotic filtering effect of soil physico-chemistry on phylogenetic community composition, with pH resulting the only significant variable. The importance of pH as influential variable over ectomycorrhizal community composition at local and regional scales has been widely described (Kivlin et al., 2014; Glassman et al., 2017; Lladó et al., 2017). However, in view of our results, it seems that pH acts as abiotic filter at both taxonomic and phylogenetic level (Rousk et al., 2010; Carrino-Kyker et al., 2016). In addition, our results showed that a left tail of *P. sylvestris* and *P. sylvestris-nigra* in the distribution of the pH values resulting in a wider niche for species adapted to low pH values (Fig. S2). In this regard, phylogenetic fungal communities were more clustered at lower values of pH (<7), thus, it seems that lower pH values might result in the occurrence of only adapted fungal species that can grow and maintain cellular function in acidic environments (Lauber et al., 2009), causing higher phylogenetic similarity (Kivlin et al., 2014; Goldmann et al., 2015; Zhang et al., 2015).

Regarding phylogenetic structure, stand structure alone explained a proportion of its variation although this was not significant. However, stand structure, soil physico-chemistry and geographic distance shared an important proportion of variance. In Mediterranean ecosystems, the influence of stand structural variables on fungi has already been assessed over mushroom yields with important effects (Tomao et al., 2020). Although weakly, differences in stand structural variables may result in the occurrence of different less phylogenetically related fungal species that are better adapted to certain local forest conditions. In view of our results, we argue that ectomycorrhizal phylogenetic structure is more importantly influenced by the combined effect of all environmental variables. Hence, phylogenetic relatedness between species decreases with increasing geographic distance, differences in stand structure and soil conditions. Finally, we hypothesize that there may be other processes influencing ectomycorrhizal phylogenetic community, such as competition for space and resource (Tucker et al., 2017). that were not directly tested, therefore further studies are needed to further disentangle whether other processes influence phylogenetic structure in these ecosystems.

4.4. Trait evolution of the exploration types

We observed that 51% of the ectomycorrhizal species had short and contact exploration types, 21% and 13% of the species had medium fringe and medium exploration types respectively and only 4% of the species had long exploration types. Similarly, Castaño et al. (2018) found that in *P. pinaster* dominated Mediterranean forests, long distance were the least abundant exploration types, while short and contact types were dominating the community. Moreover, our results showed that traits were dispersed across the phylogenetic tree (Fig. 5). Thus, the third hypothesis is accepted. In this regard, the dispersion of traits across the phylogenetic tree suggests that even more distant related species showed the same exploration type, resulting in a random trait pattern with lack of phylogenetic signal. In addition, Castaño et al. (2018) found that mycorrhizal species with long distance exploration types were less abundant under drier conditions, whereas short-distance and contact type species increased. Recent studies suggest that drier conditions may favor short-contact types (Fernandez et al., 2016; Castaño et al., 2018). Similarly, based on our results, we argue that dispersion of short and contact exploration types might be an adaptation to the Mediterranean stress conditions where the limiting factor is water and not nutrients. Therefore, having medium mat and long exploration types might be a disadvantage due their higher C demand on the host (Fernandez et al., 2016; Castaño et al., 2018). At the same time, in northern and temperate ecosystems, soil N is a limiting nutrient (Tomao et al., 2020) and previous studies have shown that species with long medium mat exploration occurs in soils where N is limiting and patchily distributed (Koide et al., 2014; Defrenne et al., 2016), while short exploration types are more efficient in up-taking soluble inorganic N (Lilleskov et al., 2002). However, despite these observed trends and since exploration types of mycorrhizae represent a distinct set of fungal traits, the use of exploration types to study fungal trait responses to environmental changes can be often misleading and further research should be addressed. In any case, previous work in this area showed a lack of N effect on mycorrhizal communities in Mediterranean pine forests (Adamo et al., 2021) therefore, as N is not limiting it can be easily captured by ectomycorrhizal fungi close to the host with no need of investing in high biomass exploration types.

Finally, we acknowledge the accuracy of ITS2 region in species identification and resolution (Read & Perez-Moreno, 2003), but also its limitation in phylogenetic applications due to its high variability (Sundersan et al. 2019; Mikryuokov et al., 2020). However, the use a backbone phylogenetic tree at family level constructed from multiple gene sequences provides a sufficient taxonomic resolution, thus can be an accurate predictor of phylogenetic diversity metrics (Liu et al., 2019). Moreover, it is known that the identification of basidiomycetes through ITS2

amplification is more efficient than in other taxa (i.e. Ascomycetes) (Badotti et al., 2017). Therefore, future studies aiming to disentangle fungal phylogenetic patterns in community structure should include a robust backbone phylogenetic tree and at least the whole ITS region.

5. Conclusions

In this study, we found no differences neither in ectomycorrhizal phylogenetic community composition nor structure and diversity, indicating that ectomycorrhizal communities at both phylogenetic and taxonomic level do not change among phylogenetically closely related tree hosts. Moreover, soil parameters only had a marginal filtering effect on ectomycorrhizal phylogenetic variation as pH resulted the only significant driver of the phylogenetic community. In this regard, our results showed that pH acts as the broadest abiotic filter of ectomycorrhizal communities at local scale.

Conversely, ectomycorrhizal phylogenetic structure was marginally influenced by the combined effect of soil, stand structure and geographic distance, indicating that phylogenetic structure is mainly influenced by their combined effect.

Finally, short and contact distance were the dominant exploration types, as they may be favored under drought stress conditions but also under high nutrient availability. Our results shed light on the drivers of ectomycorrhizal phylogenetic community variation in Mediterranean pine forests, being fundamental to get a better insight on the drivers of community assembly and ecosystem functioning. Nevertheless, further research on ectomycorrhizal phylogenetic communities is needed to better understand how changes in deterministic processes will affect ectomycorrhizal communities and forest ecosystems functioning.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Catalonia map displaying the location of the 42 plots, Figure S2: Density curves of pH values of the five tree hosts from the permutational test of density equality, Figure S3: Hierarchical clustering of ectomycorrhizal phylogenetic compositional data based on a Euclidean distance matrix, Table S1: Table summarizing the main texture and moisture properties of the study plots, Table S2: Most abundant ectomycorrhizal species detected in pure and mixed stands of *Pinus spp*, Table S3:Phylogenetic species turnover, nestedness and total beta diversity values across host tree species stands.

Author Contributions: Colinas C, Bonet JA, Martínez de Aragón J and Alday JG planned and designed the fungal research. Martínez de Aragón J sampled the soils and together with Colinas C, measured the environmental variables. Castaño C performed the lab works and the bioinformatic analyses. Adamo I analysed the data and wrote the manuscript, with inputs of Alday JG and Castaño C. All authors provided inputs on the last version.

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References

Adamo I, Piñuela Y, Bonet JA, Castaño C, Martínez de Aragón J, Parladé J, Pera J & JG Alday (2021). Sampling forest soils to describe fungal diversity and composition. Which is the optimal sampling size in Mediterranean pure and mixed pine oak forest? Fungal Biology doi: https://doi.org/10.1016/j.funbio.2021.01.005

Adamo I, Castaño C, Y, Bonet JA, Colinas C, Martínez de Aragón J, JG Alday (2021). Soil physico-chemical properties have a greater effect on soil fungi tan host species in Mediterranean pure and mixed pine forests. Soil Biology and Biochemistry, 160 doi: https://doi.org/10.1016/j.soilbio.2021.108320.

Abarenkov K, Nilsson RH, Larsson KH, et al (2010) The UNITE database for molecular identification of fungi - recent updates and future perspectives. New Phytol.

Agerer R (2001) Exploration types of ectomycorrhizae. Mycorrhiza 11:107-114. https://doi.org/10.1007/s005720100108

Alday JG, Marrs RH, Martínez-Ruiz C (2012) Soil and vegetation development during early succession on restored coal wastes: A six-year permanent plot study. Plant Soil 352:305-320. https://doi.org/10.1007/s11104-011-1033-2

Alday JG, Martínez De Aragón J, De-Miguel S, Bonet JA (2017) Mushroom biomass and diversity are driven by different spatio-Temporal scales along Mediterranean elevation gradients. Sci Rep 7:45824. https://doi.org/10.1038/srep45824

Allen SE (1989) Chemical analysis of ecological materials. Blackwell's, Oxford.

Allen MF (2007) Mycorrhizal Fungi: Highways for Water and Nutrients in Arid Soils. Vadose Zo J. 6(2):291-297. https://doi.org/10.2136/vzj2006.0068

Anderson JM, Ingram JSI (1993). Tropical soil biology and fertility: a handbook of methods, 2nd edn. C.A.B. International, Wallingford

Arraiano-Castilho R, Bidartondo MI, Niskanen T, et al (2020) Plant-fungal interactions in hybrid zones: Ectomycorrhizal communities of willows (Salix) in an alpine glacier forefield. Fungal Ecol 45:100936. https://doi.org/10.1016/j.funeco.2020.100936

Badotti F, de Oliveira FS, Garcia CF (2017) Effectiveness of ITS and sub-regions as DNA barcode markers for the identification of Basidiomycota. BMC Microbiology 17,42. https://doi.org/10.1186/s12866-017-0958-x

Baselga A, Orme CDL (2012) Betapart: An R package for the study of beta diversity. Methods Ecol Evol 3(5): 808-812. https://doi.org/10.1111/j.2041-210X.2012.00224.x

Blomberg SP, Garland T, Ives AR (2003) Testing for phylogenetic signal in comparative data: Behavioral traits are more labile. Evolution (N Y) 57(4):717-745. https://doi.org/10.1111/j.0014-3820.2003.tb00285.x

Bödeker ITM, Clemmensen KE, de Boer W, et al (2014) Ectomycorrhizal Cortinarius species participate in enzymatic oxidation of humus in northern forest ecosystems. New Phytol 203(1):245-256. https://doi.org/10.1111/nph.12791

Boeraeve M, Honnay O, Jacquemyn H (2018) Effects of host species, environmental filtering and forest age on community assembly of ectomycorrhizal fungi in fragmented forests. Fungal Ecol 36:89-98. https://doi.org/10.1016/j.funeco.2018.08.003

Bowman AW, Azzalini A. (2018) R package "sm: non-parametric smoothing methods.

Bremner JM., Mulvaney CS (1982). Nitrogen total. In: Miller AL, Keeney DR (eds) Methods of soil analysis, 2nd edn. American Society of Agronomy, Madison, pp 595–624

Carrino-Kyker SR, Kluber LA, Petersen SM, et al (2016) Mycorrhizal fungal communities respond to experimental elevation of soil pH and P availability in temperate hardwood forests. FEMS Microbiol Ecol 93(2):fiw024. https://doi.org/10.1093/femsec/fiw024

Castaño C, Lindahl BD, Alday JG, et al (2018) Soil microclimate changes affect soil fungal communities in a Mediterranean pine forest. New Phytol 220(4):1211-1221. https://doi.org/10.1111/nph.15205

Cavender-Bares J, Kozak KH, Fine PVA, Kembel SW (2009) The merging of community ecology and phylogenetic biology. Ecol Lett 12(7):693:715. https://doi.org/10.1111/j.1461-0248.2009.01314.x

Clemmensen KE, Finlay RD, Dahlberg A, et al (2015) Carbon sequestration is related to mycorrhizal fungal community shifts during long-term succession in boreal forests. New Phytol 205(4):1525-1536. https://doi.org/10.1111/nph.13208

Day PR (1965). Particle fractionation and particle-size analysis. In C.A. Black ed. Methods of soil analysis part 1. Agronomy No. 9. American Society of Agronomy, Madison, WI.

Defrenne CE, Philpott TJ, Guichon SHA, et al (2019) Shifts in ectomycorrhizal fungal communities and exploration types relate to the environment and fine-root traits across interior douglas-fir forests of western Canada. Front Plant Sci 10:643. https://doi.org/10.3389/fpls.2019.00643

Dumbrell AJ, Nelson M, Helgason T, et al (2010) Relative roles of niche and neutral processes in structuring a soil microbial community. ISME J4:337-345. https://doi.org/10.1038/ismej.2009.122

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19):2460-2461. https://doi.org/10.1093/bioinformatics/btq461

Edwards IP, Zak DR (2010) Phylogenetic similarity and structure of Agaricomycotina communities across a forested landscape. Mol Ecol 19(7):1469-1482. https://doi.org/10.1111/j.1365-294X.2010.04566.x

Egan CP, Callaway RM, Hart MM, et al (2017) Phylogenetic structure of arbuscular mycorrhizal fungal communities along an elevation gradient. Mycorrhiza 27:273-282. https://doi.org/10.1007/s00572-016-0752-x

Emerson BC, Gillespie RG (2008) Phylogenetic analysis of community assembly and structure over space and time. Trends Ecol. Evol 23(11):619-630. https://doi.org/10.1016/j.tree.2008.07.005

Erlandson SR, Savage JA, Cavender-Bares JM, Peay KG (2016) Soil moisture and chemistry influence diversity of ectomycorrhizal fungal communities associating with willow along an hydrologic gradient. FEMS Microbiol Ecol 92(1): fiv148. https://doi.org/10.1093/femsec/fiv148

Faith DP (1992) Conservation evaluation and phylogenetic diversity. Biol Conserv 61(1): 1-

10. https://doi.org/10.1016/0006-3207(92)91201-3

Fernandez CW, Kennedy PG (2016) Revisiting the “Gadgil effect”: Do interguild fungal interactions control carbon cycling in forest soils? New Phytol 209(4):1382-1394.. https://doi.org/10.1111/nph.13648

Fouquier J, Rideout JR, Bolyen E, et al (2016) Ghost-tree: Creating hybrid-gene phylogenetic trees for diversity analyses. Microbiome 4: 11. https://doi.org/10.1186/s40168-016-0153-6

Gernandt DS, Geada López G, Ortiz García S, Liston A (2005) Phylogeny and classification of Pinus. Taxon 54(1):29-42. https://doi.org/10.2307/25065300

Glassman SI, Peay KG, Talbot JM, et al (2015) A continental view of pine-associated ectomycorrhizal fungal spore banks: A quiescent functional guild with a strong biogeographic pattern. New Phytol 205(4):1619-1631. https://doi.org/10.1111/nph.13240

Glassman SI, Wang IJ, Bruns TD (2017) Environmental filtering by pH and soil nutrients drives community assembly in fungi at fine spatial scales. Mol Ecol 26(24):6960-6973. https://doi.org/10.1111/mec.14414

Goldmann K, Schöning I, Buscot F, Wubet T (2015) Forest management type influences diversity and community composition of soil fungi across temperate forest ecosystems. Front Microbiol 6:1300. https://doi.org/10.3389/fmicb.2015.01300

Gotelli NJ (2000) Null model analysis of species co-occurrence patterns. Ecology 81(9):2606-2621. https://doi.org/10.1890/0012-9658(2000)081[2606:NMAOSC]2.0.CO;2

Guo J, Ling N, Chen Z, et al (2020) Soil fungal assemblage complexity is dependent on soil fertility and dominated by deterministic processes. New Phytol 226(1):232-243. https://doi.org/10.1111/nph.16345

Hartmann M, Brunner I, Hagedorn F, et al (2017) A decade of irrigation transforms the soil microbiome of a semi-arid pine forest. Mol Ecol 26(4):1190-1206. https://doi.org/10.1111/mec.13995

Hobbie EA, Agerer R (2010) Nitrogen isotopes in ectomycorrhizal sporocarps correspond to belowground exploration types. Plant Soil 327:71-83. https://doi.org/10.1007/s11104-009-0032-z

Ihrmark K, Bödeker ITM, Cruz-Martinez K, et al (2012) New primers to amplify the fungal ITS2 region - evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiol Ecol 82:666–677. https://doi.org/10.1111/j.1574-6941.2012.01437.x

Ishida TA, Nara K, Hogetsu T (2007) Host effects on ectomycorrhizal fungal communities: Insight from eight host species in mixed conifer-broadleaf forests. New Phyto.

174(2):430-440. https://doi.org/10.1111/j.1469-8137.2007.02016.x

Kembel SW, Cowan PD, Helmus MR, et al (2010) Picante: R tools for integrating phylogenies and ecology. Bioinformatics 26(11):1463-1564. https://doi.org/10.1093/bioinformatics/btq166

Kivlin SN, Winston GC, Goulden ML, Treseder KK (2014) Environmental filtering affects soil fungal community composition more than dispersal limitation at regional scales. Fungal Ecol 12:14-25. https://doi.org/10.1016/j.funeco.2014.04.004

Koide RT, Fernandez C, Malcolm G (2014) Determining place and process: Functional traits of ectomycorrhizal fungi that affect both community structure and ecosystem function. New Phytol 201(2): 433-439. https://doi.org/10.1111/nph.12538

Koljalg U, Nilsson RH, Abarenkov K, et al (2014) Towards a unified paradigm for sequence-based identification of fungi. Mol Ecol 22:5271–5277. https://doi.org/10.1111/mec.12481

Kraft NJB, Adler PB, Godoy O, et al (2015) Community assembly, coexistence and the environmental filtering metaphor. Funct Ecol 29(5):592-599. https://doi.org/10.1111/1365-2435.12345

Kyaschenko J, Clemmensen KE, Karltun E, Lindahl BD (2017) Below-ground organic matter accumulation along a boreal forest fertility gradient relates to guild interaction within fungal communities. Ecol Lett 20:1546–1555. https://doi.org/10.1111/ele.12862

Lauber CL, Hamady L, Knight R, Fierer N (2009) Pyrosequencing-based assesment of pH as a predictor of soil bacterial community structure at continental scale. Applied and Environmental microbiology 75:5111-5120. https://doi.org/10.1128/AEM.00335-09

Lilleskov EA, Fahey TJ, Horton TR, Lovett GM (2002) Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in alaska. Ecology 83(1):104-115. https://doi.org/10.1890/0012-9658(2002)083[0104:BEFCCO]2.0.CO;2

Lilleskov EA, Hobbie EA, Horton TR (2011) Conservation of ectomycorrhizal fungi: Exploring the linkages between functional and taxonomic responses to anthropogenic N deposition. Fungal Ecol 4(2):174-183. https://doi.org/10.1016/j.funeco.2010.09.008

Liston A, Robinson WA, Piñero D, Alvarez-Buylla ER (1999) Phylogenetics ofPinus(Pinaceae) Based on Nuclear Ribosomal DNA Internal Transcribed Spacer Region Sequences. Mol Phylogenet Evol 11(1):95-109. https://doi.org/10.1006/mpev.1998.0550

Liu J, Liu J, Shan Y, Ge X, Burgess K (2019) The use of DNA barcodes to estimate phylogenetic diversity in forest communities of Southern China. Ecology and Evolution 9(9):5372-5379. https://doi.org/10.1002/ece3.5128

Maherali H, Klironomos JN (2007) Influence of phylogeny on fungal community assembly

and ecosystem functioning. Science (80-). https://doi.org/10.1126/science.1143082

Martínez de Aragón J, Bonet JA, Fischer CR, Colinas C (2007) Productivity of ectomycorrhizal and selected edible saprotrophic fungi in pine forests of the pre-Pyrenees mountains, Spain: Predictive equations for forest management of mycological resources. For Ecol Manage 252(1-3):239-256. https://doi.org/10.1016/j.foreco.2007.06.040

McMurdie PJ, Holmes S (2013) Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One 8(4):e611217. https://doi.org/10.1371/journal.pone.0061217

Mikryukov VS, Dulya O V., Modorov M V. (2020) Phylogenetic signature of fungal response to long-term chemical pollution. Soil Biol Biochem 140:107644. https://doi.org/10.1016/j.soilbio.2019.107644

Moeller H V., Peay KG, Fukami T (2014) Ectomycorrhizal fungal traits reflect environmental conditions along a coastal California edaphic gradient. FEMS Microbiol Ecol 87(3): 797-806. https://doi.org/10.1111/1574-6941.12265

Mohan JE, Cowden CC, Baas P, et al (2014) Mycorrhizal fungi mediation of terrestrial ecosystem responses to global change: Mini-review. Fungal Ecol 10:3-19. https://doi.org/10.1016/j.funeco.2014.01.005

Nagati M, Roy M, Manzi S, et al (2018) Impact of local forest composition on soil fungal communities in a mixed boreal forest. Plant Soil 432:345–357. https://doi.org/10.1007/s11104-018-3806-3

Narwani A, Matthews B, Fox J, Venail P (2015) Using phylogenetics in community assembly and ecosystem functioning research. Funct Ecol 29(5):589-591. https://doi.org/10.1111/1365-2435.12431

Nguyen NH, Song Z, Bates ST, et al (2016) FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecol 20:241–248. https://doi.org/10.1016/j.funeco.2015.06.006

Ning C, Mueller GM, Egerton-Warburton LM, et al (2019) Host phylogenetic relatedness and soil nutrients shape ectomycorrhizal community composition in native and exotic pine plantations. Forests 10(3):263. https://doi.org/10.3390/f10030263

Oksanen J, Blanchet FG, Friendly M, et al (2018) Package "vegan"

Olsen SR, Sommers L (1982). Phosphorus. In: Miller AL, Keeney DR (eds) Methods of soil analysis. American Society of Agronomy, Madison, pp 403–427

Op De Beeck M, Troein C, Peterson C, et al (2018) Fenton reaction facilitates organic nitrogen acquisition by an ectomycorrhizal fungus. New Phytol 218(1):335-343.

https://doi.org/10.1111/nph.14971

Paradis E, Claude J, Strimmer K (2004) APE: Analyses of phylogenetics and evolution in R language. Bioinformatics 20(2):289-290. https://doi.org/10.1093/bioinformatics/btg412

Peay KG, Garbelotto M, Bruns TD (2010) Evidence of dispersal limitation in soil microorganisms: Isolation reduces species richness on mycorrhizal tree islands. Ecology 91(12): 3631-3640. https://doi.org/10.1890/09-2237.1

Pena R, Lang C, Lohaus G, et al (2017) Phylogenetic and functional traits of ectomycorrhizal assemblages in top soil from different biogeographic regions and forest types. Mycorrhiza 27:233-245. https://doi.org/10.1007/s00572-016-0742-z

Pérez-Izquierdo, L., Zabal-Aguirre, M., González-Martínez, S.C., Buée, M., Verdú, M., Rincón, A., Goberna, M., 2019. Plant intraspecific variation modulates nutrient cycling through its below ground rhizospheric microbiome. Journal of Ecology 107(4), 1594-1605. doi:10.1111/1365-2745.13202

Pérez-Izquierdo, L., Zabal-Aguirre, M., Verdú, M., Rincón, A., 2020. Ectomycorrhizal fungal diversity decreases in Mediterranean pine forests adapted to recurrent fires. Molecular Ecology 29(13), 2463-2476. doi:10.1111/1365-2745.13202

Powell JR, Bennett AE (2016) Unpredictable assembly of arbuscular mycorrhizal fungal communities. Pedobiologia (Jena) 59(1-2):11-15. https://doi.org/10.1016/j.pedobi.2015.12.001

Read DJ, Perez-Moreno J (2003) Mycorrhizas and nutrient cycling in ecosystems - A journey towards relevance? New Phytol 157(3):475 - 492. https://doi.org/ 10.1046/j.1469-8137.2003.00704.x

Rousk J, Bååth E, Brookes PC, et al (2010) Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME J 4:1340-1351. https://doi.org/10.1038/ismej.2010.58

Saladin B, Leslie AB, Wüest RO, et al (2017) Fossils matter: improved estimates of divergence times in Pinus reveal older diversification. BMC Evol Biol 17:95. https://doi.org/10.1186/s12862-017-0941-z

Sardans J, Peñuelas J (2013) Plant-soil interactions in Mediterranean forest and shrublands: Impacts of climatic change. Plant Soil 365:1-33. https://doi.org/10.1007/s11104-013-1591-6

Silverman JD, Washburne AD, Mukherjee S, David LA (2017) A phylogenetic transform enhances analysis of compositional microbiota data. Elife. https://doi.org/10.7554/eLife.21887

Smith SE, Read DJ (2008) Mycorrhizal Symbiosis 3rd edn Academic Press: San Diego. Acad

Press San Diego

Somervuo P, Koskela S, Pennanen J, et al (2016) Unbiased probabilistic taxonomic classification for DNA barcoding. Bioinformatics 32(19):2920–2927. https://doi.org/10.1093/bioinformatics/btw346

Somervuo P, Yu DW, Pennanen J, Xu CCY, Ji X, Hultman J, Wirta H, Ovaskainen O (2017) Quantifying uncertainty of taxonomic placement in DNA barcoding and metabarcoding. methods in Ecology and Evolution 8(4):398–407. https://doi.org/ 10.1111/2041-210X.12721

Sterkenburg E, Clemmensen KE, Ekblad A, et al (2018) Contrasting effects of ectomycorrhizal fungi on early and late stage decomposition in a boreal forest. ISME J 12:2187–2197. https://doi.org/10.1038/s41396-018-0181-2

Sundersan N, Kumar SA, Ganeshan EJ, Pandi M (2019) Evaluation of ITS molecular morphometrics effectiveness in species delimitation of Ascomycota- a pilot study. Fungal biology 123(7): 517-527. https://doi.org/10.1016/j.funbio.2019.05.002

Suz LM, Kallow S, Reed K, et al (2017) Pine mycorrhizal communities in pure and mixed pine-oak forests: Abiotic environment trumps neighboring oak host effects. For Ecol Manage 406:370-380. https://doi.org/10.1016/j.foreco.2017.09.030

Tedersoo L, Bahram M, Põlme S, et al (2014) Global diversity and geography of soil fungi. Science (80-). https://doi.org/10.1126/science.1256688

Tedersoo L, Brundrett MC (2017) Evolution of Ectomycorrhizal Symbiosis in Plants. https://doi.org/10.1007/978-3-319-56363-3_19

Tedersoo L, Mett M, Ishida TA, Bahram M (2013) Phylogenetic relationships among host plants explain differences in fungal species richness and community composition in ectomycorrhizal symbiosis. New Phytol 199(3):822-831. https://doi.org/10.1111/nph.12328

Tedersoo L, Smith ME (2013) Lineages of ectomycorrhizal fungi revisited: Foraging strategies and novel lineages revealed by sequences from belowground. Fungal Biol. Rev 27:83-99. https://doi.org/10.1007/s00572-009-0274-x

Tomao A, Antonio Bonet J, Castaño C, de-Miguel S (2020) How does forest management affect fungal diversity and community composition? Current knowledge and future perspectives for the conservation of forest fungi. For. Ecol. Manage 457:117678. 10.1016/j.foreco.2019.117678

Tucker CM, Cadotte MW, Carvalho SB, et al (2017) A guide to phylogenetic metrics for conservation, community ecology and macroecology. Biol Rev 92(2):698-715.

https://doi.org/10.1111/brv.12252

Urbanová M, Šnajdr J, Baldrian P (2015) Composition of fungal and bacterial communities in forest litter and soil is largely determined by dominant trees. Soil Biol Biochem 84:53-64. https://doi.org/10.1016/j.soilbio.2015.02.011

Vamosi SM, Heard SB, Vamosi JC, Webb CO (2009) Emerging patterns in the comparative analysis of phylogenetic community structure. Mol. Ecol 18(4):572-92. https://doi.org/10.1111/j.1365-294X.2008.04001.x

Van Der Heijden MGA, Bardgett RD, Van Straalen NM (2008) The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol. Lett 11(3):296-310. https://doi.org/10.1111/j.1461-0248.2007.01139.x.

Van Der Linde S, Suz LM, Orme CDL, et al (2018) Environment and host as large-scale controls of ectomycorrhizal fungi. Nature 558:243–248. https://doi.org/10.1038/s41586-018-0189-9

Walkley A (1947). A critical examination of rapid method for determining organic carbon in soils. Soil Sci 63:251–254

Webb CO (2000) Exploring the phylogenetic structure of ecological communities: An example for rain forest trees. Am Nat 156:2. https://doi.org/10.1086/303378

Webb CO, Ackerly DD, McPeek MA, Donoghue MJ (2002) Phylogenies and community ecology. Annu. Rev. Ecol. Syst 33:475-505. https://doi.org/10.1146/annurev.ecolsys.33.010802.150448

Weber MG, Agrawal AA (2012) Phylogeny, ecology, and the coupling of comparative and experimental approaches. Trends Ecol. Evol 27(7):394-403. https://doi.org/10.1016/j.tree.2012.04.010.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics in: PCR Protocols. pp. 315–322. doi:10.1016/B978-0-12-372180-8.50042-1

Winter M, Devictor V, Schweiger O (2013) Phylogenetic diversity and nature conservation: Where are we? Trends Ecol Evol 28:199–204. https://doi.org/10.1016/j.tree.2012.10.015

Zhang T, Wang NF, Liu HY, et al (2016) Soil pH is a key determinant of soil fungal community composition in the Ny-Ålesund Region, Svalbard (High Arctic). Front Microbiol 7:227. https://doi.org/10.3389/fmicb.2016.00227

Zhao RL, Li GJ, Sánchez-Ramírez S, et al (2017) A six-gene phylogenetic overview of Basidiomycota and allied phyla with estimated divergence times of higher taxa and a phyloproteomics perspective. Fungal Divers 84:43–74.

CHAPTER IV

"Distinct nutrient economy within ectomycorrhizal types relates to dominance by microbial groups with contrasting ecologies in a Mediterranean forest"

In review:

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Distinct nutrient economy within ectomycorrhizal types relates to dominance by microbial groups with contrasting ecologies in a Mediterranean forest

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Abstract

Land use and climate changes can trigger shifts in vegetation types, with potential consequences for soil processes. Particularly shifts from conifers to broadleaf species are predicted in certain forested areas of the Mediterranean basin, but the consequences of these shifts for soil processes such as nitrogen (N) and carbon (C) cycling are still underexplored.

In a temperate forest, we investigated how nutrient economy and microbial communities differ among contrasting ectomycorrhizal types of vegetation (*Quercus robur, Pinus sylvestris*, and mixed *Q. Robur* and *P. Sylvestris*) in a fully factorial natural experiment.

We observed distinct patterns in soil characteristics and microbial communities among vegetation types. Notably, C:N ratios differed more profoundly with soil depth in *Q. robur* than in *P. sylvestris* stands. Compared to *Q. robur* soils, we observed higher relative abundances of ectomycorrhizal fungi with extensive mycelia in *P. sylvestris* paralleled with higher fungal biomass, higher δ15N signatures, N content and ammonium concentration. By contrast, *Q. robur* soils had higher archaeal and bacterial ammonia oxidizers, and a larger dominance by ectomycorrhizal fungi with contact exploration types. Mixed forest soils displayed transitional characteristics in terms of soil parameters and microbial communities.

We showed that changes in the nutrient economy among ectomycorrhizal types relates to shifts in microbial communities with contrasting ecologies. Replacement of conifers by broadleaf species may promote shifts towards inorganic N-cycling, while ectomycorrhizal fungi in

conifers may retain N and slow C cycling. These findings are important in the context of ongoing global shifts in vegetation types, which can lead to microbial-driven changes in soil C and N fluxes.

Keywords: *mixed forests, ectomycorrhizal, nitrification, soil fungi, N-cycling*

1. Introduction

Soil fungal communities are fundamental drivers of ecosystem processes (Bardgett and van der Putten, 2014), such as organic matter decomposition, soil nutrient release and plant nutrient uptake (Bardgett and Wardle, 2010). Mycorrhizal fungi are important regulators of soil organic matter dynamics by accessing to organically bound nitrogen (N) (Read and Perez-Moreno, 2003; Smith & Read, 2008). Conversely, saprotrophic fungi are main decomposers of soil organic matter in forest ecosystems, particularly in the uppermost litter layer (Voriskova et al., 2013; Boddy et al., 2007; Lindahl et al., 2007). Previous studies found that soil fungi contribute to C and N retention through their mycelial biomass (Clemmensen et al., 2013; Ekblad et al., 2013; Näsholm et al., 2013), but can also promote C and N losses by decomposing organic matter (Clemmensen et al., 2013; Lindahl and Tunlid, 2015). Differences in soil C and N dynamics have been observed particularly between mycorrhizal types, *i.e.* mainly ectomycorrhizal (ECM), arbuscular (AM) and ericoid (ERM) types (Averill et al., 2014; Averill and Hawkes 2016; Clemmensen et al., 2021; Castaño et al., 2022). Fungal and microbial communities with contrasting ecologies have been proposed as mediators of C and N dynamics in these mycorrhizal types (Phillips et al., 2013; Fernandez et al. 2014; Cheeke et al., 2017). However, distinct vegetation types belonging to the same mycorrhizal type can also be dominated by fungal guilds with contrasting ecologies, with potential implications for soil organic matter dynamics.

In forests, fungal communities with contrasting ecologies are often compartmentalized along a soil depth gradient, where free-living saprotrophs dominate the litter layer and ECM dominate more decomposed layers (Lindahl et al., 2017, Clemmensen et al., 2021; Voriskova et al., 2013). However, fungal communities with contrasting ecologies and particularly ectomycorrhizal fungi and fungal saprotrophs can also coexist and compete for soil N (Fernandez et al., 2016). Outcomes of these competitive interactions can alter C and N cycling, with dominance of ectomycorrhizal fungi over fungal saprotrophs resulting in the suppression of organic matter decomposition (Van der Wal et al., 2013; Fernandez et al., 2014; 2016; 2018; Sterkenburg et al., 2018). In addition, mycorrhizal-associated nutrient economy (MANE) framework predict that dominance by microorganisms with contrasting ecologies affect mode

of N cycling between mycorrhizal types (Phillips et al., 2013). Particularly, dominance by fungal saprotrophs in AM types and high plant litter qualities promote fast organic matter decomposition which can fuel N mineralization and nitrifiers, supporting inorganic-N economy. By contrast, in ECM types, lower litter qualities and dominance by ECM fungi promote organic N-cycling (Phillips et al., 2013; Lin et al., 2017), potentially slowing soil C cycling (Averill et al., 2014; Averill and Hawkes 2016). However, microbial communities and soil fungal dynamics and abundances also vary within tree species of the same mycorrhizal type (Fernandez et al., 2018; Hagenbo et al., 2020). Similarly, litter qualities can also differ among species of the same mycorrhizal type, particularly between conifers and angiosperms (Hobbie, 2005). This leads to hypothesize that mode of N cycling and microorganisms between tree species belonging to the same mycorrhizal type could also differ. Although it is known that ectomycorrhizal communities differ between coniferous and broadleaf forests (Ishida et al., 2007, Tedersoo et al., 2013), the implications for N and C cycling and the relationship with distinct microbial groups needs to be explored.

Changes in land use and warmer temperatures are predicted to induce shifts in vegetation types worldwide (Menzel et al., 2020). Particularly in the Mediterranean basin, a shift from conifers to broadleaf species have been predicted as a result of abandonment of forest management activities, but also recurrent fires (Santana et al., 2020) and increasing temperatures (Menzel et al., 2020). For example, drought and higher temperatures are expected to cause latitudinal shifts in *Pinus sylvestris*, which could potentially be replaced by broadleaf species (e. g. *Quercus* spp., Vospernik et al., 2023). Although belowground consequences of shifts from ECM to AM types have been explored (Steidinger et al., 2019; Soudzilovskaia et al., 2019), similar assessments are needed to predict microbial-driven shifts in belowground processes between species of the same mycorrhizal type. Our previous studies indicate pronounced differences in ECM biomass and dynamics between *P. sylvestris* and *Quercus* ilex forests (Hagenbo et al., 2020). Similarly, species of *Pinus* and *Quercus* differ in their root litter traits (Sardans and Peñuelas, 2013, Ishida et al 2007, Fernandez et al., 2020) but particularly in their aboveground litter traits, with higher litter qualities in *Quercus* (Vospernik et al., 2023). In addition, abundances of nitrifiers have been shown to differ between coniferous and broadleaf forests (Zhou et al., 2022).

In this study, we sampled tree distinct habitat types dominated by ectomycorrhizal tree species (*Q. robur*, *P. sylvestris* and a mix forest with *Q. robur* and *P. sylvestris*) in 3 sites in a fully factorial natural experiment. The overall aim of the study was to assess differences in C and N cycling among the three vegetation types and assess whether differences are linked with

microbial communities with contrasting ecologies. We profiled the fungal communities, quantified certain genes involved in inorganic N-cycling, and characterized soil properties. We also investigate natural abundances of δ15N, which can indicate N mobilization and transfer of N to plant hosts by mycorrhizal fungi (Hobbie & Högberg, 2012), but can also provide overall information on nitrification and denitrification processes among other processes affecting N-pools. First, we asses nutrient economy between the three vegetation types and hypothesize that i) organic N-cycling will prevail in *P. sylvestris* forests, compared to *Q. robur*. Thus, we also hypothesize that organic N-cycling in *P. sylvestris* will parallel with ii) dominance by ECM fungi in these forests, compared with *Q. robur*. According to the hyphal morphology and foraging strategies, ECM fungi are influenced by N availability (Agerer 2001, Hobbie et al., 2010, Pena et al., 2017). Therefore, we hypothesize that higher potential organic N-mining mode in *P. sylvestris* would correspond to an increase in ECM fungi with high foraging strategies in this forest. By contrast, in *Q. robur* we expect shifts toward open, inorganic N-cycling, paralleling lower abundances of ECM fungi or higher dominance by ECM fungi with lower foraging strategies. Finally, as indicators of inorganic N-cycling processes in soils (Philippot et al., 2007), we expect iii) nitrifiers, in particular bacteria and archaea ammonia oxidizers to increase in *Q. robur* forests.

2. Materials and Methods

2.1. *Study site*

The study belongs to a consortium of established triplets through Europe (Steckel et al. 2020; Pretzsch et al. 2020). We specifically selected a triplet located in the Pre-pyrenees (02°04'18.61"E, 42°15'46.42"N) with 54 years-old trees, at an elevation of 1149 a.m.s.l. and mean anual temperature of 9.9°C. De Martonne aridity index (mm °C−1) for 40-year average expanding from 1976-2015 (De Martonne, 1926) is 63.9, which is considered humid. Parental material soil is limestone/marl/sandstone, key reference soil group according to FAO WRB classification (IUSS Working Group WRB, 2015) is regosol. Soil texture is silty loam, and site index for scots at age 100 y is 17.5 for *P. sylvestris* and 16.5 for *Q. robur* (Steckel et al., 2020; Pretzsch et al., 2020).

The site consists in 5 triplets located in distinct geographical locations, which allows to test shifts in soil processes and microbial communities among vegetation types without any confounding geographically related factor, such as soil type or climate. Each triplet consists in three rectangular plots, representing a mixed *P. sylvestris*- *Q. robur* stand, and two monospecific stands of each species, respectively. Each stand is even-aged, fully stocked, with

no other tree species except for scattered shrub understory (Steckel et al., 2020; Pretzsch et al., 2020). To avoid disturbance-related effects, these stands had not been thinned at least for the last 10 years, and they are considered to have maximum stand density. Mixed plots had an average of 46 trees of *P. sylvestris* trees and 61 *Q. robur* trees (Pretzsch et al., 2020).

2.2. *Soil sampling*

In October 2018, a total of 4 soil cores were taken in each of the plots, separating the cores in three distinct soil layers until 20-30. The first layer consisted in a humus layer (approx 0-5 cm), the second layer was a transition of humus with mineral content (approx 5-10 cm), while the third layer was mainly mineral with still some organic parts (approx 10-20 cm). We used a rectangular steel drill to extract a soil core with a width of 6 ξ 4.5 cm. The four soil subsamples were pooled, and the composite sample was stored at 4°C for < 24 h, sieved through a 3-mm mesh, and stored at –20°C. A subset of the sieved sample was used to determine soil physico-chemical parameters and the remainder soil was freeze-dried at -20°C for 72 h, and homogenized using a pestle and mortar until fine powder.

2.3. Soil analyses

Total organic matter was measured following the loss on ignition (LOI). Soil pH was measured in 1:2.5 soil:deionized water slurry (Allen,1989). Total N concentration was measured using the Kjeldahl-method (Bremner & Mulvaney,1982); available P concentration using the Olsen method (Olsen & Sommers,1982); Finally, $\delta^{15}N$ in the soil were measured by UC Davis Stable Isotope Facility using a PDZ Europa ANCA-GSL elemental analyzer coupled with a PDZ Europa 20-20 isotope ratio mass spectrometer (IRMS, Sercon Ltd., Cheshire, UK).

2.4. Fungal biomass

Fungal biomass was estimated by quantifying ergosterol following Nylund and Wallander (1992). However, only free ergosterol was extracted by using pure methanol instead of 10% KOH in methanol (Wallander et al., 2010) to get a better estimation of recently produced mycelia (Wallander et al., 2013). Ergosterol was then quantified chromatographically following Hagenbo et al. (2020), using an UPLC system (Acquity UPLC, Waters, Milford, CT, USA), consisting of a triple quadrupole mass spectrometer (Xevo TQ-S; Waters, Milford, CT, USA) equipped with an atmospheric pressure chemical ionisation source (Sun et al., 2005). A

Cortecs C18 analytical column (1.6 µm, 2.1 × 100 mm) was employed for chromatographic separation.

2.5. Quantitative PCR (qPCR) of total fungi, total bacteria and ammonia oxidizing genes of bacteria and archaea.

Prior to quantification of target genes, DNA concentration was estimated on a Qubit Fluorometer (Life Technologies) and extracts were diluted (3 ng DNA μL^{-1}). Diluted extracts were checked for PCR inhibition with real-time qPCR using SYBR Green on a BioRad CFX Connect Real-Time System. Known amounts of pGEM-T plasmid (Promega, WI, USA) were spiked into PCR reactions containing template or into non-template controls and were amplified. No inhibition was detected for any of the samples.

Total fungi and bacteria were estimated separately by quantification of fragments of fungal internal transcribed spacer region 2 (ITS2), using the primers fITS7 (Ihrmark *et al.*, 2012), ITS4 (White *et al.*, 1990) and ITS4arch (Sterkenburg *et al.*, 2018), and the V3 region of the bacterial 16S rRNA gene, using the primers 341F and 534R (López-Gutiérrez *et al.*, 2004), respectively.

To evaluate potential for inorganic N-cycling, ammonia oxidizing genes (*amo*A) of archaea (AOA) were quantified using the primers crenamoA23F and crenamoA616R (Tourna *et al.*, 2008), and ammonia-oxidizing genes of bacteria (AOB), were quantified using the primers AmoA1F and AmoA2R (Rotthauwe *et al.*, 1997). *Amo*A genes are responsible for encoding the enzyme ammonia monooxygenase, which catalyzes the oxidation of ammonia to hydroxylamine and ultimately to nitrite. Quantifications were done using a CFX Connect Real-Time System (Bio-Rad, Hercules CA, USA) in 20 µl reactions with 1× iQ™ SYBR Green Supermix (Bio-Rad), 0.1 % bovine serum albumin (BSA; New England Biolabs, Ipswich MA, USA), primers and 3 ng DNA. Standard curves were obtained by serial dilutions of plasmids with cloned fragments of the specific genes. Amplicons for each targeted gene were inspected by agarose gel electrophoresis to exclude unspecific amplification.

2.6. Fungal community analyses

The fungal DNA was extracted from 150 mg of homogenized soil using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol. We amplified the fungal internal transcribed spacer 2 (ITS2) region in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) using the primers gITS7 (Ihrmark et al., 2012), ITS4 (White et al., 1990) and ITS4arch (Sterkenburg et al., 2018) following a metabarcoding

protocol to minimize size-length related biases (Castaño et al. 2020). Samples were amplified in triplicates and pooled, and negative controls were also included during the DNA extraction and PCR. Amplified products from each pooled sample were purified using NGS clean-up and size selection kit (Macherey-Nagel, Duren, Germany), and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled, and the mix was purified using the EZNA Cycle Pure kit (Omega Bio-Tek).

2.5. Bioinformatic analysis

Sequences were quality filtered and clustered using the SCATA pipeline (https://scata.mykopat.slu.se/). We first removed DNA sequences with length <200 bp and were screened for sample tags and primers defining a primer match of at least 90%. Sequences were pair-wise compared using 'usearch' (Edgar, 2010) after collapsing homopolymers to 3 bp. Sequences were quality filtered removing data with amplicon quality score of <20 (averaged per sequence) and with a score of <10 at any position. Pairwise alignments were scored as follows: mismatch penalty of 1, gap open penalty of 0 and a gap extension penalty of 1. Putative chimera sequences were removed, and the quality-filtered sequences were clustered into species hypotheses (Kõljalg et al., 2013) using single linkage clustering, with a maximum distance of 1.5% to the closest neighbour required to enter clusters. Global singletons were excluded from further analyses. Sequence data will be archived at NCBI's Sequence Read Archive (www.ncbi.nlm.nih.gov/sra).

2.6. Taxonomic and functional identification

We taxonomically identified the 1220 most abundant OTUs, which represented 93% of the total sequences. We selected the most abundant sequence from each OTU for taxonomic identification, using PROTAX software (Somervuo et al., 2016) implemented in PlutoF, using a 50% probability of correct classification (called by Somervuo et al. (2016) as "plausible identifications"). These identifications were confirmed and some of them improved using massBLASTer in PlutoF against the UNITE (Abarenkov et al. 2010). Taxonomic identities at species level were assigned based on >98.5% similarity with database references, or to other lower levels using the next criteria: genus on >97%, family on >95%, order on >92% and phylum on >90% similarity. OTUs were assigned to the following functional guilds: a) root-associated basidiomycetes, b) root-associated ascomycetes, c) moulds, d) yeasts, e) litter-

associated basidiomycetes, f) litter-associated ascomycetes, g) pathogens, h) moss-associated fungi, i) soil saprotrophs (saprotrophic taxa commonly found in N-rich mineral soils), j) unknown function, based on the UNITE database, DEEMY (www.deemy.de) or FUNGuild (Nguyen et al., 2016). Most of the root-associated fungi in this study were found to be ectomycorrhizal.

2.8. Statistical analyses

Statistical analyses were implemented in R software environment (version 3.6.0, R Development Core Team 2019), using the *vegan* package for the multivariate analyses (Oksanen et al., 2018) and the *phyloseq* package to visualize differences in fungal relative abundance (McMurdie & Holmes, 2013). First, the effects of the forest types and soil layers were analysed using linear mixed models with layer (df = 2) and site (df=2) and their interaction (df=4) defined as fixed factors, using the "lmer" function from the lme4 R package (Bates et al., 2021). Then, the effects of forest types and soil layers were visualized using the ggplot2 R package (Wickham, 2016). For all compositional analyses, the species abundance matrix was previously Hellinger transformed to account for taxa with low counts (Legendre and Gallagher 2001). Secondly, differences in overall fungal community composition between forest types were tested using permutational multivariate analyses of variance (PMAV, function *"adonis"*) on a Bray-Curtis dissimilarity matrix. After that, the overall community matrix was split by main functional guilds (i.e. mycorrhizal and saprotrophs) in two matrices and analysed individually in the same way. Then, a non-metric multidimensional scaling (NMDS, function *"metaMDS"*) was implemented in order to visualize the overall, mycorrhizal, and saprotrophic guilds compositional differences between pure pine, pure oak and mixed pine-oak forests. Here, the standard error ellipses were used to visualize the dispersion of each forest in the ordination space. Moreover, the relative strength of correlation of total fungal biomass, difference of C:N and $\delta^{15}N$ with depth $\delta^{15}N$, AOB and AOA variables was determined using the 'envfit' function in the vegan R package (Oksanen et al*.,* 2018). In addition, differences in fungal to bacteria ratio, AOB and AOA gene copies' number between forest types and soil layers, were analysed with two-way anova using the function "aov". Finally, we then used Structural Equation Modelling (SEM; Grace 2006), using the R package lavaan (Rosseel, 2012) to further clarify to further clarify the direct and indirect effect of C:N ratio and NH4 differences in depth, *amoA*, mycorrhizal and saprotrophic average abundance in soil depth on $\delta^{15}N$ difference in soil depth regardless of the forest type. Structural equation modelling

requires the development of an a priori model of the hypothesised effects and relationships among the main drivers and microbial groups (Fig. S2).

3. Results

3.1. Soil characteristics and fungal biomass differ among vegetation types

All soil properties and fungal biomass significantly differed among soil layers (Fig. 1). Although we did not find significant differences between forest types (Table 1), there were significant tree species × soil layer interactions for C:N ratios, with values particularly decreasing with depth in *Q. robur*. Thus, *Q. robur* and mixed stands had higher C:N ratios in the uppermost soil layers (O2 and MO1) than pure *P. sylvestris* stands. However, C:N ratios only decreased with depth in *Q. robur* and mixed forests (Table 1). N content (mg g dry soil^{-1}) showed opposite patterns than C:N ratios, again with strong tree species × soil layer interactions, but with values higher in *P. sylvestris* than in *Q. robur* and mixed forests at the O2 layer. By contrast, in deeper layers, N content sharply decreased in *P. sylvestris* while increased particularly in *Q. robur* (Table 1). Extractable NH_4-N, P and fungal biomass concentrations all decreased similarly among vegetation types with soil depth. However, NH_4-N and P concentrations were significantly lower in *Q. robur* (Fig. 1c, d, Table 1), while mixed forests and *P. sylvestris* had the highest NH4 values. *P. sylvestris* had also the higher PO4 values than *Q. robur.* Fungal biomass was around 8 times higher in *P. sylvestris* than *Q. robur* at the O2 layer, although values were similar in the other layers for all vegetation types. $\delta^{15}N$ signatures increased with soil depth in all forest types, but these were the highest in *P. sylvestris* forest. However, $\delta^{15}N$ signatures showed a steeper increase with depth in *P. sylvestris* forests than in mixed and *Q. robur* forests. $\delta^{15}N$ weighted across all soil layers were highest in pure *Q. robur* and lowest in *P. sylvestris,* with *Q. robur-P. sylvestris* having intermediate values (Table S1).

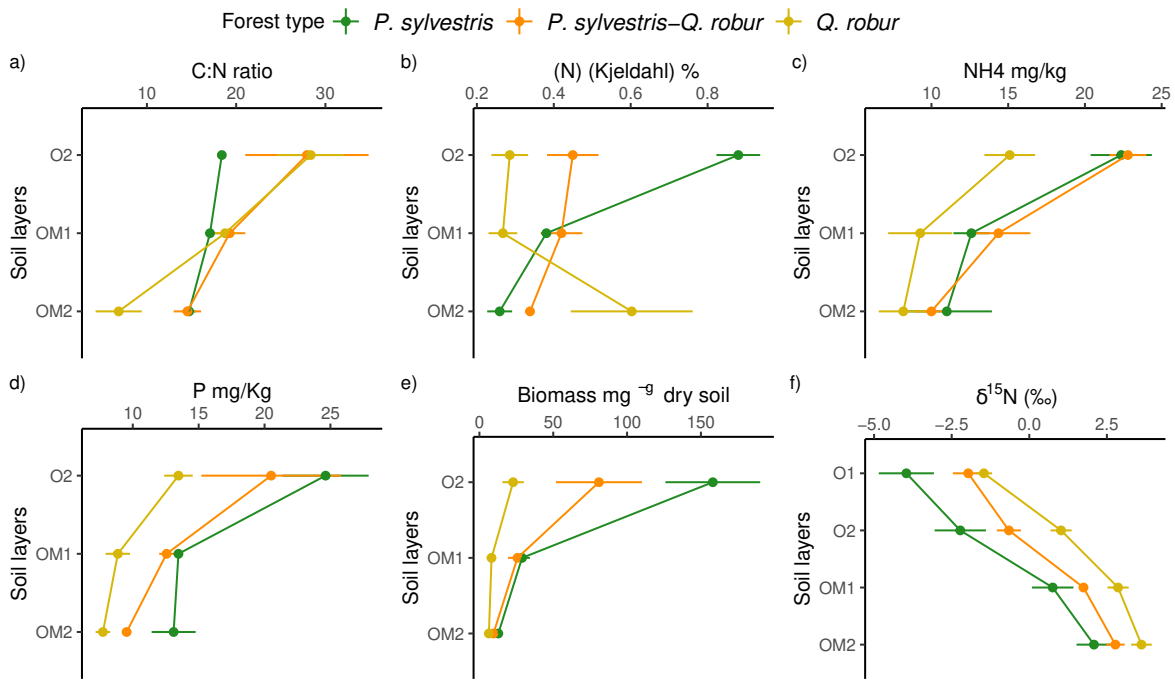

Fig. 1. Soil depth profiles of a) C:N ratio, b) total N (% of dry weight (DW)), c) extractable ammonium (mgNH_4-N/kg DW, d) total P, e) fungal biomass and f) $\delta^{15}N$ in three forest types; *P. sylvestris* (green), *P. Sylvestris-Q. robur* (orange), *Q. robur* (yellow) including the O1 layer. Soil layers are O1 = litter layer O2 = organic layer 2, OM1 =Mineral 1 and OM2 = Mineral 2. Points show means ±SE (n=3 stands).

Table 1. C:N ratio, total N, mgNH_4-N/kg DW, P, fungal biomass, and $\delta^{15}N$ differences between forest types and soil depth using Linear mixed models.

C:N ratio	**df**	**F-value**	**p-values**
Tree species	2-15	1.63	0.552
Soil layers	2-15	19.20	<0.001
Tree species * Soil layers	4-15	3.75	0.005
Total N	**df**	**F-value**	**p-values**
Tree species	2-15	14.17	<0.001
Soil layers	2-15	14.96	<0.001
Tree species * Soil layers	4-15	13.29	<0.001
mgNH_4-N/kg DW	**df**	**F-value**	**p-values**
Forest type	2-17	6.98	0.01
Soil depth	2-17	23.43	<0.001
Forest types* Soil depth	4-17	0.76	0.564
Total P (mg/Kg)	**df**	**F-value**	**p-values**
Forest type	2-17	12.44	<0.001

Soil depth	2-17	22.86	<0.001
Forest types* Soil depth	4-17	1.26	0.325
Fungal biomass	**df**	**F-value**	**p-values**
Tree host	2	7.41	0.001
Soil depth	2	2.15	0.013
Forest types* Soil depth	4	0.58	0.986
$\delta^{15}N$	**df**	**F-value**	**p-values**
Tree host	2	17.43	<0.001
Soil depth	2	41.26	0.001
Forest types* Soil depth	4	5.44	0.51

3.2 Fungal community composition changes with forest type but not soil layers

Overall, Ascomycota was the most abundant phylum (73.8 ± 1.2%) followed by Basidiomycota (25.5 ± 1.2% sequences) and Zygomycota (0.7 ± 10.12%). The most abundant guilds were root-associated basidiomycetes and moulds, representing 47.7 ± 4.2% and 20.8 ± 3.2% of the sequences, respectively, followed by saprotrophs (19.2 ± 2.2%) and-root associated ascomycetes (8.8 ± 1.3).

There were significant differences in the overall, mycorrhizal and saprotrophic fungal composition between pure *P. sylvestris,* mixed *P. sylvestris- Q. robur* and pure *Q. robur* forests. When including the O1 layer in the compositional analyses, showed overall compositional differences between soil layers along axis 1, due to O1 layer hosting a totally different community than the other soil layers (Figure S1). Therefore, to avoid any biases in the factors affecting the communities, the OM1 was removed from the downstream analyses. However, no significant soil depth or forest type-soil depth interaction effect was detected on the total fungal or mycorrhizal fungal community composition (Table 2). Conversely, significant differences were found in saprotrophic community composition among soil layers (Fig. 2c, Table 1). The NMDS ordination and SE-ellipses (Fig. 2a) clearly showed overall compositional differences between forest types along axis 1 (Table 1). Pure and mixed *P. sylvestris* stands were located to the left along the first axis, while pure *Q. robur* plots were more distinct and positioned to the right (NMDS stress=0.14, Fig. 2a). Similar patterns were detected in mycorrhizal and saprotrophic community composition (Fig. 2a-b, Table 1), although, relatively small compositional differences were detected in mycorrhizal fungal communities between pure and mixed *P. sylvestris* stands. In addition, the NMDS showed no compositional differences for overall and mycorrhizal community composition across soil layers. Conversely,

the composition saprotrophic communities differed significantly among soil depths along axis 2, with particularly clear differences between the humus and the two mineral layers (Fig. 2c). The most abundant species related to *P. sylvestris* were *Leotiomycetes spp.* (yeasts), *Hydnum spp*. (root-associated basidiomycetes and *Hygrophorum spp*. Additional mycorrhizal species related to pure *P. sylvestris* stands were: *Suillus spp.*, *Rhizpogon molhensis* and *Hydnum spp*., while the most common saprotrophic species were *Volutella spp*., *Chalara spp, and Venturia spp.*The ectomycorrhizal *Cenococcum geophylum* (root-associated basidiomycetes*) Cortinarius spp,* and *Tricholoma spp*., *Oidiodendron spp*, and *the saprotrophic Acromonium spp., Volutella spp., and Cylindrosympodium spp.* were associated with the mixed *P.sylvestris-Q. robur stands,* , Conversely, species related to pure *Q. robur* stands were: *Cortinarius spp*., *Hebeloma spp.*, and *Clavulina spp*. Finally, saprotrophic species related to *Q. robur* stands were: *Pyrenochaeta spp and Rhinocladiella spp.*

Table 2 Permanova summary table showing the forest type and soil depth effect on overall, mycorrhizal and saprophytic community composition. The pair wise PERMANOVA p-values were adjusted using the Holm-Bonferroni method.

Overall	**df**	**F-value**	**R^2**	**p-values**
Tree host	2-26	5.26	0.31	0.001
Soil depth	2-26	1.46	0.08	0.07
Forest types* Soil depth	4-26	0.05	0.06	0.99
Pairwise adonis	**df**	**F-value**	**R^2**	**Adj p-values**
P. sylvestris- Q. robur	1	8.49	0.34	*0.003*
P. sylvestris- mixed	1	2.56	0.13	0.003
Q. Robur- mixed	1	5.44	0.25	0.003
Mycorrhizal	**df**	**F-value**	**R^2**	**p-values**
Tree host	2-26	4.72	0.31	0.001
Soil depth	2-26	0.83	0.05	0.667
Forest types* Soil depth	4-26	0.38	0.05	1.000
Pairwise adonis	**df**	**F-value**	**R^2**	**Adj p-values**
P. sylvestris- Q. robur	1	7.73	0.32	0.004
P. sylvestris- mixed	1	2.82	0.15	0.010
Q. Robur- mixed	1	5.63	0.26	0.003
Saprotrophs	**df**	**F-value**	**R^2**	**p-values**
Tree host	2-26	7.41	0.36	0.001
Soil depth	2-26	2.15	0.11	0.013
Forest types* Soil depth	4-26	0.58	0.06	0.986
Pairwise adonis	**df**	**F-value**	**R^2**	**Adj p-values**
P. sylvestris- Q. robur	1	12.44	0.44	*0.003*
P. sylvestris- mixed	1	3.01	0.16	0.003
Q. Robur- mixed	1	6.95	0.30	0.003

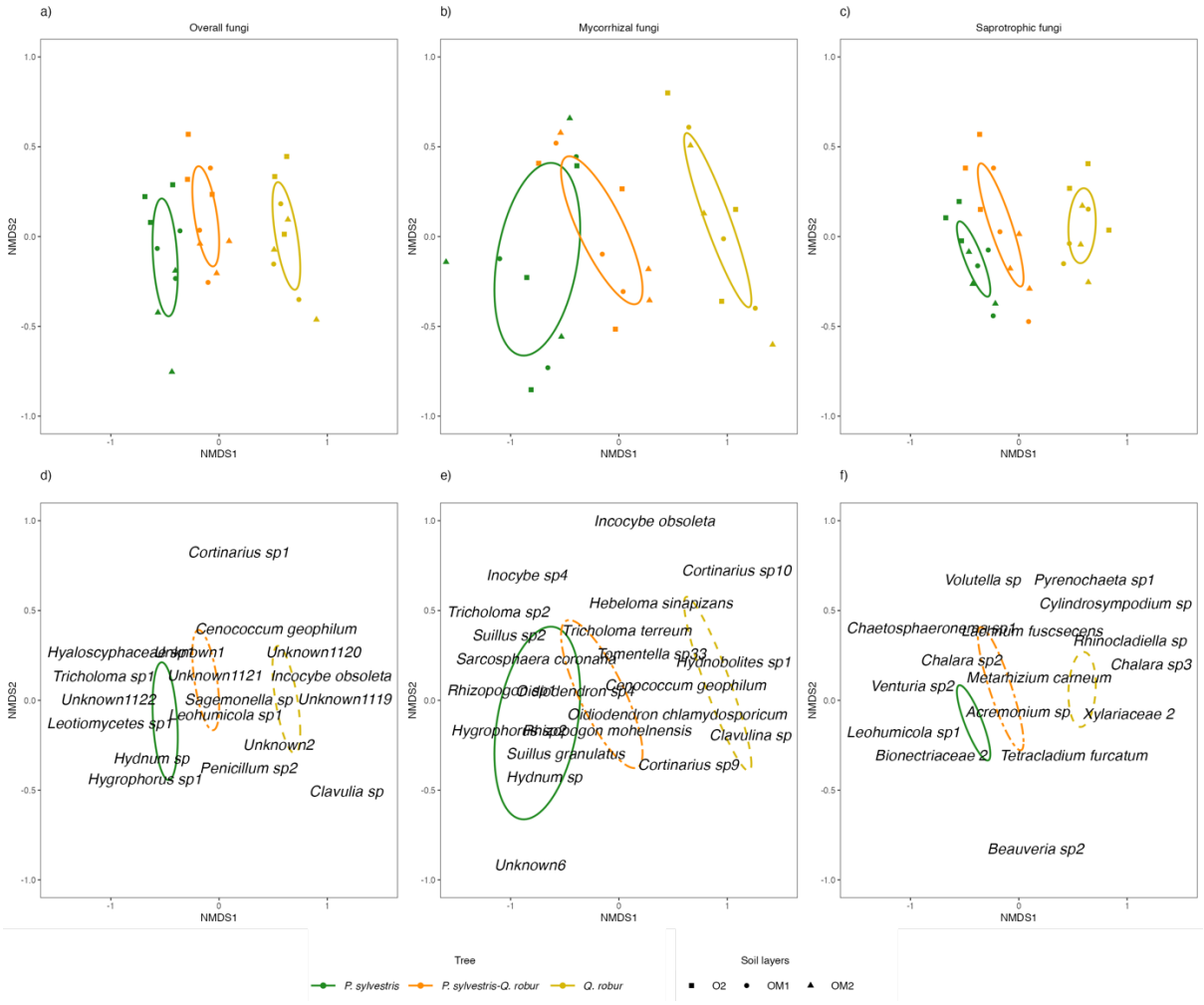

Fig. 2. Non-metric multidimensional scaling (NMDS) of (a) total fungal, (b) mycorrhizal fungal and (c) saprotrophic fungal community composition in three soil layers in three forest types: *P. sylvestris* (green; *P. Sylvestris-Q. robur* (orange); *Q. robur* (gold) The most abundant species detected in the (d) total fungal community, (e) mycorrhizal community and (f) saprotrophic fungal community.

3.3 Functional community structure changes between tree hosts and soil layers

The genetic potential for ammonia oxidation by bacteria (AOB) and archaea (AOA) and $\delta^{15}N$ signatures were significantly higher in *Q. robur* forests, while C:N ratio was significantly higher in mixed forests. In general, saprotrophic fungi were more abundant in the upper layers regardless of the forest type (Fig. 3b). Conversely, fungal communities in *P. sylvestris* forests were mainly dominated by root-associated basidiomycetes in O2 and OM1 layers (Fig.3b), while moulds were most abundant in OM2 layers. Conversely, saprotrophs were more abundant in the O2 layers of *Q. robur-P. sylvestris,* while root-associated basidiomycetes more

abundant in the OM2 layers. Moreover, the relative abundance of root-associated basidiomycetes was highest across the three layers of *Q. robur* (Fig. 3b). However, *Q. robur* forests were clearly dominated by *Clavulina spp*. as it had the highest number of reads (Fig. 3b). Moreover, root-associated basidiomycetes clearly dominated all three forest types (Fig. 3c). The abundance of root-associated basidiomycetes' exploration types was different between pure P. sylvestris and pure *Q. robur* forests. The relative abundance of long-distance exploration types (long and medium fringe types summed) was highest in *P. sylvestris* and *P. sylvestris*-*Q. robur* forests (Fig. 3c), while short distance exploration types (contact and short types summed) dominated in pure *Q. robur* forests (Fig. 3c). Finally, the relative abundance of ectomycorrhizal exploration types was not significantly different across soil layers.

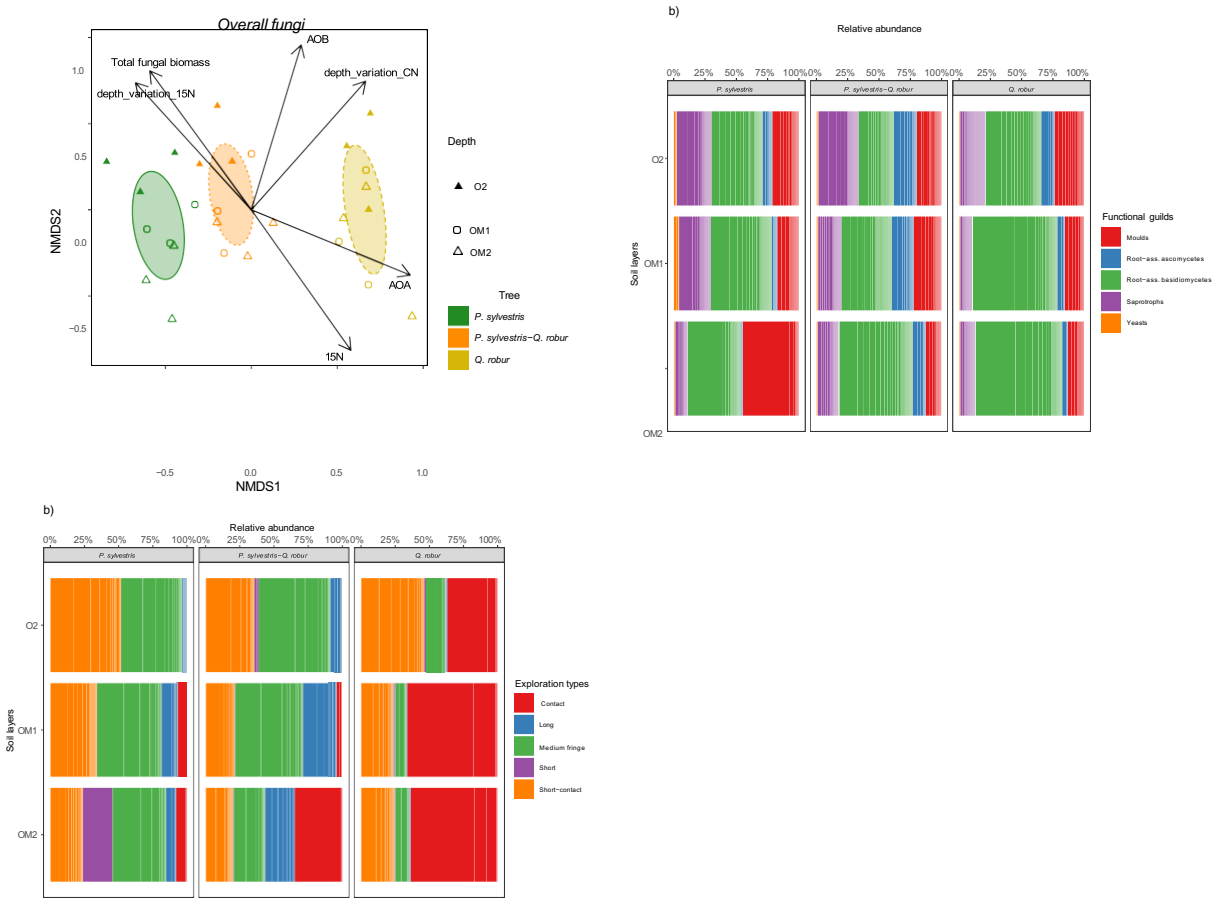

Fig. 3. a) Non-metric multidimensional scaling (NMDS) showing overall compositional differences between forest types: *P. sylvestris* (green; *P. Sylvestris-Q. robur* (orange); *Q. robur* (gold), with total fungal biomass, depth wise differences in $\delta^{15}N$ and C:N, AOA, AOB and $\delta^{15}N$ values fitted into the NMDS, b) relative abundance of functional guilds in depth-wise soil layers in three forest types, c) relative abundance of exploration types in depth-wise soil layers between the three forest types

3.4 Fungal bacteria ratio and N-cycling bacteria abundance between forest types

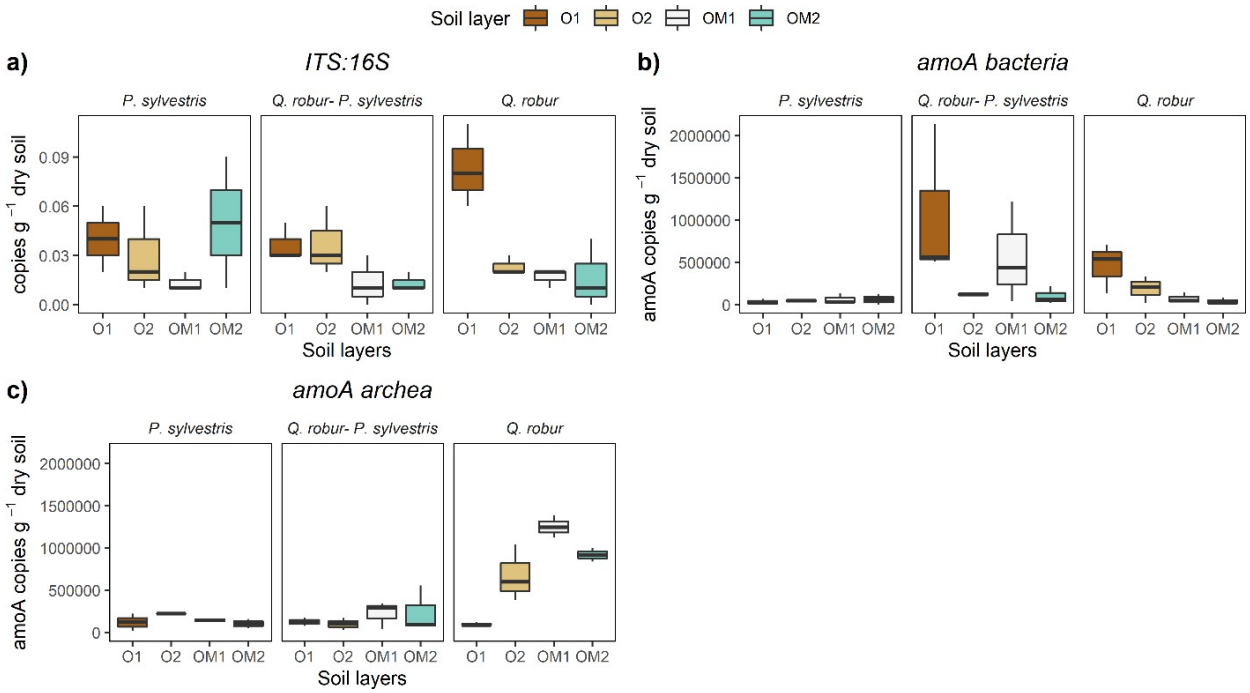

Fig. 4. a) ITS to 16S gene copies ratio in relation to forest type and across soil layers, b) *amoA* bacteria genes copies abundance in relation to forest type and across soil layers, c) *amoA* archea genes copies abundance in relation to forest type and across soil layers.

There was no significant difference in fungal to bacteria ratio between forest types ($F_{2,22}$ = 0,710, p = 0.502), as well as no significant interaction effect (forest type x soil layer, $F_{6,22}$ = 2.09, p = 0.095). However, there were significant differences across soil layers ($F_{3,22}$ = 5.88, p = 0.004). Fungal to bacteria ratio was highest in the O1 layer and lowest in the OM1 layer in *Q. robur* forests, while it was highest in the OM2 layers in *P. sylvestris* forests. Conversely, fungal to bacteria ratio was highest in O2 layers in *Q. robur- P. sylvestris* forests. Finally, fungal to bacteria ratio was highest in OM2 layers (Fig. 4a). Regarding amoA gene copies, forests (Fig. 4b).

3.5 Relations of the ecosystem's microbial community and C, N pools

The structural equation model showed that in Mediterranean forest ecosystems depth differences in C:N ratio had a significant negative relationship on mycorrhizal and saprotrophic biomass across soil depth, while non-significant relationship with *amoA* bacterias (Fig. 5). Conversely, NH_4-N had a significant negative effect on amoA bacterias but a positive effect on saprotrophic biomass (Fig. 5). Finally, mycorrhizal and saprotrophic biomass had a significant negative effect on $\delta^{15}N$ depth differences, while the effect of amoA bacterias was non-significant.

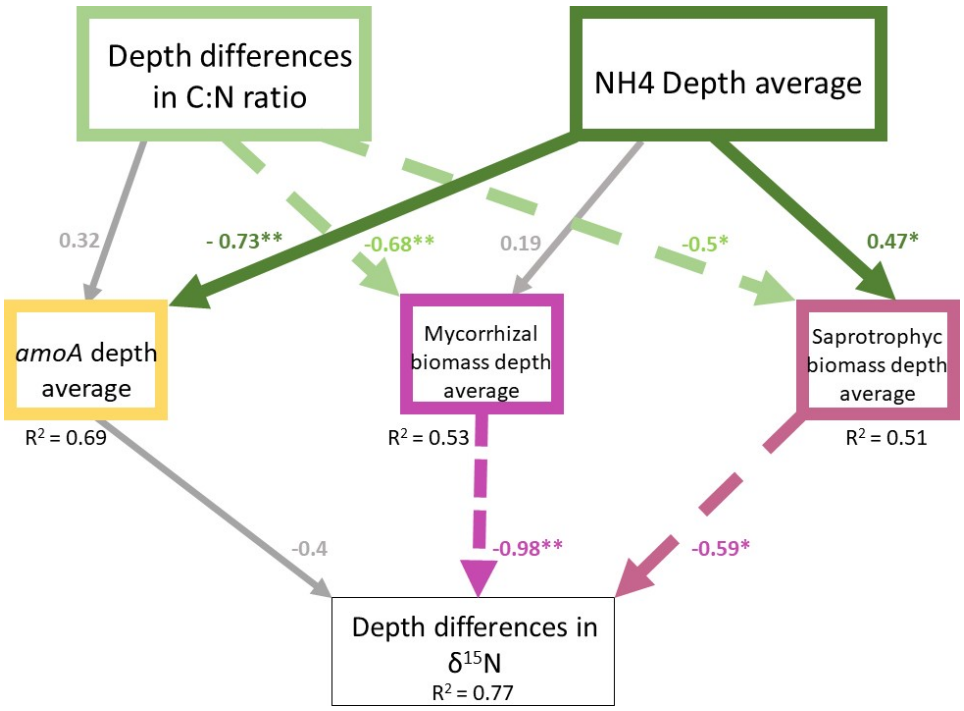

Fig. 5. A structural equation model showing the direct and indirect effects of C:N ratio and NH4 differences in depth, *amoA*, mycorrhizal and saprotrophic average abundance in soil depth on $\delta^{15}N$ difference in soil depth. Solid and dashed arrows indicate positive and negative relationships, respectively, while grey arrows indicate non-significant relationships.

4.- Discussion

The results of our study indicate that soil characteristics and fungal biomass vary significantly among different vegetation types. We observed significant differences in all soil properties and fungal biomass across different soil layers. While we did not find significant differences between forest types overall, there were significant interactions between tree species and soil layers for C:N ratios. Specifically, we observed that C:N ratios decreased with depth in *Q. robur* stands, suggesting a shift towards lower carbon-to-nitrogen ratios in deeper soil layers in these stands. In contrast, C:N ratios remained relatively constant with depth in pure *P. sylvestris* stands. This suggests that the nutrient dynamics and organic matter decomposition processes differ between these two tree species, with *Q. robur* stands potentially retaining more carbon in deeper soil layers.

We also found contrasting patterns in N content across the soil layers and vegetation types. N content was higher in *P. sylvestris* stands compared to *Q. robur* and mixed forests in the uppermost soil layers, but sharply decreased in deeper layers in *P. sylvestris* stands while increasing particularly in *Q. robur* stands. This indicates differences in nitrogen availability and cycling among the vegetation types (Sardans and Peñuelas, 2013), with *P. sylvestris* stands potentially having higher nitrogen availability in the upper layers but depleting nitrogen in deeper layers. In contrast, *Q. robur* stands may exhibit a more consistent increase in nitrogen content with depth, potentially due to the deeper root system and nutrient uptake strategy of this species. Regarding the concentrations of extractable NH_4-N and P, we found similar decreasing trends with soil depth among all vegetation types. However, *Q. robur* stands had significantly lower NH_4-N and P concentrations compared to mixed forests and P. sylvestris stands. This suggests that *Q. robur* stands may have a lower nutrient availability, particularly for ammonium and phosphorus, compared to the other vegetation types (Hobbie & Högberg, 2012). The higher NH_4-N values in mixed forests and P. sylvestris stands indicate a potentially higher microbial activity and nutrient turnover in these areas (Högberg et al. 2014).

Fungal biomass also exhibited variation among vegetation types and soil layers. At the uppermost soil layer (O2), fungal biomass was approximately eight times higher in *P. sylvestris* stands compared to *Q. robur* stands, but similar in the other layers for all vegetation types. This suggests that *P. sylvestris* stands may have a higher fungal abundance and activity near the soil surface, which could be attributed to the presence of ectomycorrhizal fungi associated with this tree species (Adamo et al 2021). The higher fungal biomass in *P. sylvestris* stands may also

contribute to the observed differences in nutrient dynamics, as fungi play a crucial role in organic matter decomposition and nutrient cycling processes (Zhou et al. 2022). In contrast the $\delta^{15}N$ signatures, which indicate nitrogen isotope ratios, increased with soil depth in all forest types, but were highest in *P. sylvestris* forests. Furthermore, the $\delta^{15}N$ signatures showed a steeper increase with depth in *P. sylvestris* forests compared to mixed and *Q. robur* forests. This suggests that *P. sylvestris* forests may have a higher input of isotopically enriched nitrogen, potentially due to nitrogen fixation or nitrogen deposition from the atmosphere (Phillips et al. 2013). The higher $\delta^{15}N$ signatures in *P. sylvestris* forests can also be attributed to the higher fungal biomass in these stands, as fungi can fractionate nitrogen isotopes during nutrient uptake and cycling (Phillips et al. 2013).

In terms of fungal community composition, we observed significant differences among the vegetation types. The overall composition of fungal communities showed distinct patterns between pure *P. sylvestris*, mixed *P. sylvestris*-*Q. robur*, and pure *Q. robur* forests, being pure *P. sylvestris* stands significantly different compared to the other forest types. Similarly, mycorrhizal, and saprotrophic fungal communities showed similar compositional patterns, with relatively small differences detected between pure and mixed *P. sylvestris* stands in terms of mycorrhizal fungi. The compositional differences observed in saprotrophic communities among soil layers suggest variations in decomposition processes and substrate availability across different depths (Hobbie, 2005). In any case, the dominant phyla in the fungal communities were Ascomycota, Basidiomycota, and Zygomycota, with Ascomycota being the most abundant. The most abundant guilds were root-associated basidiomycetes and moulds, followed by saprotrophs and root-associated ascomycetes. These results highlight the importance of both mycorrhizal and saprotrophic fungi in these forest ecosystems, as they play key roles in nutrient cycling and organic matter decomposition processes (Fernandez et al., 2014; Phillips et al., 2013).

Our study also revealed significant differences in the genetic potential for ammonia oxidation by bacteria (AOB) and archaea (AOA), as well as $\delta^{15}N$ signatures, among the different forest types. The *Q. robur* forests exhibited higher genetic potential for ammonia oxidation and higher $\delta^{15}N$ signatures compared to the other forest types. This suggests that *Q. robur* forests may have a higher abundance and activity of nitrifying microorganisms, potentially contributing to increased nitrogen availability and transformation processes in these ecosystems (Hagenbo et al., 2020). The higher C:N ratio observed in mixed forests indicates a relatively higher carbon

content compared to nitrogen content in the soil, which could be attributed to the specific litter inputs and decomposition dynamics in these forests (Ishida et al., 2007).

Regarding the fungal communities, we observed differences in their abundance and composition across the soil layers and forest types. Saprotrophic fungi were generally more abundant in the upper layers, regardless of the forest type. This is expected as saprotrophic fungi play a vital role in organic matter decomposition, and the availability of organic substrates tends to be higher in the surface layers of the soil (Boddy et al., 2007; Lindahl et al., 2007). In *P. sylvestris* forests, the fungal communities were predominantly dominated by root- associated basidiomycetes in the O2 and OM1 layers, while moulds were most abundant in the OM2 layers. This suggests a stratification of fungal communities along the soil depth gradient, with different fungal guilds occupying distinct niches within the soil profile. In contrast, in *Q. robur* forests, the relative abundance of root-associated basidiomycetes was highest across all three soil layers, indicating their importance in these ecosystems. *Clavulina spp.* emerged as the dominant species in *Q. robur* forests, highlighting its ecological significance in these environments. Moreover, root-associated basidiomycetes were found to be dominant across all three forest types, suggesting their widespread association with tree roots and potential roles in nutrient uptake and cycling (Hobbie et al., 2010, Pena et al., 2013).

The exploration types of root-associated basidiomycetes showed variation between pure *P. sylvestris* and pure *Q. robur* forests. The relative abundance of long-distance exploration types (long and medium fringe types) was highest in *P. sylvestris* and *P. sylvestris*-*Q. robur* forests, indicating a potential strategy of these fungi to explore larger areas in search of nutrients (Castaño et al., 2018). In contrast, short-distance exploration types (contact and short types) dominated in pure *Q. robur* forests, suggesting a more localized nutrient acquisition strategy in these stands. These differences in exploration types reflect the adaptability and resource acquisition strategies of root-associated basidiomycetes in different forest ecosystems.

To further explore the relationships between soil properties, fungal biomass, and ammonia-oxidizing bacteria, we constructed a structural equation model (SEM). The results of the SEM demonstrated that depth differences in C:N ratio had a significant negative relationship with mycorrhizal and saprotrophic biomass across soil depth. This suggests that changes in the C:N ratio with depth influence the biomass of these fungal guilds, potentially due to variations in nutrient availability and substrate quality (Phillips et al., 2013). NH_4-N had a significant negative effect on amoA bacteria, indicating that high ammonium concentrations may suppress the abundance of these nitrifying microorganisms. However, NH_4-N had a positive effect on

saprotrophic biomass, suggesting that higher ammonium levels may enhance the decomposition activity of saprotrophic fungi. Furthermore, mycorrhizal and saprotrophic biomass showed a significant negative effect on $\delta^{15}N$ depth differences, indicating that these fungal guilds may contribute to the stabilization and cycling of nitrogen isotopes in the soil.

5. Conclusions

Overall, our findings demonstrate that different vegetation types exhibit distinct soil characteristics and fungal community compositions. The variations in soil properties, such as C:N ratios, N content, extractable NH_4-N and P concentrations, and fungal biomass, suggest differences in nutrient availability and cycling among the vegetation types. These variations can be attributed to the specific traits and interactions of tree species, microbial communities, and soil processes. At the same time, the genetic potential for ammonia oxidation, C:N ratios, and fungal community composition varied among the forest types, suggesting unique nutrient cycling processes and ecological functions in each ecosystem. Understanding these differences is crucial, especially in the context of global vegetation shifts, as they can have significant implications for soil carbon and nitrogen fluxes. Further research is needed to explore the underlying mechanisms driving these patterns and their potential long-term impacts on ecosystem functioning and nutrient dynamics.

6. References

Abarenkov, K., Nilsson, R.H., Larsson, K.H., Alexander, I.J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A.F.S., Tedersoo, L., Ursing, B.M., Vrålstad, T., Liimatainen, K., Peintner, U., Kõljalg, U., 2010. The UNITE database for molecular identification of fungi - recent updates and future perspectives. New Phytologist. doi:10.1111/j.1469-8137.2009.03160.x

Adamo, I., Castaño, C., Bonet, J.A., Colinas, C., Martínez de Aragón, J., Alday, J.G., 2021. Soil physico-chemical properties have a greater effect on soil fungi than host species in Mediterranean pure and mixed pine forests. Soil Biology and Biochemistry 160. doi:10.1016/j.soilbio.2021.108320

Agerer, R., 2001. Exploration types of ectomycorrhizae. Mycorrhiza. doi:10.1007/s005720100108

Averill, C., Hawkes, C. V., 2016a. Ectomycorrhizal fungi slow soil carbon cycling. Ecology Letters 19, 937–947. doi:10.1111/ele.12631

Averill, C., Hawkes, C. V., 2016b. Ectomycorrhizal fungi slow soil carbon cycling. Ecology Letters 19, 937–947. doi:10.1111/ele.12631

Averill, C., Turner, B.L., Finzi, A.C., 2014. Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. Nature. doi:10.1038/nature12901

Castaño, C., Alday, J.G., Lindahl, B.D., Martínez de Aragón, J., de-Miguel, S., Colinas, C., Parladé, J., Pera, J., Bonet, J.A., 2018. Lack of thinning effects over inter-annual changes in soil fungal community and diversity in a Mediterranean pine forest. Forest Ecology and Management. doi:10.1016/j.foreco.2018.05.004

Castaño, C., Berlin, A., Brandström Durling, M., Ihrmark, K., Lindahl, B.D., Stenlid, J., Clemmensen, K.E., Olson, Å., 2020a. Optimized metabarcoding with Pacific biosciences enables semi-quantitative analysis of fungal communities. New Phytologist 228, 1149–1158. doi:10.1111/NPH.16731

Castaño, C., Berlin, A., Brandström Durling, M., Ihrmark, K., Lindahl, B.D., Stenlid, J., Clemmensen, K.E., Olson, Å., 2020b. Optimized metabarcoding with Pacific biosciences enables semi-quantitative analysis of fungal communities. New Phytologist 228, 1149–1158. doi:10.1111/nph.16731

Castaño, C., Hallin, S., Egelkraut, D., Lindahl, B.D., Olofsson, J., Clemmensen, K.E., 2022. Contrasting plant–soil–microbial feedbacks stabilize vegetation types and uncouple topsoil C and N stocks across a subarctic–alpine landscape. New Phytologist 2621–2633. doi:10.1111/nph.18679

Cheeke, T.E., Phillips, R.P., Brzostek, E.R., Rosling, A., Bever, J.D., Fransson, P., 2017. Dominant mycorrhizal association of trees alters carbon and nutrient cycling by selecting for microbial groups with distinct enzyme function. New Phytologist 214, 432–442. doi:10.1111/nph.14343

Clemmensen, K.E., Bahr, A., Ovaskainen, O., Dahlberg, A., Ekblad, A., Wallander, H., Stenlid, J., Finlay, R.D., Wardle, D.A., Lindahl, B.D., 2013. Roots and associated fungi drive long-term carbon sequestration in boreal forest. Science 340, 1615–1618. doi:10.1126/science.1231923

Clemmensen, K.E., Durling, M.B., Michelsen, A., Hallin, S., Finlay, R.D., Lindahl, B.D., 2021. A tipping point in carbon storage when forest expands into tundra is related to mycorrhizal recycling of nitrogen. Ecology Letters 24, 1193–1204. doi:10.1111/ele.13735

Create, T., Data, E., Using, V., Description, G., 2023. Package 'ggplot2.'

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. doi:10.1093/bioinformatics/btq461

Ekblad, A., Wallander, H., Godbold, D.L., Cruz, C., Johnson, D., Baldrian, P., Björk, R.G., Epron, D., Kieliszewska-Rokicka, B., Kjøller, R., Kraigher, H., Matzner, E., Neumann, J., Plassard, C., 2013. The production and turnover of extramatrical mycelium of ectomycorrhizal fungi in forest soils: Role in carbon cycling. Plant and Soil 366, 1–27. doi:10.1007/s11104-013-1630-3

Fernandez, C.W., Kennedy, P.G., 2018. Melanization of mycorrhizal fungal necromass structures microbial decomposer communities. Journal of Ecology 106, 468–479. doi:10.1111/1365-2745.12920

Fernandez, C.W., Koide, R.T., 2014. Initial melanin and nitrogen concentrations control the decomposition of ectomycorrhizal fungal litter. Soil Biology and Biochemistry 77, 150–157. doi:10.1016/j.soilbio.2014.06.026

Fernandez, C.W., See, C.R., Kennedy, P.G., 2020. Decelerated carbon cycling by ectomycorrhizal fungi is controlled by substrate quality and community composition. New Phytologist 226, 569–582. doi:10.1111/nph.16269

Grace, J.B, Keeley, J.E., 2006. A structural equation model analysis of postfire plant diversity in California shrublands. Ecological Applications 16(2), 503–514. doi: 10.1890/1051-0761(2006)016[0503:ASEMAO]2.0.CO;2

Hagenbo, A., Piñuela, Y., Castaño, C., Martínez de Aragón, J., de-Miguel, S., Alday, J.G., Bonet, J.A., 2021. Production and turnover of mycorrhizal soil mycelium relate to variation in drought conditions in Mediterranean Pinus pinaster, Pinus sylvestris and

Quercus ilex forests. New Phytologist 230, 1609–1622. doi:10.1111/nph.17012 Hagenbo, A., Piñuela, Y., Castaño, C., Martínez de Aragón, J., de-Miguel, S., Alday, J.G., Bonet, J.A., 2020. Production and turnover of mycorrhizal soil mycelium relate to variation in drought conditions in Mediterranean *Pinus pinaster* , *Pinus sylvestris* and *Quercus ilex* forests. New Phytologist nph.17012. doi:10.1111/nph.17012

Hobbie, E.A., Agerer, R., 2010. Nitrogen isotopes in ectomycorrhizal sporocarps correspond to belowground exploration types. Plant and Soil. doi:10.1007/s11104-009-0032-z

Högberg, P., Johannisson, C., Högberg, M.N., 2014. Is the high 15N natural abundance of trees in N-loaded forests caused by an internal ecosystem N isotope redistribution or a change in the ecosystem N isotope mass balance? Biogeochemistry 117, 351–358. doi:10.1007/s10533-013-9873-x

Ihrmark, K., Bödeker, I.T.M., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., Strid, Y., Stenlid, J., Brandström-Durling, M., Clemmensen, K.E., Lindahl, B.D., 2012. New primers to amplify the fungal ITS2 region - evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiology Ecology 82, 666–677. doi:10.1111/j.1574-6941.2012.01437.x

Ishida, T.A., Nara, K., Hogetsu, T., 2007. Host effects on ectomycorrhizal fungal communities: Insight from eight host species in mixed conifer-broadleaf forests. New Phytologist. doi:10.1111/j.1469-8137.2007.02016.x

Koljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F.S., Bahram, M., 2014. Towards a unified paradigm for sequence-based identification of fungi. Molecular Ecology 22, 5271–5277. doi:10.1111/mec.12481

Legendre, P., Gallagher, E.D., 2001. Ecologically meaningful transformations for ordination of species data. Oecologia. doi:10.1007/s004420100716

Liesack, Werner; Jan-Henrich, R., 1997. The Ammonia Monooxygenase Structural Gene. Microbiology 63, 4704–4712.

Lin, G., McCormack, M.L., Ma, C., Guo, D., 2017. Similar below-ground carbon cycling dynamics but contrasting modes of nitrogen cycling between arbuscular mycorrhizal and ectomycorrhizal forests. New Phytologist 213, 1440–1451. doi:10.1111/nph.14206

Lin, J., Wang, Y., Sun, S., Mu, C., Yan, X., 2017. Effects of arbuscular mycorrhizal fungi on the growth, photosynthesis and photosynthetic pigments of Leymus chinensis seedlings under salt-alkali stress and nitrogen deposition. Science of the Total Environment 576, 234–241. doi:10.1016/j.scitotenv.2016.10.091

Lindahl, B.D., Ihrmark, K., Boberg, J., Trumbore, S.E., Högberg, P., Stenlid, J., Finlay, R.D.,

2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytologist 173, 611–620. doi:10.1111/j.1469-8137.2006.01936.x

Lindahl, B.D., Tunlid, A., 2015. Ectomycorrhizal fungi - potential organic matter decomposers, yet not saprotrophs. New Phytologist 205, 1443–1447. doi:10.1111/nph.13201

McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS ONE. doi:10.1371/journal.pone.0061217

Menzel, A., Yuan, Y., Matiu, M., Sparks, T., Scheifinger, H., Gehrig, R., Estrella, N., 2020. Climate change fingerprints in recent European plant phenology. Global Change Biology 26, 2599–2612. doi:10.1111/gcb.15000

Näsholm, T., Högberg, P., Franklin, O., Metcalfe, D., Keel, S.G., Campbell, C., Hurry, V., Linder, S., Högberg, M.N., 2013. Are ectomycorrhizal fungi alleviating or aggravating nitrogen limitation of tree growth in boreal forests? New Phytologist 198, 214–221. doi:10.1111/nph.12139

Nguyen, N.H., Song, Z., Bates, S.T., Branco, S., Tedersoo, L., Menke, J., Schilling, J.S., Kennedy, P.G., 2016. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecology 20, 241–248. doi:10.1016/j.funeco.2015.06.006

Nylund, J.E., Wallander, H., 1992. Ergosterol Analysis as a Means of Quantifying Mycorrhizal Biomass. Methods in Microbiology. doi:10.1016/S0580-9517(08)70088-6

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P.R., O ’hara, R.B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H., Szoecs, E., Wagner, H., Oksanen, M.J., 2018. Package “vegan.”

Pena, R., Lang, C., Lohaus, G., Boch, S., Schall, P., Schöning, I., Ammer, C., Fischer, M., Polle, A., 2017. Phylogenetic and functional traits of ectomycorrhizal assemblages in top soil from different biogeographic regions and forest types. Mycorrhiza. doi:10.1007/s00572-016-0742-z

Philippot, L., Hallin, S., Schloter, M., 2007. Ecology of Denitrifying Prokaryotes in Agricultural Soil. Advances in Agronomy 96, 249–305. doi:10.1016/S0065-2113(07)96003-4

Phillips, R.P., Brzostek, E., Midgley, M.G., 2013. The mycorrhizal-associated nutrient economy: A new framework for predicting carbon-nutrient couplings in temperate forests. New Phytologist 199, 41–51. doi:10.1111/nph.12221

Pretzsch, H., Steckel, M., Heym, M., Biber, P., Ammer, C., Ehbrecht, M., Bielak, K., Bravo,

F., Ordóñez, C., Collet, C., Vast, F., Drössler, L., Brazaitis, G., Godvod, K., Jansons, A., de-Dios-García, J., Löf, M., Aldea, J., Korboulewsky, N., Reventlow, D.O.J., Nothdurft, A., Engel, M., Pach, M., Skrzyszewski, J., Pardos, M., Ponette, Q., Sitko, R., Fabrika, M., Svoboda, M., Černý, J., Wolff, B., Ruíz-Peinado, R., del Río, M., 2020. Stand growth and structure of mixed-species and monospecific stands of Scots pine (Pinus sylvestris L.) and oak (Q. robur L., Quercus petraea (Matt.) Liebl.) analysed along a productivity gradient through Europe. European Journal of Forest Research 139, 349–367. doi:10.1007/S10342-019-01233-Y/FIGURES/6

Robinson, D., 2001. δ15N as an integrator of the nitrogen cycle. Trends in Ecology and Evolution 16, 153–162. doi:10.1016/S0169-5347(00)02098-X

Rosseel, Y., 2011. lavaan: An R package for equation modeling. Journal of Statistical Software 48(2). doi:10.18637/jss.v048.i02.

Santana, V.M., Alday, J.G., Adamo, I., Alloza, J.A., Baeza, M.J., 2020. Climate, and not fire, drives the phylogenetic clustering of species with hard-coated seeds in Mediterranean Basin communities. Perspectives in Plant Ecology, Evolution and Systematics 45, 1–34. doi:10.1016/j.ppees.2020.125545

Sardans, J., Peñuelas, J., 2013. Plant-soil interactions in Mediterranean forest and shrublands: Impacts of climatic change. Plant and Soil. doi:10.1007/s11104-013-1591-6

Somervuo, P., Koskela, S., Pennanen, J., Henrik Nilsson, R., Ovaskainen, O., 2016. Unbiased probabilistic taxonomic classification for DNA barcoding. Bioinformatics. doi:10.1093/bioinformatics/btw346

Soudzilovskaia, N.A., van Bodegom, P.M., Terrer, C., Zelfde, M. van't, McCallum, I., Luke McCormack, M., Fisher, J.B., Brundrett, M.C., de Sá, N.C., Tedersoo, L., 2019. Global mycorrhizal plant distribution linked to terrestrial carbon stocks. Nature Communications 2019 10:1 10, 1–10. doi:10.1038/s41467-019-13019-2

Steckel, M., del Río, M., Heym, M., Aldea, J., Bielak, K., Brazaitis, G., Černý, J., Coll, L., Collet, C., Ehbrecht, M., Jansons, A., Nothdurft, A., Pach, M., Pardos, M., Ponette, Q., Reventlow, D.O.J., Sitko, R., Svoboda, M., Vallet, P., Wolff, B., Pretzsch, H., 2020. Species mixing reduces drought susceptibility of Scots pine (Pinus sylvestris L.) and oak (Quercus robur L., Quercus petraea (Matt.) Liebl.) – Site water supply and fertility modify the mixing effect. Forest Ecology and Management 461, 117908. doi:10.1016/J.FORECO.2020.117908

Steidinger, B.S., Crowther, T.W., Liang, J., Van Nuland, M.E., Werner, G.D.A., et al. 2019. Climatic controls of decomposition drive the global biogeography of forest-tree

symbioses. Nature 569, 404–408. doi:10.1038/s41586-019-1128-0

Sterkenburg, E., Clemmensen, K.E., Ekblad, A., Finlay, R.D., Lindahl, B.D., 2018. Contrasting effects of ectomycorrhizal fungi on early and late stage decomposition in a boreal forest. ISME Journal. doi:10.1038/s41396-018-0181-2

Sun, S., Gao, Y., Ling, X., Lou, H., 2005. The combination effects of phenolic compounds and fluconazole on the formation of ergosterol in Candida albicans determined by high-performance liquid chromatography/tandem mass spectrometry. Analytical Biochemistry 336, 39–45. doi:10.1016/j.ab.2004.06.038

Tedersoo, L., Mett, M., Ishida, T.A., Bahram, M., 2013. Phylogenetic relationships among host plants explain differences in fungal species richness and community composition in ectomycorrhizal symbiosis. New Phytologist. doi:10.1111/nph.12328

Tourna, M., Freitag, T.E., Nicol, G.W., Prosser, J.I., 2008. Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environmental Microbiology 10, 1357–1364. doi:10.1111/j.1462-2920.2007.01563.x

Van der Wal, A., Geydan, T.D., Kuyper, T.W., De Boer, W., 2013. A thready affair: Linking Fungal diversity and community dynamics to terrestrial decomposition processes. FEMS Microbiology Reviews 37, 477–494. doi:10.1111/1574-6976.12001

Voriskova, J., Baldrian, P., 2013. Fungal community on decomposing leaf litter undergoes rapid successional changes. ISME Journal 7, 477–486. doi:10.1038/ismej.2012.116

Vospernik, S., Heym, M., Pretzsch, H., Pach, M., Steckel, M., Aldea, J., Brazaitis, G., Bravo-Oviedo, A., Del Rio, M., Löf, M., Pardos, M., Bielak, K., Bravo, F., Coll, L., Černý, J., Droessler, L., Ehbrecht, M., Jansons, A., Korboulewsky, N., Jourdan, M., Nord-Larsen, T., Nothdurft, A., Ruiz-Peinado, R., Ponette, Q., Sitko, R., Svoboda, M., Wolff, B., 2023. Tree species growth response to climate in mixtures of Quercus robur/Quercus petraea and Pinus sylvestris across Europe - a dynamic, sensitive equilibrium. Forest Ecology and Management 530. doi:10.1016/j.foreco.2022.120753

Wallander, H., Ekblad, A., Godbold, D.L., Johnson, D., Bahr, A., Baldrian, P., Björk, R.G., Kieliszewska-Rokicka, B., Kjøller, R., Kraigher, H., Plassard, C., Rudawska, M., 2013. Evaluation of methods to estimate production, biomass and turnover of ectomycorrhizal mycelium in forests soils - A review. Soil Biology and Biochemistry 57, 1034–1047. doi:10.1016/j.soilbio.2012.08.027

Wallander, H., Johansson, U., Sterkenburg, E., Brandström Durling, M., Lindahl, B.D., 2010. Production of ectomycorrhizal mycelium peaks during canopy closure in Norway spruce forests. New Phytologist 187, 1124–1134. doi:10.1111/j.1469-8137.2010.03324.x

Supplementary Material

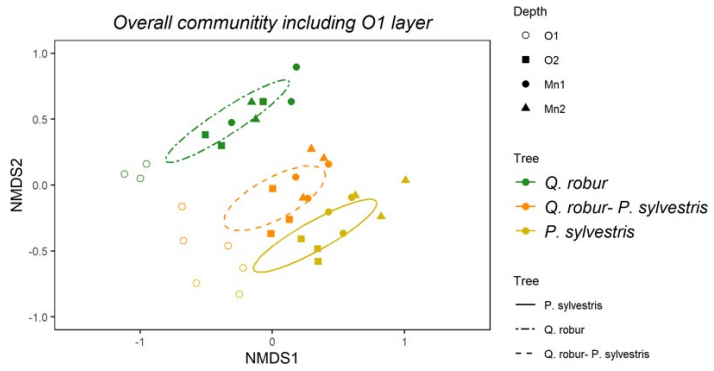

Fig. S1. Non-metric multidimensional scaling (NMDS) of total fungal community composition including the O1 soil layer in three forest types: *P. sylvestris* (gold; *P. Sylvestris-Q. robur* (orange); *Q. robur* (forest green).

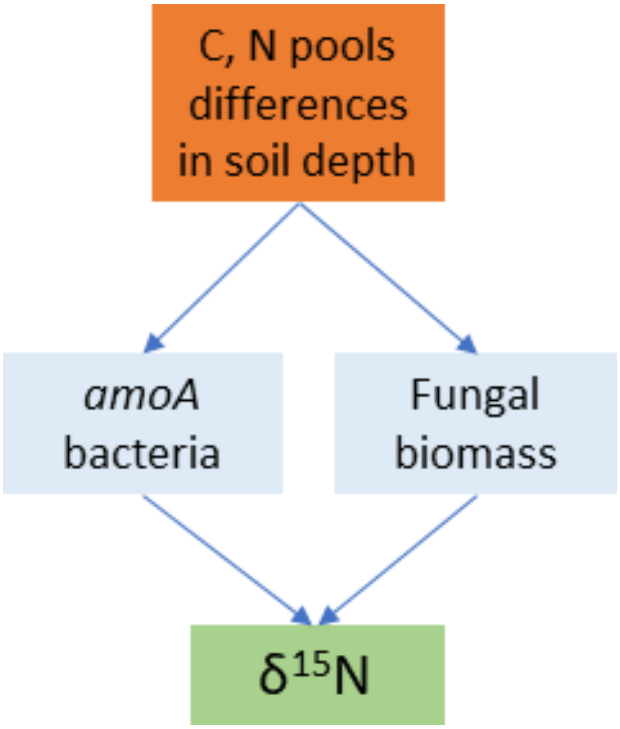

Fig S2. A conceptual structural equation model showing the expected relationships between C and N pools in soil depth and the microbial soil components and the effects on $\delta^{15}N$ difference in soil depth.

Table S1. $\delta^{15}N$ weighted mean values between forest types.

P. sylvestris	$\delta^{15}N$ **weighted mean**
P1	0.402
P2	1.027
P3	0.161
Q. robur	$\delta^{15}N$ **weighted mean**
Q1	1.340
Q2	1.011
Q3	1.523
P. sylvestris-Q.robur	$\delta^{15}N$ **weighted mean**
M1	0.845
M2	1.118
M3	1.328

GENERAL DISCUSSION

The results obtained in this thesis provide new insights into the soil fungal communities inhabiting Mediterranean forest ecosystems, identifying the soil physicochemical factors that filter them and their relationships with host and nutrient cycles. In addition, this thesis provides information on the ecology of soil fungi very relevant to understand the ecosystem functioning and to adapt the forest management to the future changes. The work also provides results on phylogenetics and assemblage processes of the fungal communities in Mediterranean pine forest. To finish, different nutrient cycles related with the soil fungal communities have been described for forest ecosystems dominated by different host species. The integration of these four studies allows for a more comprehensive overview of the intricate relationships between fungi, soils, and the surrounding environment in these ecologically significant Mediterranean forests. Such knowledge is crucial for effective forest management practices and the preservation of these vital ecosystems in the face of ongoing environmental changes.

Which is the optimal sampling size in Mediterranean pure and mixed pine oak forests?

This study underlines the importance of sample pool size for accurate soil fungal diversity estimation in Mediterranean pure and mixed pine-oak forests, as increasing the number of soil sample pools, i.e. sampled volume, more reliable diversity predictions can be made with a positive species/area relationship. However, it seems not possible to standardise sampling pool protocols across distinct forest types, as our richness results showed that optimal soil sample pool size depended on forest type (e.g. pure or mixed forests). Moreover, increasing number of soil sample pools led to an increase in community similarity in pure forests, but not in mixed forests. Consequently, pools that represented less than three soil samples led to significant increases in ß-diversity values in pure forests, while values did not change in mixed forests. Finally, increasing the number of sample pools had no significant effect over species composition for any forest type, as we increased the sample pools while repeatedly sampling the same sites.

In this study, increasing number of sample pools had a significant effect on fungal richness in all the three forest types, indicating a positive species/area relationship. Moreover, our results indicate that the minimum number of sample pools to adequately estimate fungal richness and species composition will be lower in monospecific stands, three in our case, than for more diverse mixed forest where the optimal pooling will be almost six samples. Our results

shed light on best soil sample monitoring implementations to be applied for characterizing pure and mixed forests ecosystems.

Soil physico-chemical properties have a greater effect on soil fungi than host species.

At regional spatial scales, our results revealed that niche processes such as environmental filtering (i.e., soil parameters) and neutral processes (geographical distance) largely determined the fungal community assemblages rather than other niche processes as interspecific differences between *Pinus* species. However, the relative importance of soil variables on fungal community assembly varied between mycorrhizal and saprotrophic guilds because the mycorrhizal communities were primarily shaped by pH and P effects, whereas the saprotrophic communities were shaped mainly by the C:N ratio and N. Thus, these results suggest that different assembly mechanisms are involved in the structuring of mycorrhizal and saprotrophic communities. Interestingly, soil fungal communities are not influenced by closely related congeneric host species but are primarily affected by soil properties, with pH, P and the C:N ratio the strongest predictors shaping fungal communities in these forest ecosystems. Importantly, mycorrhizal communities are significantly affected by P but not N, therefore being the limiting nutrient in these ecosystems. Conversely, saprotrophic communities are significantly influenced by the C:N ratio and N.

Lack of phylogenetic differences in ectomycorrhizal fungi among distinct pine forest.

The results of our phylogenetic study on ectomycorrhizal communities in Mediterranean pine forests, showed that phylogenetic composition, structure, and diversity were similar among habitats with distinct pine tree hosts. However, significant differences were found in nearest taxon index values between *P. nigra-halepensis* and *P. sylvestris-nigra*, probably not directly caused by differences in tree hosts but due to higher differences in the local abiotic conditions in *P. sylvestris-nigra* than in *P. halepensis-nigra* sites. Moreover, we detected a weak abiotic filtering effect on ectomycorrhizal phylogenetic compositional variation, being pH the only variable among soil variables that significantly influence ectomycorrhizal phylogenetic community. This finding suggests that pH acts as a strong abiotic filter on ectomycorrhizal community at both phylogenetic and taxonomic level. In contrast, ectomycorrhizal phylogenetic structure variation was marginally influenced only by the shared effect of stand structure, soil and geographic distance. Therefore, phylogenetic structure may be indirectly influenced by other processes (i.e. competition) not directly tested in this study. Finally, short and contact exploration types were the most abundant in these forest ecosystems.

Conversely, long exploration types were the least abundant, although, there was no phylogenetic signal since exploration types were dispersed across the phylogenetic tree. In this study, there were no differences neither in ectomycorrhizal phylogenetic community composition nor structure and diversity, indicating that ectomycorrhizal communities at both phylogenetic and taxonomic level do not change among phylogenetically closely related tree hosts. Moreover, soil parameters only had a marginal filtering effect on ectomycorrhizal phylogenetic variation as pH resulted the only significant driver of the phylogenetic community. In this regard, our results showed that pH acts as the broadest abiotic filter of ectomycorrhizal communities at local scale. Conversely, ectomycorrhizal phylogenetic structure was marginally influenced by the combined effect of soil, stand structure and geographic distance, indicating that phylogenetic structure is mainly influenced by their combined effect. Finally, short and contact distance were the dominant exploration types, as they may be favoured under drought stress conditions but also under high nutrient availability.

Distinct nutrient economy within ectomycorrhizal types relates to dominance by microbial groups with contrasting ecologies.

There were distinct patterns in soil characteristics and microbial communities among vegetation types. Notably, C:N ratios differed more profoundly with soil depth in *Q. robur* than in *P. sylvestris* stands. Compared to *Q. robur* soils, higher relative abundances of ectomycorrhizal fungi with extensive mycelia in *P. sylvestris* paralleled with higher fungal biomass, higher $\delta^{15}N$ signatures, N content and ammonium concentration. By contrast, *Q. robur* soils had higher archaeal and bacterial ammonia oxidizers, and a larger dominance by ectomycorrhizal fungi with contact exploration types. Mixed forest soils displayed transitional characteristics in terms of soil parameters and microbial communities. It seems that there are changes in the nutrient economy among ectomycorrhizal types relates to shifts in microbial communities with contrasting ecologies. Replacement of conifers by broadleaf species may promote shifts towards inorganic N-cycling, while ectomycorrhizal fungi in conifers may retain N and slow C cycling. These findings are important in the context of ongoing global shifts in vegetation types, which can lead to microbial-driven changes in soil C and N fluxes.

CONCLUSIONS

- The sample pool size significantly influences the estimation of soil fungal diversity, and increasing the number of soil sample pools improves diversity predictions. Moreover, increasing the number of sample pools leads to increased community similarity in pure forests but not in mixed forests, affecting ß-diversity values differently. Therefore, the minimum number of sample pools to adequately estimate fungal richness and species composition will be lower in monospecific stands, three in our case, than for more diverse mixed forest where the optimal pooling will be almost six samples (Paper 1).

- Soil physicochemical properties have a greater influence on soil fungi than host species, and environmental filtering plays a significant role in shaping fungal communities. (Paper 2)

- Different assembly mechanisms are involved in structuring mycorrhizal and saprotrophic fungal communities, with mycorrhizal communities primarily shaped by pH and P effects and saprotrophic communities influenced by the C:N ratio and N. (Paper 2)

- Phylogenetic composition, structure, and diversity of ectomycorrhizal fungi are similar among distinct pine forest habitats, indicating a lack of phylogenetic differences related to tree hosts. However, local abiotic conditions and soil pH act as important factors in shaping ectomycorrhizal phylogenetic communities. (Paper 3)

- Different vegetation types exhibit distinct patterns in soil characteristics and microbial communities, with notable differences in C:N ratios, fungal biomass, nitrogen content, and abundance of specific microbial groups. This suggests differences in nutrient availability and cycling among the vegetation types. (Paper 4)

- The replacement of conifers by broadleaf species can lead to shifts in microbial communities and nutrient cycling, promoting inorganic nitrogen cycling and potentially

altering soil carbon and nitrogen fluxes. This highlights the importance of understanding microbial-driven processes in the context of global vegetation shifts. (Paper 4)

REFERENCES

Adamczyk, B., Ahvenainen, A., Sietiö, O.M., Kanerva, S., Kieloaho, A.J., Smolander, A., Kitunen, V., Saranpää, P., Laakso, T., Straková, P., Heinonsalo, J., 2016. The contribution of ericoid plants to soil nitrogen chemistry and organic matter decomposition in boreal forest soil. Soil Biology and Biochemistry. doi:10.1016/j.soilbio.2016.09.016

Anslan, S., Bahram, M., Hiiesalu, I., Tedersoo, L., 2017. PipeCraft: Flexible open-source toolkit for bioinformatics analysis of custom high-throughput amplicon sequencing data. Molecular Ecology Resources. doi:10.1111/1755-0998.12692

Averill, C., Hawkes, C. V., 2016. Ectomycorrhizal fungi slow soil carbon cycling. Ecology Letters 19, 937–947. doi:10.1111/ele.12631

Averill, C., Turner, B.L., Finzi, A.C., 2014. Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. Nature. doi:10.1038/nature12901

Awad, A., Majcherczyk, A., Schall, P., Schröter, K., Schöning, I., Schrumpf, M., Ehbrecht, M., Boch, S., Kahl, T., Bauhus, J., Seidel, D., Ammer, C., Fischer, M., Kües, U., Pena, R., 2019. Ectomycorrhizal and saprotrophic soil fungal biomass are driven by different factors and vary among broadleaf and coniferous temperate forests. Soil Biology and Biochemistry. doi:10.1016/j.soilbio.2018.12.014

Bahram, M., Kõljalg, U., Courty, P.E., Diédhiou, A.G., Kjøller, R., Põlme, S., Ryberg, M., Veldre, V., Tedersoo, L., 2013. The distance decay of similarity in communities of ectomycorrhizal fungi in different ecosystems and scales. Journal of Ecology 101, 1335–1344. doi:10.1111/1365-2745.12120

Baldrian, P., Zrůstová, P., Tláskal, V., Davidová, A., Merhautová, V., Vrška, T., 2016. Fungi associated with decomposing deadwood in a natural beech-dominated forest. Fungal Ecology 23, 109–122. doi:10.1016/j.funeco.2016.07.001

Bardgett, R.D., Van Der Putten, W.H., 2014. Belowground biodiversity and ecosystem functioning. Nature. doi:10.1038/nature13855

Bello, J., Hasselquist, N.J., Vallet, P., Kahmen, A., Perot, T., Korboulewsky, N., 2019. Complementary water uptake depth of Quercus petraea and Pinus sylvestris in mixed stands during an extreme drought. Plant and Soil. doi:10.1007/s11104-019-03951-z

Bjørnsgaard Aas, A., Davey, M.L., Kauserud, H., 2017. ITS all right mama: investigating the formation of chimeric sequences in the ITS2 region by DNA metabarcoding analyses of fungal mock communities of different complexities. Molecular Ecology Resources. doi:10.1111/1755-0998.12622

Boddy, L., Büntgen, U., Egli, S., Gange, A.C., Heegaard, E., Kirk, P.M., Mohammad, A., Kauserud, H., 2014. Climate variation effects on fungal fruiting. Fungal Ecology 10, 20–33. doi:10.1016/j.funeco.2013.10.006

Bravo-Oviedo, A., Pretzsch, H., Ammer, C., Andenmatten, E., Barbati, A., Barreiro, S., Brang, P., Bravo, F., Coll, L., Corona, P., Den Ouden, J., Ducey, M.J., Forrester, D.I., Giergiczny, M., Jacobsen, J.B., Lesinski, J., Löf, M., Mason, B., Matovic, B., Metslaid, M., Morneau, F., Motiejunaite, J., O'Reilly, C., Pach, M., Ponette, Q., Del Rio, M., Short, I., Skovsgaard, J.P., Soliño, M., Spathelf, P., Sterba, H., Stojanovic, D., Strelcova, K., Svoboda, M., Verheyen, K., Von Lüpke, N., Zlatanov, T., 2014. European mixed forests: Definition and research perspectives. Forest Systems. doi:10.5424/fs/2014233-06256

Cao, M. wen, Jia, T., Mi, J., Jing, J. hui, Chai, B. feng, 2019. Relative roles of niche and neutral processes on turnover of plant, fungal and bacterial communities in arid and semi-arid areas at the regional scale. Basic and Applied Ecology. doi:10.1016/j.baae.2019.08.005

Cavard, X., Macdonald, S.E., Bergeron, Y., Chen, H.Y.H., 2011. Importance of mixedwoods for biodiversity conservation: Evidence for understory plants, songbirds, soil fauna, and ectomycorrhizae in northern forests. Environmental Reviews. doi:10.1139/A11-004

Clemmensen, K.E., Bahr, A., Ovaskainen, O., Dahlberg, A., Ekblad, A., Wallander, H., Stenlid, J., Finlay, R.D., Wardle, D.A., Lindahl, B.D., 2013. Roots and associated fungi drive long-term carbon sequestration in boreal forest. Science 340, 1615–1618. doi:10.1126/science.1231923

Clemmensen, K.E., Durling, M.B., Michelsen, A., Hallin, S., Finlay, R.D., Lindahl, B.D., 2021. A tipping point in carbon storage when forest expands into tundra is related to mycorrhizal recycling of nitrogen. Ecology Letters 24, 1193–1204. doi:10.1111/ele.13735

De Filippis, F., Laiola, M., Blaiotta, G., Ercolini, D., 2017. Different amplicon targets for sequencing-based studies of fungal diversity. Applied and Environmental Microbiology 83, 1–9. doi:10.1128/AEM.00905-17

Dickie, I.A., Boyer, S., Buckley, H.L., Duncan, R.P., Gardner, P.P., Hogg, I.D., Holdaway, R.J., Lear, G., Makiola, A., Morales, S.E., Powell, J.R., Weaver, L., 2018. Towards robust and repeatable sampling methods in eDNA-based studies. Molecular Ecology Resources. doi:10.1111/1755-0998.12907

Dopheide, A., Xie, D., Buckley, T.R., Drummond, A.J., Newcomb, R.D., 2019. Impacts of DNA extraction and PCR on DNA metabarcoding estimates of soil biodiversity. Methods in Ecology and Evolution. doi:10.1111/2041-210X.13086

Du, E., Terrer, C., Pellegrini, A.F.A., Ahlström, A., van Lissa, C.J., Zhao, X., Xia, N., Wu, X., Jackson, R.B., 2020. Global patterns of terrestrial nitrogen and phosphorus limitation. Nature Geoscience. doi:10.1038/s41561-019-0530-4

Ekblad, A., Wallander, H., Godbold, D.L., Cruz, C., Johnson, D., Baldrian, P., Björk, R.G., Epron, D., Kieliszewska-Rokicka, B., Kjøller, R., Kraigher, H., Matzner, E., Neumann, J., Plassard, C., 2013. The production and turnover of extramatrical mycelium of ectomycorrhizal fungi in forest soils: Role in carbon cycling. Plant and Soil 366, 1–27. doi:10.1007/s11104-013-1630-3

Emerson, B.C., Gillespie, R.G., 2008. Phylogenetic analysis of community assembly and structure over space and time. Trends in Ecology and Evolution. doi:10.1016/j.tree.2008.07.005

Fernandez, C.W., Kennedy, P.G., 2018. Melanization of mycorrhizal fungal necromass structures microbial decomposer communities. Journal of Ecology 106, 468–479. doi:10.1111/1365-2745.12920

Fernandez, C.W., Kennedy, P.G., 2016. Revisiting the “Gadgil effect”: Do interguild fungal interactions control carbon cycling in forest soils? New Phytologist. doi:10.1111/nph.13648

Fernandez, C.W., Koide, R.T., 2014. Initial melanin and nitrogen concentrations control the decomposition of ectomycorrhizal fungal litter. Soil Biology and Biochemistry 77, 150–157. doi:10.1016/j.soilbio.2014.06.026

Glassman, S.I., Wang, I.J., Bruns, T.D., 2017. Environmental filtering by pH and soil nutrients drives community assembly in fungi at fine spatial scales. Molecular Ecology. doi:10.1111/mec.14414

Goldmann, K., Schöning, I., Buscot, F., Wubet, T., 2015. Forest management type influences diversity and community composition of soil fungi across temperate forest ecosystems. Frontiers in Microbiology. doi:10.3389/fmicb.2015.01300

Gravel, D., Canham, C.D., Beaudet, M., Messier, C., 2006. Reconciling niche and neutrality: The continuum hypothesis. Ecology Letters. doi:10.1111/j.1461-0248.2006.00884.x

Green, J., Bohannan, B.J.M., 2006. Spatial scaling of microbial biodiversity. Trends in Ecology and Evolution. doi:10.1016/j.tree.2006.06.012

Guo, J., Ling, N., Chen, Z., Xue, C., Li, L., Liu, L., Gao, L., Wang, M., Ruan, J., Guo, S.,

Vandenkoornhuyse, P., Shen, Q., 2020. Soil fungal assemblage complexity is dependent on soil fertility and dominated by deterministic processes. New Phytologist. doi:10.1111/nph.16345

Hagenbo, A., Piñuela, Y., Castaño, C., Martínez de Aragón, J., de-Miguel, S., Alday, J.G., Bonet, J.A., 2020. Production and turnover of mycorrhizal soil mycelium relate to variation in drought conditions in Mediterranean *Pinus pinaster* , *Pinus sylvestris* and *Quercus ilex* forests. New Phytologist nph.17012. doi:10.1111/nph.17012

Hibbett, D., Abarenkov, K., Kõljalg, U., Öpik, M., Chai, B., Cole, J., Wang, Q., Crous, P., Robert, V., Helgason, T., Herr, J.R., Kirk, P., Lueschow, S., O'Donnell, K., Nilsson, R.H., Oono, R., Schoch, C., Smyth, C., Walker, D.M., Porras-Alfaro, A., Taylor, J.W., Geiser, D.M., 2016. Sequence-based classification and identification of Fungi. Mycologia 108, 1049–1068. doi:10.3852/16-130

Hibbett, D.S., Ohman, A., Glotzer, D., Nuhn, M., Kirk, P., Nilsson, R.H., 2011. Progress in molecular and morphological taxon discovery in Fungi and options for formal classification of environmental sequences. Fungal Biology Reviews 25, 38–47. doi:10.1016/j.fbr.2011.01.001

Hobbie, E.A., Agerer, R., 2010. Nitrogen isotopes in ectomycorrhizal sporocarps correspond to belowground exploration types. Plant and Soil. doi:10.1007/s11104-009-0032-z

Högberg, P., Johannisson, C., Högberg, M.N., 2014. Is the high 15N natural abundance of trees in N-loaded forests caused by an internal ecosystem N isotope redistribution or a change in the ecosystem N isotope mass balance? Biogeochemistry 117, 351–358. doi:10.1007/s10533-013-9873-x

Ishida, T.A., Nara, K., Hogetsu, T., 2007. Host effects on ectomycorrhizal fungal communities: Insight from eight host species in mixed conifer-broadleaf forests. New Phytologist. doi:10.1111/j.1469-8137.2007.02016.x

Joergensen, R.G., Wichern, F., 2008. Quantitative assessment of the fungal contribution to microbial tissue in soil. Soil Biology and Biochemistry 40, 2977–2991. doi:10.1016/j.soilbio.2008.08.017

Kraft, N.J.B., Adler, P.B., Godoy, O., James, E.C., Fuller, S., Levine, J.M., 2015. Community assembly, coexistence and the environmental filtering metaphor. Functional Ecology. doi:10.1111/1365-2435.12345

Kyaschenko, J., Ovaskainen, O., Ekblad, A., Hagenbo, A., Karltun, E., Clemmensen, K.E., Lindahl, B.D., 2019. Soil fertility in boreal forest relates to root-driven nitrogen retention and carbon sequestration in the mor layer. New Phytologist. doi:10.1111/nph.15454

Lin, J., Wang, Y., Sun, S., Mu, C., Yan, X., 2017. Effects of arbuscular mycorrhizal fungi on the growth, photosynthesis and photosynthetic pigments of Leymus chinensis seedlings under salt-alkali stress and nitrogen deposition. Science of the Total Environment 576, 234–241. doi:10.1016/j.scitotenv.2016.10.091

Lindahl, B.D., Nilsson, R.H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., Kõljalg, U., Pennanen, T., Rosendahl, S., Stenlid, J., Kauserud, H., 2013. Fungal community analysis by high-throughput sequencing of amplified markers - a user's guide. New Phytologist. doi:10.1111/nph.12243

Lindahl, B.D., Tunlid, A., 2015. Ectomycorrhizal fungi - potential organic matter decomposers, yet not saprotrophs. New Phytologist 205, 1443–1447. doi:10.1111/nph.13201

Maherali, H., Klironomos, J.N., 2007. Influence of phylogeny on fungal community assembly and ecosystem functioning. Science. doi:10.1126/science.1143082

Mohan, J.E., Cowden, C.C., Baas, P., Dawadi, A., Frankson, P.T., Helmick, K., Hughes, E., Khan, S., Lang, A., Machmuller, M., Taylor, M., Witt, C.A., 2014. Mycorrhizal fungi mediation of terrestrial ecosystem responses to global change: Mini-review. Fungal Ecology. doi:10.1016/j.funeco.2014.01.005

Nagati, M., Roy, M., Manzi, S., Richard, F., Desrochers, A., Gardes, M., Bergeron, Y., 2018. Impact of local forest composition on soil fungal communities in a mixed boreal forest. Plant and Soil 432, 345–357. doi:10.1007/s11104-018-3806-3

Näsholm, T., Högberg, P., Franklin, O., Metcalfe, D., Keel, S.G., Campbell, C., Hurry, V., Linder, S., Högberg, M.N., 2013. Are ectomycorrhizal fungi alleviating or aggravating nitrogen limitation of tree growth in boreal forests? New Phytologist 198, 214–221. doi:10.1111/nph.12139

Nilsson, R.H., Wurzbacher, C., Bahram, M., Coimbra, V.R.M., Larsson, E., Tedersoo, L., Eriksson, J., Ritter, C.D., Svantesson, S., Sánchez-García, M., Ryberg, M., Kristiansson, E., Abarenkov, K., 2016. Top 50 most wanted fungi. MycoKeys. doi:10.3897/mycokeys.12.7553

Pauvert, C., Buée, M., Laval, V., Edel-Hermann, V., Fauchery, L., Gautier, A., Lesur, I., Vallance, J., Vacher, C., 2019. Bioinformatics matters: The accuracy of plant and soil fungal community data is highly dependent on the metabarcoding pipeline. Fungal Ecology 41, 23–33. doi:10.1016/j.funeco.2019.03.005

Peay, K.G., Bruns, T.D., 2014. Spore dispersal of basidiomycete fungi at the landscape scale is driven by stochastic and deterministic processes and generates variability in plant-fungal interactions. New Phytologist. doi:10.1111/nph.12906

Peay, K.G., Garbelotto, M., Bruns, T.D., 2010. Evidence of dispersal limitation in soil microorganisms: Isolation reduces species richness on mycorrhizal tree islands. Ecology. doi:10.1890/09-2237.1

Peay, K.G., Schubert, M.G., Nguyen, N.H., Bruns, T.D., 2012. Measuring ectomycorrhizal fungal dispersal: Macroecological patterns driven by microscopic propagules. Molecular Ecology. doi:10.1111/j.1365-294X.2012.05666.x

Phillips, R.P., Brzostek, E., Midgley, M.G., 2013. The mycorrhizal-associated nutrient economy: A new framework for predicting carbon-nutrient couplings in temperate forests. New Phytologist 199, 41–51. doi:10.1111/nph.12221

Read, D.J., Perez-Moreno, J., 2003. Mycorrhizas and nutrient cycling in ecosystems - A journey towards relevance? New Phytologist. doi:10.1046/j.1469-8137.2003.00704.x

Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4, e2584. doi:10.7717/peerj.2584

Sardans, J., Peñuelas, J., 2013. Plant-soil interactions in Mediterranean forest and shrublands: Impacts of climatic change. Plant and Soil. doi:10.1007/s11104-013-1591-6

Smith, S.E., Read, D.J., 2008. Mycorrhizal Symbiosis 3rd edn Academic Press: San Diego. Academic Press: San Diego.

Somervuo, P., Koskela, S., Pennanen, J., Henrik Nilsson, R., Ovaskainen, O., 2016. Unbiased probabilistic taxonomic classification for DNA barcoding. Bioinformatics. doi:10.1093/bioinformatics/btw346

Soudzilovskaia, N.A., van Bodegom, P.M., Terrer, C., Zelfde, M. van't, McCallum, I., Luke McCormack, M., Fisher, J.B., Brundrett, M.C., de Sá, N.C., Tedersoo, L., 2019. Global mycorrhizal plant distribution linked to terrestrial carbon stocks. Nature Communications 10, 1–10. doi:10.1038/s41467-019-13019-2

Steidinger, B.S., Crowther, T.W., Liang, J., Van Nuland, M.E., Werner, G.D.A., et al., 2019. Climatic controls of decomposition drive the global biogeography of forest-tree symbioses. Nature 569, 404–408. doi:10.1038/s41586-019-1128-0

Sterkenburg, E., Clemmensen, K.E., Ekblad, A., Finlay, R.D., Lindahl, B.D., 2018. Contrasting effects of ectomycorrhizal fungi on early and late stage decomposition in a boreal forest. ISME Journal. doi:10.1038/s41396-018-0181-2

Tedersoo, L., Anslan, S., Bahram, M., Drenkhan, R., Pritsch, K., Buegger, F., Padari, A., Hagh-Doust, N., Mikryukov, V., Gohar, D., Amiri, R., Hiiesalu, I., Lutter, R., Rosenvald, R., Rähn, E., Adamson, K., Drenkhan, T., Tullus, H., Jürimaa, K., Sibul, I., Otsing, E., Põlme, S., Metslaid, M., Loit, K., Agan, A., Puusepp, R., Varik, I., Kõljalg, U., Abarenkov, K.,

2020. Regional-Scale In-Depth Analysis of Soil Fungal Diversity Reveals Strong pH and Plant Species Effects in Northern Europe. Frontiers in Microbiology 11, 1–31. doi:10.3389/fmicb.2020.01953

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N.S., Wijesundera, R., Ruiz, L.V., Vasco-Palacios, A.M., Thu, P.Q., Suija, A., Smith, M.E., Sharp, C., Saluveer, E., Saitta, A., Rosas, M., Riit, T., Ratkowsky, D., Pritsch, K., Põldmaa, K., Piepenbring, M., Phosri, C., Peterson, M., Parts, K., Pärtel, K., Otsing, E., Nouhra, E., Njouonkou, A.L., Nilsson, R.H., Morgado, L.N., Mayor, J., May, T.W., Majuakim, L., Lodge, D.J., Lee, S., Larsson, K.H., Kohout, P., Hosaka, K., Hiiesalu, I., Henkel, T.W., Harend, H., Guo, L.D., Greslebin, A., Grelet, G., Geml, J., Gates, G., Dunstan, W., Dunk, C., Drenkhan, R., Dearnaley, J., De Kesel, A., Dang, T., Chen, X., Buegger, F., Brearley, F.Q., Bonito, G., Anslan, S., Abell, S., Abarenkov, K., 2014. Global diversity and geography of soil fungi. Science. doi:10.1126/science.1256688

Tedersoo, L., Smith, M.E., 2013. Lineages of ectomycorrhizal fungi revisited: Foraging strategies and novel lineages revealed by sequences from belowground. Fungal Biology Reviews. doi:10.1016/j.fbr.2013.09.001

Thuiller, W., Albert, C., Araújo, M.B., Berry, P.M., Cabeza, M., Guisan, A., Hickler, T., Midgley, G.F., Paterson, J., Schurr, F.M., Sykes, M.T., Zimmermann, N.E., 2008. Predicting global change impacts on plant species' distributions: Future challenges. Perspectives in Plant Ecology, Evolution and Systematics. doi:10.1016/j.ppees.2007.09.004

Tucker, C.M., Cadotte, M.W., Carvalho, S.B., Jonathan Davies, T., Ferrier, S., Fritz, S.A., Grenyer, R., Helmus, M.R., Jin, L.S., Mooers, A.O., Pavoine, S., Purschke, O., Redding, D.W., Rosauer, D.F., Winter, M., Mazel, F., 2017. A guide to phylogenetic metrics for conservation, community ecology and macroecology. Biological Reviews. doi:10.1111/brv.12252

Vamosi, S.M., Heard, S.B., Vamosi, J.C., Webb, C.O., 2009. Emerging patterns in the comparative analysis of phylogenetic community structure. Molecular Ecology. doi:10.1111/j.1365-294X.2008.04001.x

Van der Wal, A., Geydan, T.D., Kuyper, T.W., De Boer, W., 2013. A thready affair: Linking Fungal diversity and community dynamics to terrestrial decomposition processes. FEMS Microbiology Reviews 37, 477–494. doi:10.1111/1574-6976.12001

Větrovský, T., Kohout, P., Kopecký, M., Machac, A., Man, M., Bahnmann, B.D., Brabcová, V., Choi, J., Meszárošová, L., Human, Z.R., Lepinay, C., Lladó, S., López-Mondéjar, R.,

Martinović, T., Mašínová, T., Morais, D., Navrátilová, D., Odriozola, I., Šťursová, M., Švec, K., Tláskal, V., Urbanová, M., Wan, J., Žifčáková, L., Howe, A., Ladau, J., Peay, K.G., Storch, D., Wild, J., Baldrian, P., 2019. A meta-analysis of global fungal distribution reveals climate-driven patterns. Nature Communications. doi:10.1038/s41467-019-13164-8

Voriskova, J., Baldrian, P., 2013. Fungal community on decomposing leaf litter undergoes rapid successional changes. ISME Journal 7, 477–486. doi:10.1038/ismej.2012.116

Webb, C.O., 2000. Exploring the phylogenetic structure of ecological communities: An example for rain forest trees. American Naturalist. doi:10.1086/303378

Webb, C.O., Ackerly, D.D., McPeek, M.A., Donoghue, M.J., 2002. Phylogenies and community ecology. Annual Review of Ecology and Systematics. doi:10.1146/annurev.ecolsys.33.010802.150448

Zhang, T., Wang, N.F., Liu, H.Y., Zhang, Y.Q., Yu, L.Y., 2016. Soil pH is a key determinant of soil fungal community composition in the Ny-Ålesund Region, Svalbard (High Arctic). Frontiers in Microbiology. doi:10.3389/fmicb.2016.00227