Forest management and abiotic parameters effect on soil fungal communities inhabiting Mediterranean forest ecosystems

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Universitat de Lleida

TESI DOCTORAL

Forest management and abiotic parameters effect on soil fungal communities inhabiting Mediterranean forest ecosystems

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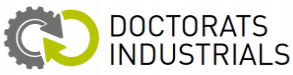

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RELATED WORKS AND MANUSCRIPTS

The following are the manuscripts derived from this thesis:

i. Castaño C, Parladé J, Pera J, Martínez de Aragón J, Alday JG., Bonet JA, 2016. Soil drying procedure affects the DNA quantification of *Lactarius vinosus* but does not change the fungal community composition. Mycorrhiza, 26, 799-808. https://doi.org/10.1007/s00572-016-0714-3.

ii. Castaño C, Alday JG, Parladé J, Pera J, Martínez de Aragón J, Bonet JA. 2017. Seasonal dynamics of the ectomycorrhizal fungus *Lactarius vinosus* are altered by changes in soil moisture and temperature. Soil Biology and Biochemistry, 115C, 253-260. https://doi.org/10.1016/j.soilbio.2017.08.021.

iii. Castaño C, Lindahl BD, Alday JG, Hagenbo A, Martínez de Aragón J, Parladé J, Pera J, Bonet JA. Temporal and spatial changes in soil fungal communities across moisture and temperature gradients in a Mediterranean pine forest. *Under review in New Phytologist: Second revision.*

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i. Liu, B.; Fischer, CR.; Bonet, J.A.; Castaño, C.; Colinas, C. 2016. Shifts in soil fungal communities in *Tuber melanosporum* plantations over a 20-year transition from agriculture fields to oak woodlands. Forest Systems, 25(1), eSC05, 1-5. https://doi.org/10.5424/fs/2016251-08353.

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i. Adamo, R.; Castaño, C.; Fischer, C.; Bonet, J.A. 2014. Detection of fungal DNA in Mediterranean forests soils: Optimization of molecular techniques for high-throughput sequencing. International Congress on Mycorrhizae. 15-17 October. Marrakech (Morocco). 2014.

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iii. Carles Castaño; Javier Parladé; Joan Pera; Juan Martínez de Aragón; José Antonio Bonet. Drying treatment of soil samples affects DNA recovery but does not change the fungal community structure by metagenomic analysis. *Poster*. Ecology of Soil Microorganisms 2015. 29 November - 3 December 2015. Prague. Czech Republic.

iv. Castaño, C.; Lindahl, BD. Could the molecular techniques predict mushroom productivity? *Poster*. COST Action FP1203 Meeting. 17-19 February 2016. Antalya, Turkey.

v. Sergio de-Miguel; Juan Martínez de Aragón; Antonio Tomao; Carles Castaño; José Antonio Bonet; Josu G. Alday. Effect of forest thinning on mushroom productivity and diversity. Wild Forest Products in Europe Congress. 13-14 October 2016. Barcelona, Spain.

vi. Carles Castaño; Jonàs Oliva; Juan Martínez de Aragón; Josu G. Alday; Javier Parladé; Joan Pera; José Antonio Bonet. Trapping fungal spores to study airborne dispersion and mushroom emergence. *Poster*. 9th International Workshop on Edible Mycorrhizal Mushrooms. 10-14

July 2017. México City, México.

vii. Daniel Oliach; Carles Castaño; Christine Fischer; Carlos Colinas. Effect of *T. melanosporum* on surrounding soil mycological diversity. *Poster*. 9th International Workshop on Edible Mycorrhizal Mushrooms. 10-14 July 2017. México City, México.

viii. José Antonio Bonet; Carles Castaño; Juan Martínez de Aragón. Producción y diversidad de hongos en masas de *Quercus ilex* del Noreste Peninsular. VII Congreso Forestal Español. 26-30 June 2017. Plasencia, Spain.

RESUM EN CATALÀ

Les comunitats fúngiques dels sòls juguen un paper vital en molts processos ecosistèmics forestals, així com en el manteniment de les poblacions d'espècies vegetals. Amb l'aparició de noves tècniques moleculars és possible estimar la diversitat i composició d'aquestes comunitats de forma més precisa i conèixer la seva resposta a pertorbacions com la gestió forestal o els canvis en el clima. L'objectiu principal d'aquesta tesi va ser descriure l'efecte de dos factors en les comunitats fúngiques de sòls forestals: la gestió forestal (concretament les aclarides) i el clima. A més, hem desenvolupat un mètode per analitzar la diversitat i composició d'espores de fongs a l'aire, així com per estudiar els seus canvis en el temps i en l'espai.

Aquesta tesi doctoral es va realitzar sobre un dispositiu experimental consistent en 28 parcel·les forestals localitzades en el Paratge Natural d'Interès Nacional de Poblet i dominades per *Pinus pinaster* de 60 anys, on es van aplicar aclarides de diverses intensitats. Mitjançant l'ús de diverses tècniques moleculars com la PCR en temps real (qPCR), seqüenciació massiva d'ADN (PacBio RS II, Illumina MiSeq) i l'extracció d'ergosterol, hem descrit les dinàmiques de biomassa i composició fúngica d'aquests sòls forestals, així com de la comunitat aèria (espores). Les dades obtingudes s'han analitzat en un gradient d'humitat i temperatura del sòl, així com també en les aclarides realitzades l'any 2009.

Pel que fa a la part metodològica d'aquesta tesi, observem com liofilitzant els sòls es va recuperar el màxim d'ADN i es va evitar el creixement de floridures. Un cop establert el millor tractament d'assecat, es va observar com la sequera d'estiu afectava negativament la biomassa fúngica del sòl i causava importants canvis funcionals i estructurals en les comunitats de fongs. Entre les espècies més resistents a la sequera figuraven els fongs ectomicorízics, probablement per la seva relació simbiòtica amb arbres hoste. Els màxims valors de biomassa fúngica en sòl es van observar a la tardor, coincidint amb majors abundàncies de fongs ectomicorízics, molt probablement a causa d'una redistribució del carboni al sòl per part de la planta hoste. D'altra banda, la majoria de fongs sapròfits es van veure afavorits sota condicions d'alta humitat i baixes temperatures al sòl. A diferència dels factors climàtics, les aclarides realitzades l'any 2009 no van afectar a la composició de fongs als sòls, possiblement degut a que es va deixar un nombre d'arbres suficients per mantenir la comunitat. Finalment, les comunitats d'espores en l'aire variaven a escala espaial però sobretot a escala temporal. Les variacions temporals d'aquesta comunitat es veien condicionades en gran part per la fenologia de la comunitat de bolets. Moltes espècies que produïen cossos fructífers van poder ser detectades a les trampes d'espores, i per tant aquesta tècnica es podria utilitzar per detectar l'emergència de bolets.

En base a aquests resultats es pot concloure que 1.- La liofilització és el mètode d'assecat que millor preserva l'ADN de fongs específics i evita el creixement de floridures. 2.- El cicle anual de certes espècies de fongs es pot veure modificat en un context de canvi climàtic, amb menor biomassa fúngica a l'estiu però major biomassa durant l'hivern i primavera. 3.- Les condicions climàtiques afecten les comunitats de fongs segons els seus trets funcionals, essent els fongs ectomicorrízics els que suporten millor les condicions de sequera estiuenca. 4.- Increments d'humitat afavoreixen la proliferació de sapròfits i fongs degradadors de miceli. 5.- Les aclarides no afecten de manera significativa les poblacions de fongs del sòl si es deixen en peu el nombre suficient d'arbres. 6.- Mitjançant l'ús de tècniques moleculars i trampes d'espores, és possible estudiar la comunitat d'espores a l'aire, essent aquesta altament afectada per la fenologia dels cossos fructífers de fongs i mostrant variabilitat espaial i temporal.

RESUMEN EN CASTELLANO

Las comunidades fúngicas de los suelos juegan un papel vital en muchos procesos ecosistémicos forestales, así como en el mantenimiento de las poblaciones de especies vegetales. Con la aparición de nuevas técnicas moleculares es posible estimar la diversidad y composición de estas comunidades de forma más precisa y conocer su respuesta a perturbaciones como la gestión forestal o los cambios en el clima. El objetivo principal de esta tesis fue describir el efecto de dos factores en las comunidades fúngicas de suelos forestales: la gestión forestal (concretamente las claras forestales) y el clima. Además, hemos desarrollado un método para analizar la diversidad y composición de esporas de hongos en el aire, así como sus cambios espacio-temporales.

Esta tesis doctoral se realizó sobre un dispositivo experimental consistente en 28 parcelas forestales localizadas en el Paraje Natural de Interés Nacional de Poblet y dominadas por *Pinus pinaster* de 60 años, en las que se aplicaron diferentes intensidades de claras. Mediante el uso de varias técnicas moleculares como la PCR en tiempo real (qPCR), secuenciación masiva de ADN (PacBio RS II, Illumina MiSeq) y la extracción de ergosterol, hemos descrito las dinámicas de biomasa y composición fúngica de estos suelos forestales, así como de la comunidad aérea (esporas). Los datos obtenidos se han analizado en un gradiente de humedad y temperatura del suelo, así como también en las claras realizadas el año 2009.

En cuanto a la parte metodológica de esta tesis, observamos como liofilizando los suelos se recuperó el máximo de ADN y se evitó el crecimiento de mohos. Una vez establecido el mejor tratamiento de secado, se observó como la sequía de verano afectaba negativamente la biomasa fúngica del suelo y causaba importantes cambios funcionales y estructurales en las comunidades de hongos. Entre las especies más resistentes a la sequía figuraban los hongos ectomicorrízicos, probablemente por su relación simbiótica con los árboles huésped. Los máximos valores de biomasa fúngica en suelo se observaron en otoño, coincidiendo con mayores abundancias de hongos ectomicorrízicos, muy probablemente debido a una redistribución del carbono en el suelo por parte del arbol huésped. Por otro lado, la mayoría de hongos saprófitos se vieron favorecidos en condiciones de alta humedad y bajas temperaturas en suelo. A diferencia de los factores climáticos, las claras realizadas en 2009 no afectaron a la composición de hongos en suelos, posiblemente debido a que se dejó un número de árboles suficiente para mantener la comunidad. Finalmente, las comunidades de esporas en el aire variaban a escala espacial, pero sobre todo a escala temporal. Asimismo, comprobamos como las variaciones temporales de estas comunidades se veían condicionadas en gran parte por la fenología de la comunidad de setas. Muchas de las especies que producían cuerpos fructíferos pudieron ser

detectadas en las trampas de esporas, y por lo tanto creemos que esta técnica podría ser utilizada para detectar la emergencia de setas.

En base a estos resultados se puede concluir que 1.- La liofilización es el método de secado de muestras de suelo que mejor preserva el ADN de hongos específicos y previene el crecimiento de mohos. 2.- El ciclo anual de ciertas especies de hongos se puede ver modificado en un contexto de cambio climático, con menor biomasa fúngica en verano, pero mayor durante invierno-primavera. 3.- Las condiciones climáticas afectan a las comunidades de hongos según sus rasgos funcionales, siendo los hongos ectomicorrízicos los que soportan mejor las condiciones de sequía estival. 4.- Incrementos de humedad favorecen la proliferación de saprófitos y hongos degradadores de micelio. 5.- Las claras no afectan de manera significativa las poblaciones de hongos del suelo si se dejan en pie el número suficiente de árboles. 6.- Mediante el uso de técnicas moleculares y trampas de esporas, es posible estudiar la comunidad de esporas en el aire, siendo esta altamente afectada por la fenología de los cuerpos fructíferos de hongos y mostrando una elevada variabilidad espacio-temporal.

ABSTRACT IN ENGLISH

Fungal communities inhabiting soils play a vital role in many forest ecosystem processes, as well as in the maintenance of plant species. With the emergence of new molecular techniques, it is possible to estimate the diversity and composition of these communities more precisely and study their responses to disturbances such as forest management or changes in climate. The main objective of this thesis was to describe the effect of two factors in the soil fungal communities: forest management (specifically forest thinnings) and climate. In addition, we have developed a method to study the diversity and composition of airborne fungal spores, as well as their spatio-temporal changes.

This doctoral thesis was carried out in an experimental set-up consisting of 28 forest plots located in the Natural Park of Poblet, dominated by 60-year-old *Pinus pinaster* trees, where thinnings were applied under different intensities. Using several molecular techniques such as real-time PCR (qPCR), high-throughput DNA sequencing (PacBio RS II, Illumina MiSeq) and ergosterol extraction, we have described the fungal biomass dynamics and fungal composition of these forest soils, as well as from the airborne community (spores). The data obtained have been analyzed in a gradient of soil moisture and temperature, as well as in the forest thinnings made in 2009.

Regarding the methodological part of this thesis, we observed how the maximum DNA recovery and the lowest growth of molds occurs under freeze-drying treatments. Once the best drying treatment was established, we observed how summer drought negatively affected the soil fungal biomass and caused important functional and structural changes in the fungal community. Ectomycorrhizal fungi were the most resistant species to drought, probably because of their symbiotic relationship with host trees. The maximum soil fungal biomass was observed in autumn, coinciding with higher abundances of ectomycorrhizal fungi, most likely due to a redistribution of soil carbon by the host plant. Moreover, most saprotrophic fungi were promoted under conditions of high soil moisture and low temperatures. Unlike climatic factors, the forest thinnings made in 2009 did not affect the soil fungal community, likely because a sufficient number of trees were left to support the community. Finally, the airborne spore community changed at spatial scale, but especially at temporal scale. We verified how the temporal variations of these communities were largely determined by fruiting body emergence. Many of the species that produced fruiting bodies could be detected in the spore traps, and therefore this technique could be used to detect fruiting body emergence

Based on these results, it can be concluded that 1.- Freeze-drying is the method that best preserves the DNA of specific fungi and prevents the growth of molds. 2.- The annual cycle of specific fungal

species can be modified in a context of climate change, with predicted lower fungal biomass in summer, but higher biomass during winter-spring. 3.- Micro-climatic soil conditions affect fungal communities according to their functional traits, with ectomycorrhizal fungi being the ones that better resist summer drought conditions. 4.- High soil moisture promote the proliferation of saprotrophs. 5.- Forest thinnings do not affect significantly the soil fungal communities if enough trees are left standing. 6.- It is possible to study the airborne spore community using spore traps and molecular techniques, being these communities highly affected by the phenology of the fruiting bodies and showing spatio-temporal variability.

INTRODUCTION

The importance of soil fungal communities inhabiting forest ecosystems

Fungi inhabiting forest soils determine many ecological processes that are responsible of ecosystem functioning (Baldrian, 2016). For instance, mycorrhizal fungi are essential drivers of soil organic matter (SOM) dynamics, and nutrient release or uptake in forest ecosystems (Clemmensen et al., 2013; 2015; Averill et al., 2014). In addition, many free-living fungi are considered crucial decomposers and are key organisms recycling organic matter in soils (Baldrian, 2016). Soil fungi, also, represent an important part of the total carbon budget in forest soils, with fungal biomass accumulations in boreal forests fluctuating from 700 to 5,800 kg ha^{-1} (Wallander et al., 2001, 2004) and annual biomass productions around 200-350 kg ha^{-1} (Högberg and Högberg, 2002; Wallander et al., 2010; Ekblad et al., 2013), although similar accurate estimations are still lacking in Mediterranean ecosystems.

Mycorrhizal species are symbiotic organisms to many plant species, providing nitrogen and phosphorus to their hosts and receiving back photosynthetically fixed carbon (Smith and Read, 2008; Van Der Heijden et al., 2008; Fig. 1). This carbon is used by the fungi to build the extrametrical mycelia (EMM) but also to build the fruiting bodies (Fig. 1). The EMM is the main driver of this symbiotic interaction: the EMM colonizes the surrounding soil to forage for nutrients and to encounter new root tips to colonize (Cairney, 2012; Fig. 1; Fig. 2). EMM accumulation is considered an important terrestrial carbon sink process as a result of impaired EMM degradation, especially in boreal ecosystems (Clemmensen et al., 2015, 2013; Hagenbo et al., 2016; Fernandez et al., 2016), contributing to soil organic matter (SOM) formation in forest soils (Clemmensen et al., 2013; Fernandez et al., 2016). However, the relevance of the EMM in Mediterranean forests soils still needs to be properly quantified. EMM may also be important for alleviation of drought stress for host trees in Mediterranean climates (Mohan et al., 2014), both directly by increasing access to soil water (Allen, 2007) and by improving soil structure and porosity through the formation and stabilization of soil aggregates and SOM (Querejeta, 2017). Moreover, with the predicted increase of drought events in Mediterranean forests, it is important to analyze the role of fungal EMM and ectomycorrhizal (ECM) species increasing plant drought resistance. Thus, describing ECM species resposes to climate changes may be fundamental to predict future changes in ecosystem functioning and nutrient dynamics.

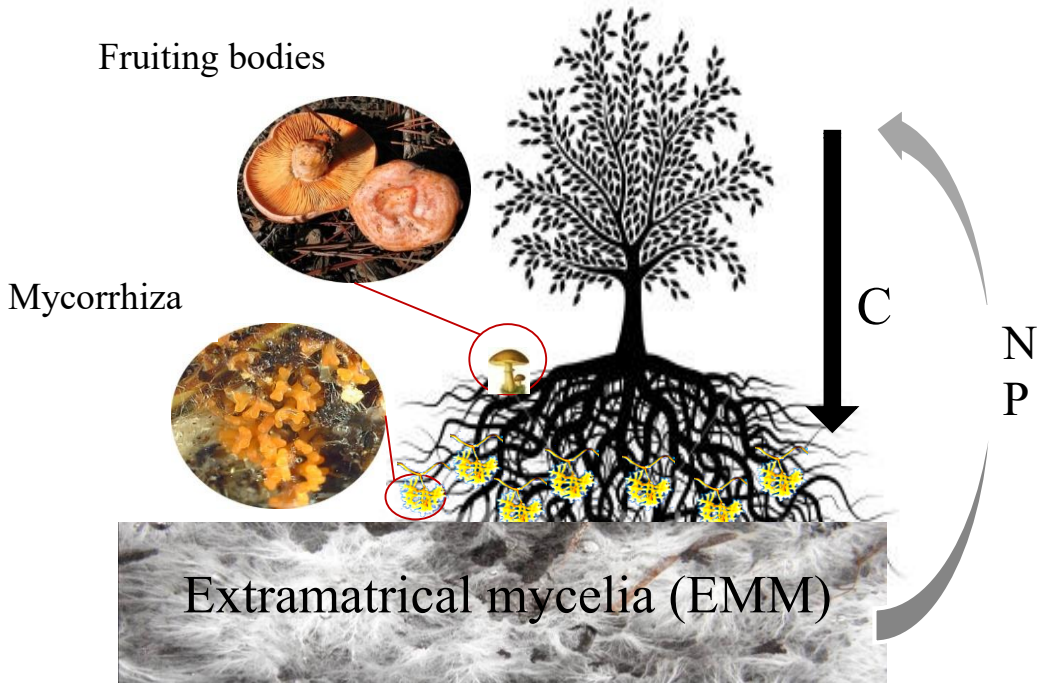


Fig. 1. Scheme showing the mutualistic relationship between host tree and their associated root symbionts (ECM fungi). Host tree transfer photosynthetically fixed carbon (C) to their mutualists through roots and mycorrhizas, and this carbon is then transferred to colonize the surrounding soil via EMM and to reproduce via fruiting bodies. In turn, ECM fungi transfer nutrients such as Nitrogen (N) or Phosphorus (P) to the host tree. Picture: Courtesy of Xavier Parladé (IRTA).

In contrast to ECM species, many free-living fungi (i.e. saprotrophic species) are considered essential decomposers of forest residues such as litter (Baldrian, 2016; Lindahl et al., 2007), mycelia (Lindahl et al., 2010) and wood (Kubartová et al., 2012). These saprotrophic fungi have shown a degree of specialization in organic substrate use, as each species may degrade different types of litter (Prescott and Grayston, 2013; Urbanová et al., 2015) or wood (Kubartova et al., 2012). In addition to litter or wood degradation, specific saprotrophs such as moulds can use a variety of carbohydrates, such as cellulose, pectin and starch (Thormann et al., 2001), and are likely contributing to the turnover of dead ECM mycelium (Lindahl et al., 2010; Jumpponen et al., 2010), being also essential for ecosystem functioning and SOM recycling.

To sum up, many of these saprotrophic and ECM fungi produce wild edible fruiting bodies that are highly important non-wood forest products and are increasingly in demand at food markets worldwide (Boa, 2004). For example, up to 268 fungal taxa have been authorized to be commercialized in Europe (Peintner et al., 2013), of which the most important marketed mushrooms are *Boletus edulis*, *Cantharellus cibarius*, *Lactarius deliciosus*, *Morchella esculenta*, and *Agaricus campestris* (Peintner et al., 2013). In Spain, *Lactarius* group *deliciosus*

is amongst the most important edible ectomycorrhizal species (Fig. 3). This species group is commonly sold in markets from Europe, Asia and North Africa (Boa, 2004). The economic impact of *L.* group *deliciosus* trade, based on data from the three most important markets in Spain, was 5.3 M € $year^{-1}$ for the period 2002–2008, with on average almost 500 t of *L. deliciosus* sold every year (Voces et al., 2012). Thus, studying specific targets of fungal groups is also important to understand how particular forest services or goods may be affected by both changes in climate and forest management regimes.

Therefore, the study of soil fungi in Mediterranean forest ecosystems has both practical and theoretical importance. From a practical perspective, information on fungal responses to forest management might help guide future sylvicultural treatments to preserve fungal communities and increase sporocarps yields, hence making more integrative non-wood forest products (de-Miguel et al., 2014). Theoretically, a scientific approach to Mediterranean fungal communities can provide insights into fundamental ecological theory, revealing SOM cycles and dynamics, and how soil fungal communities may respond to climatic events or may alleviate trees from drought (Querejeta et al., 2013; 2017).

Fig. 2. Example of EMM (white color mycelia) from *Suillus* sp. colonizing organic soil. Photo: Carles Castaño.

Tools and methods to study belowground fungal communities and biomass

Belowground communities have been difficult to study due to the inconspicuous nature of these organisms and the associated high fungal diversity found in most of forest soils (Buée et al., 2009). To circumvent these problems, new molecular technologies such as next generation DNA sequencing platforms or quantitative PCR (qPCR) are used to study fungal community

composition (Lindahl et al., 2013) or biomass (Filion et al., 2003; Parladé et al., 2007; Fig. 4), respectively. Next generation DNA sequencing platforms allow to obtain a fungal community profile in soils, normally expressed in relative abundance of each operation taxonomic unit (OTUs) for each sample (Amend et al., 2010; Lindahl et al., 2013). In contrast, Real-Time PCR (qPCR) is employed to quantify the biomass of specific ECM species such as *Lactarius deliciosus* (Parladé et al. 2007) or *Tuber melanosporum* (Suz et al., 2008), whereas the biomass of the whole ECM community has been typically determined using ergosterol analyses or PLFA (Hagenbo et al., 2016, Ekblad et al., 2016; Wallander et al., 2013). However, as new emerging tools, there are still some biases and technological limitations that need to be addressed and corrected in the close future, such as the lack of reference DNA sequences data (Bueé et al., 2009) or biases caused by the primers used or bioinformatics analysis parameters like clustering threshold (Lindahl et al., 2013). In any case, the use of these molecular techniques such as next generation DNA sequencing or qPCR brings a good opportunity to study, for the first time, fungal community composition and diversity in forest soils, which may help to disentangle the main biotic or abiotic factors influencing soil fungal communities in Mediterranean ecosystems.

Fig. 3. Great *Lactarius vinosus* production during autumn 2016. The effects of precipitation irregularity in fruiting body production has been widely studied in Mediterranean forests, with clear effects on production (Alday et al., 2017a; 2017b), but little is known about these effects on the overall soil fungal community. Photo: Carles Castaño.

Spatio-temporal changes and seasonality of soil fungal communities

Soil fungal communities are known to be primarily affected by soil chemistry, such as nitrogen forms and availability (Lilleskov et al., 2002; Sterkenburg et al., 2015) or pH (Rincón et al., 2015). It has been also shown that vegetation composition and stand age influence over soil

fungal communities (Urbanová et al., 2015; Kyaschenko et al., 2017). In addition, microclimate changes in temperature or rainfall (Fernandez et al., 2016; Hartmann et al., 2017; Solly et al., 2017) have been observed to affect these communities, together with several forest disturbances such as forest management (Varenius et al., 2016; 2017; Kyaschenko et al., 2017). However most of these studies have been focused on boreal or temperate forests and similar studies in Mediterranean forests are still scarce. In addition, most studies have observed high stochasticity in soil fungal communities (Bahram et al., 2013; 2016), since most of the variation remain unexplained by environmental variables, therefore, more studies in different ecosystems are necessary to disentangle the stochasticity of these communities. In Mediterranean forest ecosystems, drought stress and low nutrient availability are important determinants of functional and structural traits of plants (e.g. sclerophylly and low growth rate; Sardans and Peñuelas, 2013), thus, it is likely that both parameters are also important components structuring soil fungal communities.

Soil fungal composition and fungal mycelia biomass show a marked seasonality (i.e. seasonal fluctuations; Jumpponen et al., 2010; Andreetta et al., 2011; Voříšková et al., 2014). For example, studies in boreal forest ecosystems revealed higher EMM biomass during spring and autumn seasons related with greater host carbon allocation belowground (Wallander et al., 2001; Högberg et al., 2010). Thus, in temperate or boreal ecosystems, ECM fungi normally increase in relative abundance during summer or autumn (Wallander et al., 2001; Jumpponen et al., 2010; Voříšková et al., 2014; Santalahti et al., 2016). However, saprotrophs and opportunistic moulds increase under colder conditions of autumn or winter (Jumpponen et al., 2010; Voříšková et al., 2014; Santalahti et al., 2016). In contrast, in Mediterranean ecosystems, some specific ECM taxa increase during late autumn and early winter (e.g. *Boletus edulis* or *Lactarius deliciosus;* De la Varga et al., 2013) or during spring (e.g. *Tuber magnatum*; Iotti et al., 2014). It seems that host activity is also an important driver in both regions since decreasing abundance of ECM fungi during winter may be the consequence of reduced carbon reallocation of host to roots (Högberg et al., 2010; Jumpponen et al., 2010). In any case, most studies addressing seasonality of whole fungal community have been focused on boreal or temperate ecosystems with few studies in Mediterranean forests (Mohan et al., 2014).

Climate change such as warming or rainfall irregularity also modify the composition of soil fungal communities (Fernandez et al., 2016; Solly et al., 2017; Hartmann et al., 2017). Climate have important effect selecting for specific groups of fungal traits (Fernandez et al., 2016; Treseder & Lennon, 2015), such as exploration types (Agerer, 2001; 2006). Warming has generally been observed to increase ECM biomass in boreal or arctic ecosystems (Clemmensen

et al., 2006; Mohan et al., 2014), because it stimulates nutrient cycling and plant production (Clemmensen et al., 2006; Solly et al., 2017). By contrast, in Mediterranean ecosystems recent studies have suggested that mycelial production of ECM species is often arrested during warmer months such as summer but also during winter (Iotti et al., 2014; Queralt et al., 2017). In addition, drought hinders the function of hydrolytic enzymes (Sardans and Peñuelas, 2013), which may imply that other fungi such as saprotrophs may fail to establish and grow during summer months. Thus, in order to predict potential climate change-driven shifts in soil fungal communities, new studies are needed for Mediterranean forest ecosystems considering the whole soil fungal community, and to describe its response to recent warming events and adaptation to severe drought conditions.

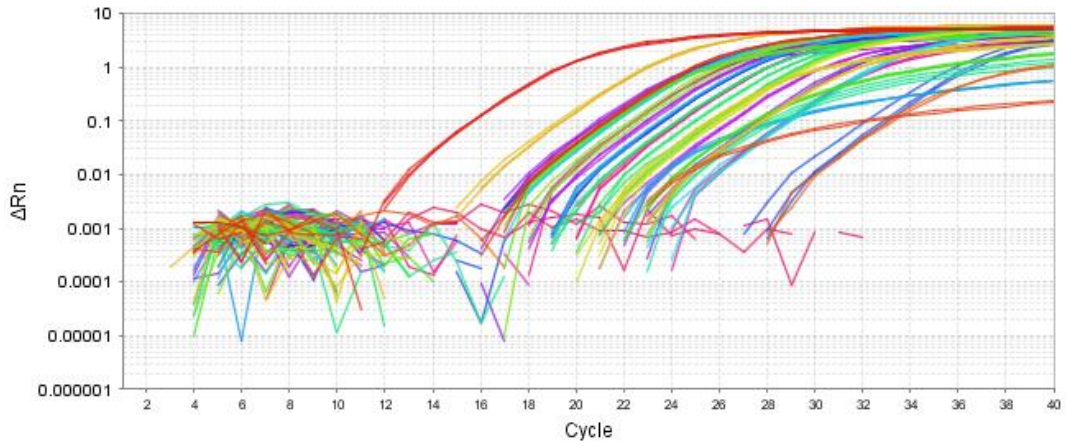


Fig. 4. qPCR allows absolute quantification of the biomass of a given species. Here, the abundance of *Lactarius vinosus* was quantified for each soil sample (each identified with a unique colour). Biomass is obtained by interpolation in a standard curve using known amounts of initial biomass.

Forest management operations such as clear-cutting usually causes important losses of ECM species (Jones et al., 2003, Parladé et al., 2017). However, logging effects on ECM communities are dependent on whether soil fungal communities can survive in symbiosis with the non-thinned trees (Amaranthus and Perry, 1987; Rosenvald and Lõhmus, 2008). Also, tree removal effect on fungal communities may be caused by changes on environmental conditions after logging, such as microclimate or soil biochemistry (Hartmann et al., 2012; Jones et al., 2003). For example, Varenius et al. (2017) observed that forest patches were more efficient to preserve the ECM diversity than dispersed trees. Despite the clear negative effects of clear-cutting operations on ECM fungi, the less intense management effects on ECM species such as forest thinning, have not been properly evaluated yet.

Fungal dispersion and fungal colonization in new environments

Spores are believed to represent a very important fraction of airborne biological aerosol in the atmosphere (Després et al., 2012). For instance, only a single mushroom has been estimated to produce and release $1.1 \times 10^8 – 1 \times 10^{10}$ basidiospores (Dahlberg and Stenlid, 1994; Kadowaki et al., 2010). In ascomycetes, spores are normally ejected from asci after the turgor pressure inside the sacs cause a hole (Trail, 2007), with efficient spore ejections due to the drag-minimizing spore shapes (Roper et al., 2008). In basidiomycetes, spore discharge is promoted by a droplet which acts as a surface-tension catapult (Stolze-Rybczynski et al., 2009). Then, a proportion of these spores will be vertically transported through convective airflows promoted by the pileus until they reach a dispersive wind (Brown and Hovmoeller, 2002; Dam, 2013; Dressaire et al., 2016). Contrary to wind, wash-out of spores by rainfall may promote spore deposition (Grinn-Gofrón and Mika, 2008; Oliveira et al., 2009) whereas other abiotic factors such as UV and solar radiation (Peay and Bruns, 2014) may negatively affect the permanence of airborne spores (Burch and Levetin, 2002; Troutt and Levetin, 2001). Although the mechanisms of spore ejection are known, studies addressing atmospheric deposition of spores and clear descriptions of spore communities are still lacking, being one of the less described microbiomes worldwide.

Spatial and temporal variation in fungal propagule abundance or composition have been reported, especially in ECM taxa (Peay and Bruns, 2014; Peay et al., 2012). Spatial autocorrelation in some species may be driven by fruiting body communities being similar across sites (Peay et al., 2010a; Talbot et al., 2014), but may also suggest that dispersal limitation may be restricted to specific groups of fungi (Peay and Bruns, 2014). ECM basidiomycetes seem to disperse the spores less abundantly (Galante et al., 2011; Kivlin et al., 2014) than other highly-sporulating fungi such as puffballs or moulds (Adams et al., 2013). Current studies suggest potential dispersal limitation of spores from ECM taxa (Peay et al., 2012; 2014), which affects community composition and establishment in many terrestrial ecosystems (Peay et al., 2012). Thus, new studies integrating both the spatial and the temporal changes in relation with fruiting body emergence are necessary to understand the whole fungal cycle and dynamics, as well as to open new research lines using spores as proxy to estimate fruiting-body emergence and yields.

OBJECTIVES

The general objectives of this thesis are:

1. To study how the most common soil drying treatments affect the soil fungal community and the biomass of specific fungal species.

2. To study the annual cycle of the soil fungal biomass of *Lactarius vinosus* and how this annual cycle is affected by changes in soil moisture and temperature.

3. To study the intra-annual dynamics of the soil fungal community composition and biomass, and how fungal functional traits are affected by changes in soil moisture and temperature.

4. To study the effect of forest thinning to the soil fungal community composition and diversity.

5. To study how the inter-annual changes in precipitation and temperature affect the soil fungal community composition.

6. To evaluate new tools to study the airborne spore community and their changes in a temporal and spatial scale.

THESIS STRUCTURE

Current literature on molecular analyses of soil fungal communities has some methodological gaps and questions. Thus, our first objective was to develop a methodological study focused on understanding the effects of the soil drying procedures on fungal community composition and biomass (Chapter I). Second, climate change is expected to impact negatively in Mediterranean forests, hence, we have focused in evaluating the seasonal changes and the effect of weather conditions on *Pinus pinaster* soil fungal community, both from an intra-annual (Chapter II, III) and inter-annual perspective (Chapter IV) and considering single-species (Chapter II) and the overall fungal community composition (Chapter III). Third, we also studied the effects of thinning on soil fungal communities (Chapter IV), since it is a currently recommended practice to mitigate climate change impacts on forests, however, the potential effects on soil fungal community of its application are still unknown. Finally, we assessed a methodology to detect and describe fungal spores and the spatio-temporal changes of fungal propagules in the airborne (Chapter V). In these chapters, we use next-generation DNA sequencing techniques to profile the fungal composition in the soils (Chapter I, II, III, IV) or spore trap samples (Chapter V). We also combine these techniques with qPCR (Chapter I, II, V) or ergosterol extractions (Chapter III, IV), which allow us to obtain absolute abundances of fungal biomass or spores. Finally, community studies were also analyzed in the context of functional traits:

i. **CHAPTER I: Soil drying procedure affects the DNA quantification of *Lactarius vinosus* but does not change the fungal community composition.**

ii. **CHAPTER II: Seasonal dynamics of the ectomycorrhizal fungus *Lactarius vinosus* are altered by changes in soil moisture and temperature.**

iii. **CHAPTER III: Temporal and spatial changes in soil fungal communities across moisture and temperature gradients in a Mediterranean pine forest.**

iv. **CHAPTER IV: Weather variation but not thinning drives inter-annual changes in fungal composition and diversity in a Mediterranean pine forest.**

v. **CHAPTER V: Mushroom emergence detected by combining spore trapping with molecular techniques.**

METHODOLOGY

Site selection

All the chapters are based in samplings carried out at the Natural Park of Poblet (Northeast Spain, 41° 21' 6.4728" latitude and 1° 2' 25.7496" longitude; Fig. 5, Fig. 6a). The average annual temperature at the study site is 11.8°C. The average annual rainfall is 666.5 mm, with the average minimum and maximum rainfall occurring in July (16 mm) and October (79.8 mm), respectively, with two peaks occurring in spring and autumn and a pronounced summer drought that usually lasts for three months. Mushroom production has been studied in 28 plots at the study site since 2008 as part of a long-term experimental set-up, where thinned and un-thinned plots were established (Fig 6b, Fig.7). The plots are composed of even-aged (60-year-old) *Pinus pinaster* plantations (Fig. 6a), with isolated *Quercus ilex* trees sometimes forming scrubs. The plots are characterized by having a range of altitudes (from 594 to 1013 m.a.s.l.), slopes (3–23%), stand densities (350 to 2,657 trees ha^{-1}) and basal areas (16.5 to 81.7 m^2 ha^{-1}). This range of plot conditions was specifically selected to capture as much variability as possible. The soils are siliceous with franc-sandy textures, pHs ranging from 6.1 to 6.6 and organic matter contents ranging from 2.95 to 10.51.

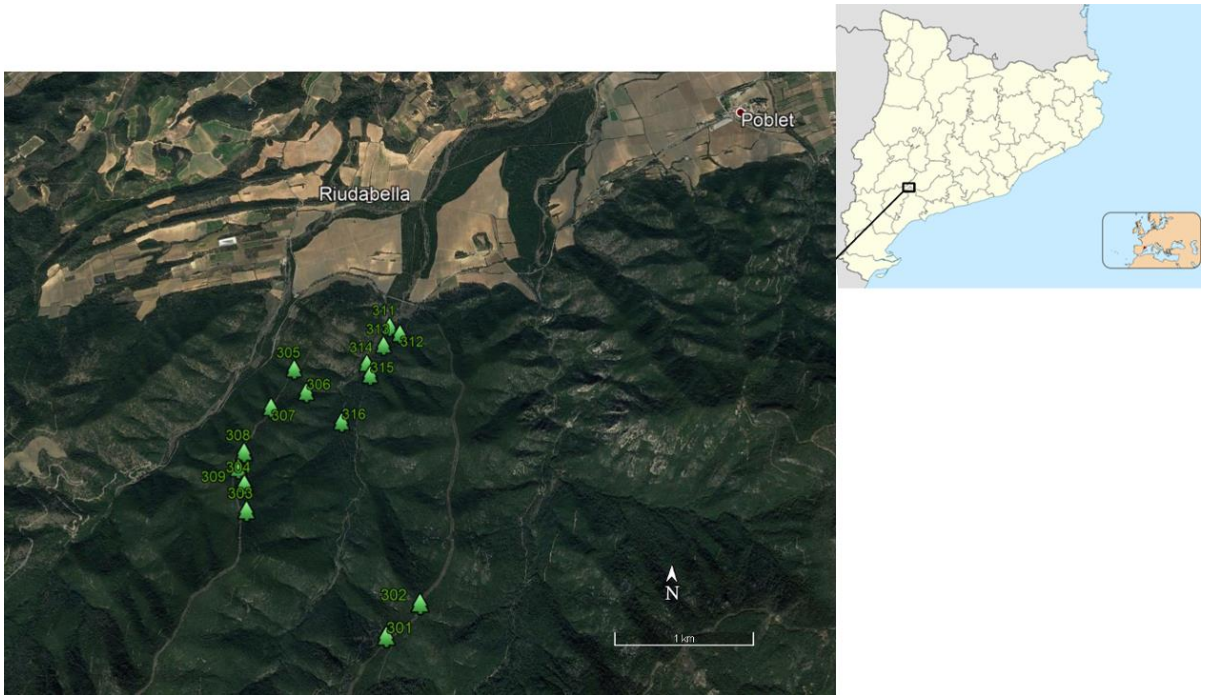


Fig. 5. Geographical localization of the plots. Each of these plots consisted in two paired plots, one thinned and another one un-thinned, except for the plots 311 and 314, amounting a total of 28 plots. Map obtained from Google earth V 6.2.2.6613 (November 2017).

Fig 6. Forests belonging to the Natural Park of Poblet. These forests were deforested and planted again 60 years ago using *P. pinaster* seedlings. This view is from one of the plots located at highest altitude and is nearby a firebreak (a). One of the long-term experimental unthinned plots. Plots are fenced to prevent the recollection of fruiting bodies from mushroom pickers (b).

Fig 7. View from one of the thinned plots. Here, *P. pinaster* trees were thinned on 2009, with the minimum recorded basal area around 16.5 m^2 ha^{-1}

Samplings

Soil samplings were carried out in Chapter I, II, III and IV, whereas spore trap samplings were carried out only in Chapter V. We followed the same soil sampling scheme in all the chapters (Fig 8), either if it was for mycelia quantification using qPCR or ergosterol, or if it was for fungal community studies. Soil samples were systematically collected from the plots, which

were each 100 m^2 (10 × 10 m) (Fig. 8a, Fig. 8b). Eight soil cores (12 cm deep and 5 cm in diameter), two cores from each side of the plot (Fig. 8b), and at least 10 m apart to avoid spatial autocorrelation, were extracted from each plot every month. We discarded the litter but included humus and mineral soil to obtain soil profiles with a depth of 12 cm and a composite sample of approximately 500 g for each plot (Fig. 8c).

Spore traps used in Chapter V were placed in the center of the plots. Two types of spore traps were installed: filter and funnel traps. Both traps were placed one meter apart in the center of each plot and captured spores 30 cm above ground level (Fig 8d). Finally, climate sensors measuring soil moisture and soil temperature every 2 h were also placed in each plot, close to the spore traps (Fig 8d).

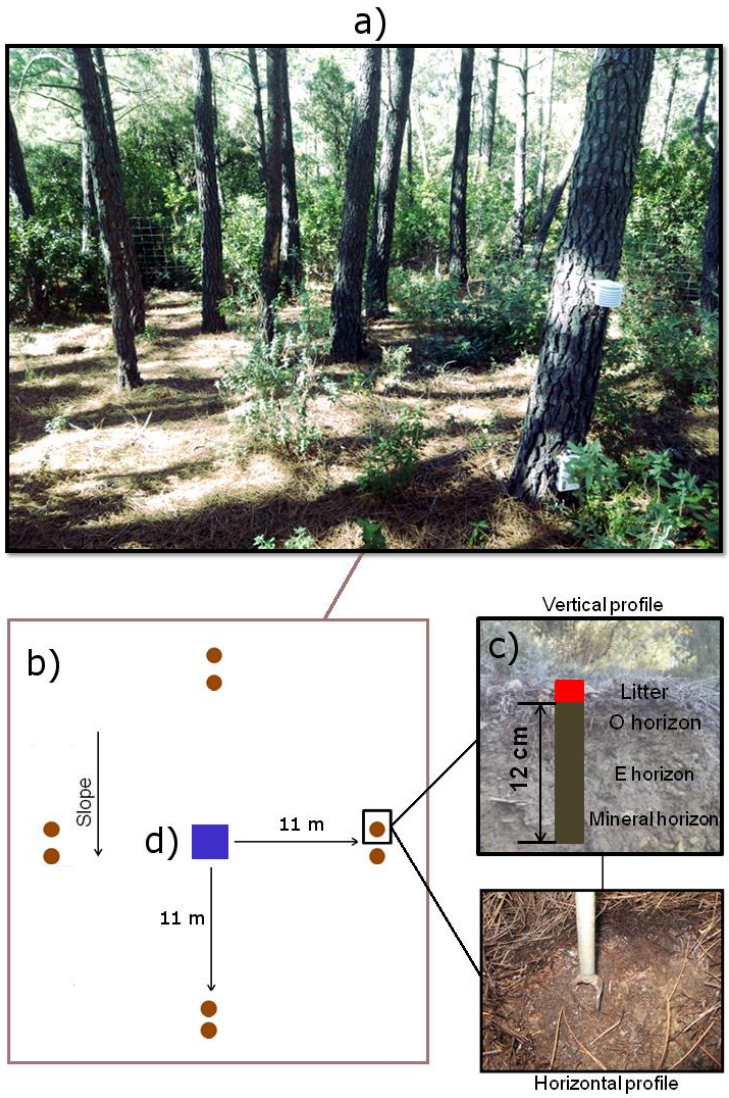


Fig. 8. Sampling scheme used. Forest floor from the plots (11 × 11) may have some heterogeneity due to the planting scheme used during the reforestation (a). Thus, 2 core samples (In light-brown colour) are obtained in each side of the plot (b). Litter (In red colour) is discarded but humus (O horizon), E horizon and mineral horizon down to 12 cm are included (in light-brown colour) (c). Also, spore traps and soil climate sensors measuring soil moisture and temperature are located at the centre of each plot,

30 cm aboveground and 10 cm belowground, respectively (d).

For all the studies, soil samples were stored at 4°C for <48 h and then sieved through a 3-mm mesh. Sieved soil samples were freeze-dried and pooled to obtain a composite sample for each plot. For DNA and ergosterol ergosterol, less than 0.5 g and 1 g are recommended, respectively. Thus, the homogenization of the soil samples is a very important and critical step. Accordingly, each composite soil sample was homogenized using a pestle and mortar during at least 1 min., resulting in a very fine powder (Fig. 9).

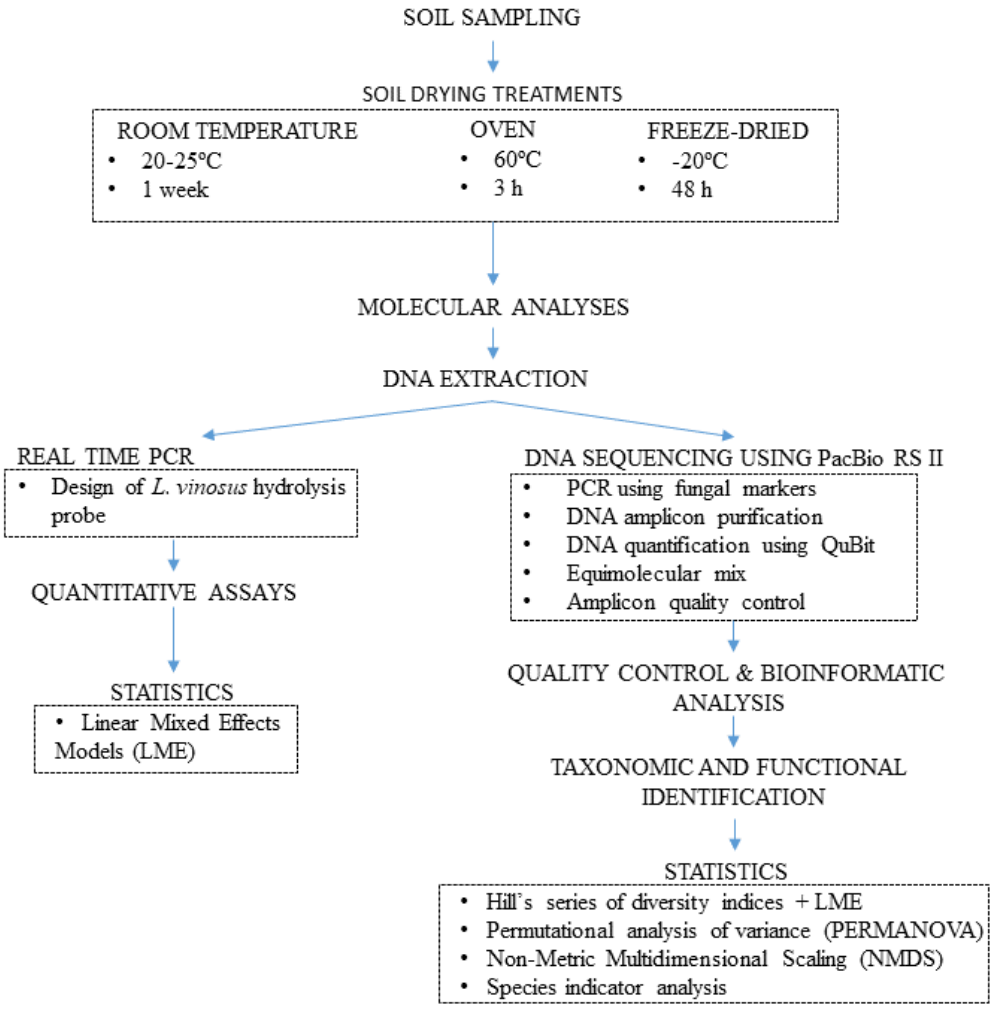
Fig. 9. The homogenization of soil samples is critical to obtain a representative soil sample and avoid sampling biases. The freeze-dried soil was homogenized usig sterile pestles and mortars during at least 1 min. or until obtainining very fine powder in a flow chamber to prevent contaminations.

Below, the methodological schemes followed in each chapter are described:

CHAPTER I: Soil drying procedure affects the DNA quantification of *Lactarius vinosus* but does not change the fungal community composition.

This work evaluates the effect of three different soil drying procedures (Freeze-drying, FD; oven at 60 °C, O; room temperature at 20–25 °C, RT) on the recovery of DNA from *Lactarius* vinosus (EMM species) and the overall fungal diversity and community composition.

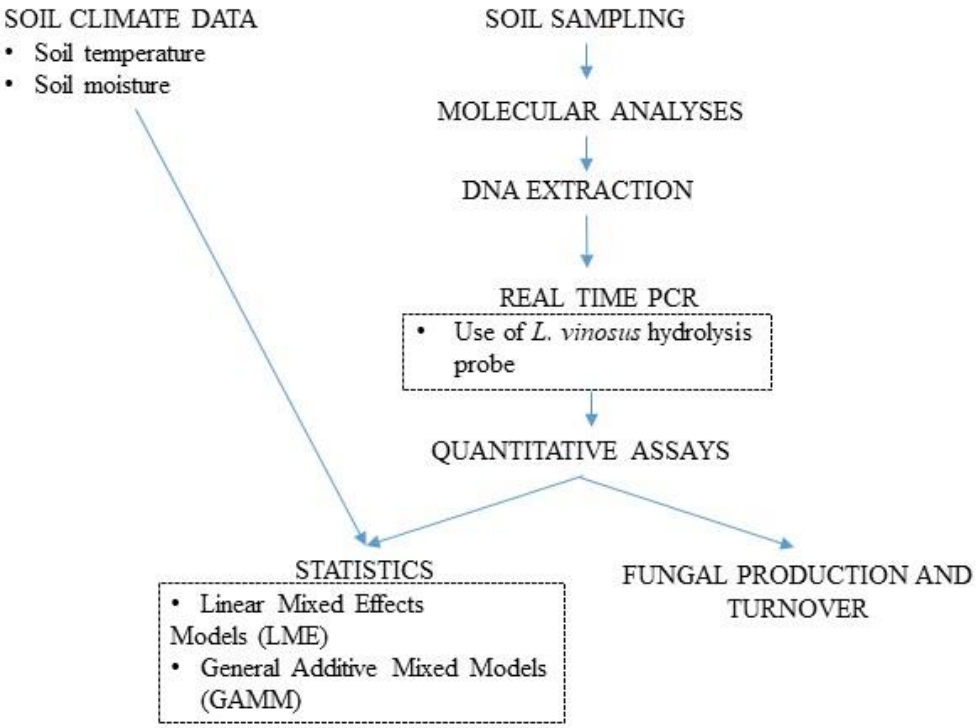
Here, we used three different soil drying procedures on 12 soil samples from *Pinus pinaster* plots to quantify how these treatments affected the EMM of *Lactarius vinosus* and the overall fungal community composition by using qPCR and PacBio, respectively. Drying treatment effects were tested on the overall fungal community permutational multivariate analysis of variance based on distancematrices. Drying treatment effects on EMM biomass of *L. vinosus* were tested by Hill's diversity index and linear mixed models (LME). Species indicator analysis was used to test which OTUs were affected by each drying treatment.



CHAPTER II: Seasonal dynamics of the ectomycorrhizal fungus *Lactarius vinosus* are altered by changes in soil moisture and temperature.

This work evaluates the annual cycle of *Lactarius vinosus*, a soil EMM, and investigates how soil temperature and soil moisture may be correlated with intra-annual changes in *L. vinosus* biomass, an important edible ectomycorrhizal species.

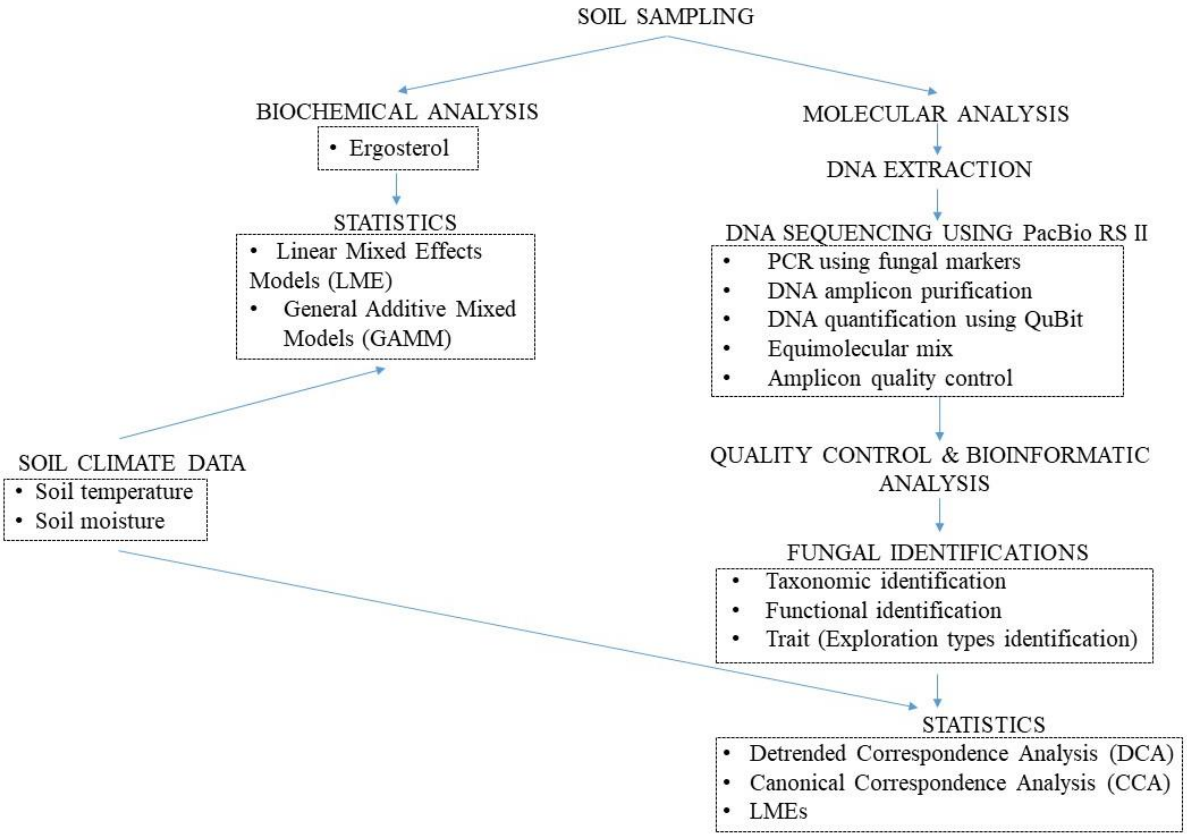
Here, we quantified the EMM of *L. vinosus* by using qPCR in 336 soil samples monthly collected in the 28 *P. pinaster* plots. Soil climate data was obtained from soil climate sensors measuring microclimate parameters such as temperature or moisture every 2 h. We used LMEs and generalized additive mixed models (GAMM) to test the effect of climatic factors on *L. vinosus* EMM.



CHAPTER III: Temporal and spatial changes in soil fungal communities across moisture and temperature gradients in a Mediterranean pine forest.

This work analyzes the intra-annual soil fungal compositional change in a *Pinus pinaster* forest and describe how summer drought affects these communities from a fungal functional and trait perspective. In addition, we use also ergosterol to study how fungal biomass change across months related with soil temperature and moisture patterns.

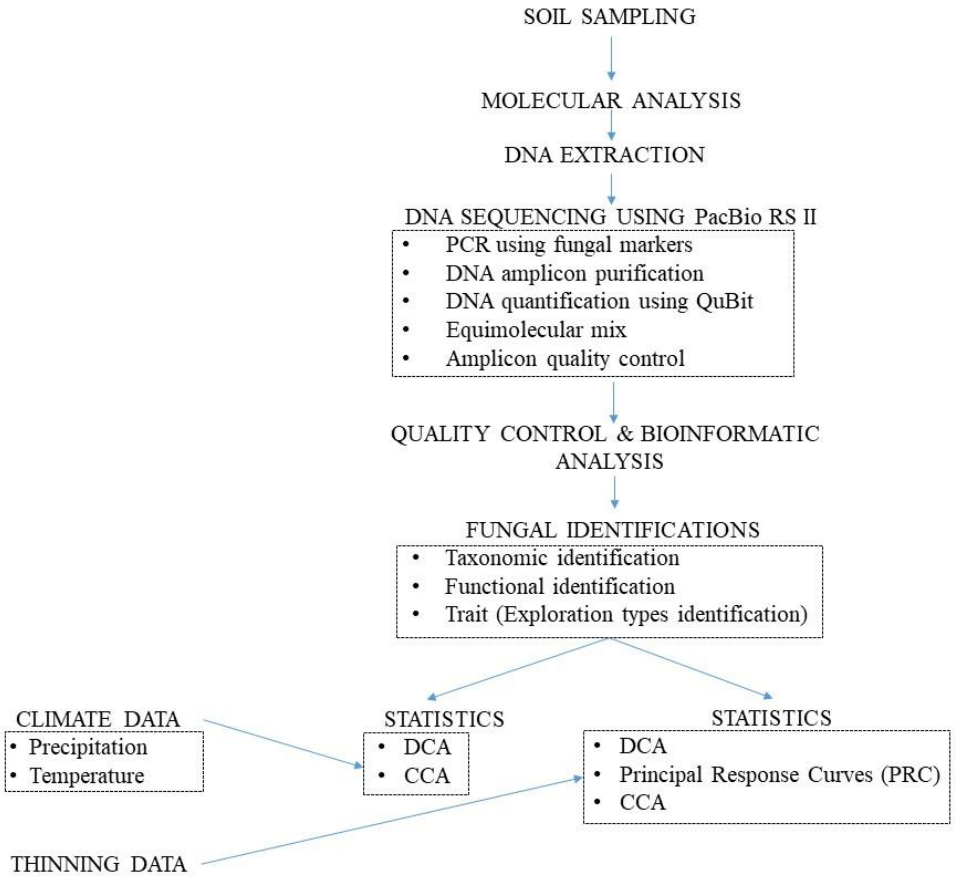
Here, we studied the fungal community composition in 336 soil samples monthly collected at the 28 *P. pinaster* plots using PacBio sequencing platform. From these samples we also quantified total soil EMM by quantifying ergosterol. Soil climate data was obtained from soil climate sensors measuring microclimate parameters such as temperature or moisture every 2 h. Decontrended Correspondence analyses (DCAs) and Canonical Correspondence Analyses (CCAs) were used to study the correlation between the climatic parameters and the fungal community composition, from species, functional and fungal trait (exploration types) level. LMEs were used to test the correlation between climatic parameters and specific functional groups and exploration types. Significance of the effect of soil moisture and temperature to the soil EMM biomass was tested by LMEs.



CHAPTER IV: Weather variation but not thinning drives inter-annual changes in fungal composition and diversity in a Mediterranean pine forest.

In this study we analyzed the short-term inter-annual dynamics (4 years) of soil fungal communities in response to *Pinus pinaster* forest thinning in a Mediterranean climate. In addition, we analyzed, in both thinned and un-thinned plots, whether there was a correlation between yearly changes in autumn precipitation and temperature with fungal community composition from a functional perspective.

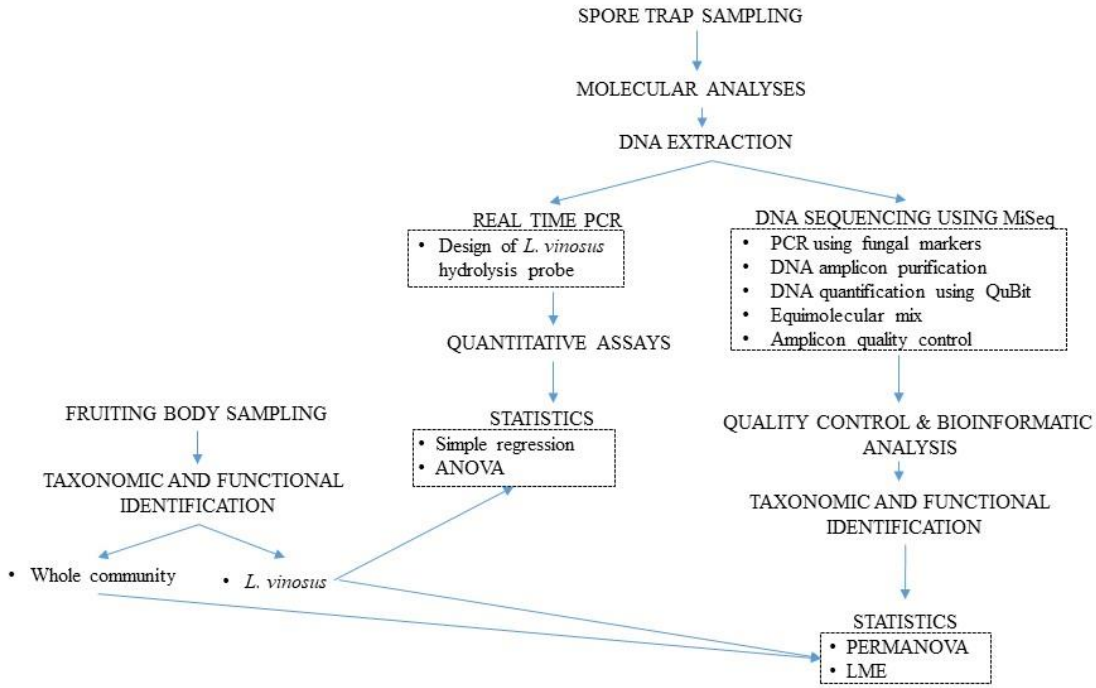
Here, we studied the fungal community composition using PacBio sequencing platform in 96 soil samples collected during 4 years in 24 *P. pinaster* plots. Thus, in this study only plots with paired thinned and un-thinned plots were considered. We also obtained precipitation and temperature data of each plot. Principal response curves (PRC) and CCAs were used to test the effect of thinnings over the 4 years of the study. DCAs and CCAs were used to correlate climatic parameters and fungal community composition, both from species, functional and fungal trait (exploration types) levels. LMEs were used to test significance between climatic parameters and specific functional groups.



CHAPTER V: Mushroom emergence detected by combining spore trapping with molecular techniques.

We assessed the use of funnel and filter paper traps in combination with next generation DNA sequencing techniques and qPCR to describe the airbone fungal community and spore abundance. We also described the spatio-temporal changes in fungal community composition and the relationship between species-specific spore abundance and fruiting body yields from temporal (week) and spatial (plot) perspectives.

Here, we used two simple spore traps (Funnel and filter traps) to capture the airborne fungal community in 8 plots weekly sampled during 8 weeks. We quantified the spores of *L. vinosus* by qPCR and the fungal community composition by Illumina MiSeq in these traps. We also sampled all the fruiting bodies found in each plot during 8 weeks. Temporal (week) and spatial (plot) effects on community data were tested using permutational multivariate analysis of variance (PERMANOVA). Temporal (week) and spatial (plot) linear relations between spore abundance and the mushroom yields using LME models.



CHAPTER I

Soil drying procedure affects the DNA quantification of *Lactarius vinosus* but does not change the fungal community composition.

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Soil drying procedure affects the DNA quantification of *Lactarius vinosus* but does not change the fungal community composition

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Abstract

Drying soil samples before DNA extraction is commonly used for specific fungal DNA quantification and metabarcoding studies, but the impact of different drying procedures on both the specific fungal DNA quantity and the fungal community composition has not been analysed. We tested three different drying procedures (freeze-drying, oven-drying and room temperature) on 12 different soil samples to determine: a) the soil mycelium biomass of the ectomycorrhizal species *Lactarius vinosus* using qPCR with a specifically designed TaqMan® probe, and b) the fungal community composition and diversity using the PacBio® RS II sequencing platform. Mycelium biomass of *L. vinosus* was significantly greater in the freeze-dried soil samples than in samples dried at oven and room temperature. However, drying procedures had no effect on fungal community composition or on fungal diversity. In addition, there were no significant differences in the proportions of fungi according to their functional roles (moulds vs. mycorrhizal species) in response to drying procedures. Only for six out of 1139 OTUs had increased their relative proportions after soil drying at room temperature, with five of these OTUs classified as mould or yeast species. However, the magnitude of these changes was small, with an overall increase in relative abundance of these OTUs of approximately 2%. These results suggest that DNA degradation may occur especially after drying soil samples at room temperature, but affecting equally nearly all fungi and therefore causing no significant differences in diversity and community composition. Despite the minimal effects caused by the drying procedures at the fungal community composition, freeze-drying resulted in higher concentrations of *L. vinosus* DNA and prevented potential colonization from opportunistic species.

Keywords: *Drying treatment, Ectomycorrhizal biomass, Fungal community, qPCR, Metabarcoding, Lactarius.*

Introduction

The use of high-throughput sequencing platforms such as 454 pyrosequencing, Illumina, PacBio or Ion Torrent in fungal ecology has increased during the last years (Lindahl and Kuske 2013). The latest reported accuracy values of PacBio data (>99%) together with higher average read lengths (Roberts et al. 2013), make this platform a promising tool for fungal community studies. Thus, data obtained from this platform will allow undertaking deep and comprehensive ecological studies about soil borne fungal species and communities. Real-Time PCR (qPCR) is also a commonly used molecular technique, which has been employed to study the seasonal dynamics and the persistence of extraradical mycelia of the ectomycorrhizal species *Lactarius deliciosus* (Parladé et al. 2007; Hortal et al. 2008), *Boletus edulis* (De la Varga et al. 2013) and *Tuber melanosporum* (Parladé et al. 2013). Due to increasing relevance and application of these molecular techniques, it is important to establish standardized protocols that will enable meaningful comparison of final results generated by different metabarcoding studies. Although there have been important advances in establishing a standard protocol for fungal metabarcoding studies (Lindahl et al. 2013), the impact of different commonly used procedures for drying soil samples have not been quantified and tested yet.

The extraction of DNA is one of the most studied and well-known critical steps for DNA recovery from soil (Plassard et al. 2012), despite the fact that no bias-free DNA extraction method is available (Feinstein et al. 2009). Drying soil samples before DNA extraction has important advantages as it facilitates soil homogenization (Lindahl et al. 2013), reduces colonization by opportunistic microbes and permits equalization of soil sample size by weight by eliminating the variable water content. However, drying soil samples has some important disadvantages. Storing soil at room temperature (also referred to as "soil incubation") can alter the DNA yield of certain species (Gryndler et al. 2013; Herdina et al. 2004). There are also evidences that soil incubation in the field may lead to saprotrophic organisms feeding on ectomycorrhizal mycelia after mycelia death (Lindahl et al. 2010), which could distort the community composition of the soil samples. Community distortion can also occur after long exposure to airborne communities or under the presence of abundantly sporulating fungi (Adams et al. 2013a). All these problems can occur during sample preparation and, consequently, influence the

fungal community structure and possibly distort the final ecological interpretation.

Presently, the most common soil drying procedures reported in the literature are: i) drying at room temperature (RT; 20-25 ºC), ii) drying in an oven (O; 40-60ºC) and iii) freeze-drying (FD). Drying at 40-60 ºC (Parladé et al. 2007) allows a rapid soil drying with negligible thermal DNA degradation (Karni et al. 2013). However, potential DNases (enzymes that break down the single or double-stranded DNA molecule into its component nucleotides) may affect the DNA to a different degree, depending on the amount of DNA bound to humic acids and the amount of free-DNA in soil (Crecchio and Stotzky 1998). Drying at room temperature (De la Varga et al. 2013; Parladé et al. 2013; Tedersoo et al. 2014) has insignificant heat impact on soil samples but requires more drying time. This increases the risk of microorganisms feeding on mycorrhizal mycelia and may facilitate DNA degradation caused by DNase activity (Crecchio and Stotzky 1998). Freeze-drying (Clemmensen et al. 2015; Sterkenburg et al. 2015) is a more expensive drying technique but, theoretically, allows for a more efficient drying, preventing the growth of opportunistic fungal species. Despite the potential effects reported for specific fungal yields and the potential distortion of the fungal community structure, no comparative studies of these different drying techniques applied to the same soil samples have been reported to date.

This work evaluates the effect of three different soil drying procedures described above (Freeze-drying, FD; Oven at 60ºC, O; Room temperature at 20-25 ºC, RT) on the recovery of DNA from the ectomycorrhizal species *Lactarius vinosus* (species-specific analysis). In addition, we tested the differences in diversity and community composition of the whole DNA pool (community analysis). To assess the impact of the drying procedures on the quantity of DNA, we applied Real-Time PCR with a TaqMan® probe designed to specifically amplify the ITS rDNA region of *Lactarius vinosus*, one of the most important ectomycorrhizal species collected in this study area with regard to fruit body yield (Bonet et al. 2012). To examine the differential impact of the drying procedures on the fungal community, we sequenced multiplexed fungal amplicons using the PacBio® RS II system based on the Real-Time (SMRT®) technology, and analyzed the diversity indices as well as the fungal community composition. We hypothesized that: (1) DNA yield of *L. vinosus* will be lower in samples dried at O and RT in comparison with FD samples. In addition, expecting colonization of opportunistic fungal species in the RT samples, both (2) fungal diversity and (3) fungal community composition are expected to be different among drying procedures. Finally, colonization or growth of

opportunistic species can be expected, and (4) the number or the relative proportion of opportunistic species are expected to increase, especially in the samples dried at room temperature.

Material and methods

Soil sampling

Soil samples were taken in 12 plots (10×10 m) from the long-term experimental site established in the protected forest (Paratge Natural d'Interès Nacional, PNIN) of Poblet (Tarragona, Northeastern Spain, 41º 21' 6.4728'' lat, 1º 2' 25.7496'' long), where fungal fruit body production has been monitored for 7 years with weekly mushroom sampling during the autumn season since 2008 (Bonet et al. 2012). Our soil samples were taken from even-aged *Pinus pinaster* plots (approx. 50 years old) and were analyzed for organic matter (OM), pH and soil humidity. These parameters are shown in Table 1 with plot altitude and corresponding *Lactarius vinosus* fruit body yields. Organic matter content in soil samples was obtained with the Walkley-Black method and expressed as percentage of dry soil sample (Walkley and Black, 1934). The pH was measured in a 1:2.5 H_2O solution. Previous studies of *Lactarius vinosus* fruit body productivity in this area have shown an average yield of 35.9 kg fruit bodies · ha^{-1} (Table 1).

Plot No.	Organic Matter (%)	pH	Altitude (m.a.s.l.)	Humidity of soil samples (%)	Averaged *L. vinosus* mushroom yield (kg/ha)
303c	7.54	6.8	903	23.60	0.64
304c	7.79	6.9	879	24.87	8.52
307c	5.79	6.7	796	23.69	115.57
312	4.08	6.6	633	24.46	9.06
312c	2.98	6.5	633	22.23	30.77
316c	2.95	6.8	644	24.08	5.59
304	10.53	6.6	879	12.21	0.22
305c	3.97	7	744	9.33	140.47
306	4.13	6.7	759	13.25	27.09
306c	5.06	6.8	759	8.94	36.53
309	4.49	7	852	11.32	3.39
309c	5.31	6.9	852	12.25	53.09
	5.38±2.24	6.77±0.16	777.15±99.6	17.5±6.71	35.91±46.36

Table 1. List of sampled plots with corresponding soil Organic Matter, pH, altitude, humidity of soil samples and *Lactarius vinosus* fruit body yields averaged for the period 2008-2014. Average values are shown with their standard deviation.

In order to obtain a representative sample from the selected plots, 8 cylinders of soil

(12 cm deep and 5 cm in diameter) were collected randomly in each of the 12 plots. Soil samples from each plot were then homogenized and pooled *in situ* to obtain a composite sample. We discarded the litter but included humus and mineral soil, obtaining approximately 500 g composite soil sample from each plot. Soil samples were sieved (3 mm mesh) and stored a maximum of 24 hours at 4ºC. Individual soil samples were homogenized, separated in four different subsamples and kept at -20ºC until use. Three of the four subsamples were subjected separately to each of the three drying procedures and the fourth subsample was used to calculate the soil moisture, which was determined by weighting the soil before and after 48 hours at 105 ºC.

Drying procedures

Subsamples selected for drying procedures were subjected to a) drying at room temperature (RT procedure, 20-25ºC), b) drying in the oven (O procedure, 60ºC) or c) freeze-drying (FD procedure during 48 hours). Subsamples dried at room temperature were placed on a table within an isolated room, free from evident fungal contamination during 5 days. Subsamples dried in the oven were subjected to 60ºC during 3 hours. Freeze-dried subsamples were dried during 48 hours to ensure complete water removal. Once dried to constant weight, subsamples were homogenized using mortar and pestle, resulting in a very fine powder. Each homogenized subsample from each of the three drying procedures was used for DNA extraction.

DNA extraction

Total DNA was extracted from an aliquot (500 mg) of each soil subsample using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol, with one modification: Lysis buffer (SL1) was increased up to 900 µl. Total DNA extracts were quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE USA).

Design and validation of Lactarius vinosus primers and TaqMan® probe

We designed species-specific primers and a TaqMan® probe to amplify an 88 bp fragment of *L. vinosus* DNA using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA): LVIN-F 5'(TCGACGAGACAACGTTTGG)3', LVIN-R 5'(GGTAGTCTCACCCGATTTGAG)3' and the TaqMan® probe LVIN-TQ 5'(6*FAM*-TCCCTTCTCGGGAAACACACTCAAC-*MGB*)3'. The probe was designed based on the variability of the rDNA internal transcribed spacer ITS1 region detected in the

alignments among different *L. vinosus* sequences. A search for highly similar sequences (MEGABLAST) was performed in the GenBank database to test the specificity of the designed oligonucleotides. Probe specificity was validated using template DNA obtained from 8 fruit bodies of *Lactarius vinosus* collected in the study area and DNA obtained from fruit bodies of different related fungal species: *Lactarius deliciosus, L. sanguifluus, L. volemus, L. sphagnetti, L. bertollini, L. chrysorreus, Russula aeruginea, R. drimeia, R. aurea* and *R. delica*. The DNA obtained from fruit bodies to test the specificity of probe and primers was extracted from 20 mg of fruit body using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the protocol established by the manufacturer.

Quantitative assays

Standard curves (R^2= 0.998, Efficiency= 99.7%) for mycelium quantification were constructed according the procedure described by Parladé et al. (2007) and Hortal et al. (2008). In short, a mixture of soil and mycelium was prepared using 0.480 g of a soil obtained nearby the sampled forest plots mixed with 0.020 g of fungal tissue obtained from the inner part of a dried *L. vinosus* fruit body. Soil used in these mixtures was previously checked for the absence of *L. vinosus* by Real Time PCR using the probe and primers described in the previous section. Total DNA was purified from soil with the added *L. vinosus* using NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany), as previously specified. Serial tenfold dilutions from the purified DNA were prepared to obtain standards of 4, 0.4, 0.04, 0.004 and 0.0004 mg *L. vinosus*·g $soil^{-1}$.

Real-time PCR reactions were prepared using 2X Premix Ex Taq™ (Probe qPCR) (Takara Bio Inc. Otsu, Shiga, Japan) according to the manufacturer's instructions, with 5 µl. of DNA template, 800 nM of each oligo, 200 nM of TaqMan probe, 0.8 µL ROX and HPLC water to adjust a final reaction volume of 20 µl. Real-Time cycling conditions for the StepOnePlus instrument (Applied Biosystems) were 30 s at 95 °C, followed by 40 cycles at 95ºC for 5 s and 34 s at 60 °C. Three replicates from each sample and the standards were included in the analysis, as well as a negative control with water instead of template. Data processing and fungal quantification were performed as described by Parladé et al. (2007).

PacBio RS II sequencing

Each subsample was subjected to PCR-amplification using 25 ng of genomic DNA

and gITS7 and ITS4 primers (Ihrmark et al. 2012) to amplify the ITS2 region. A unique tag composed of eight bases was added to both primers. The number of necessary PCR cycles was optimized until a visible band was obtained. PCR amplifications of samples and both negative controls from DNA extraction and PCR were conducted in a 2720 Thermal Cycler (Life Technologies) in solutions of 50 µL. Final concentrations in the PCR reaction mixtures were: 25 ng template, 200 µM of each nucleotide, 2.75 mM MgCl2, primers at 200 nM, 0.025 U μL^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA) in 1X buffer PCR. PCR cycling conditions was as follows: 5 min at 95ºC, followed by 24-30 cycles of 30 s at 95ºC, 30 s at 56 ºC, 30 s at 72 ºC and a final extension step at 72 ºC for 7 min before storage at 4 ºC. Each sample was amplified by triplicate, purified using AMPure kit (Beckman Coulter Inc. Brea, CA, USA) and quantified using Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled before sequencing. The final equimolar mix was purified using EZNA Cycle Pure kit (Omega Bio-Tek). Quality control of purified amplicons was carried out using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA) and a 7500 DNA chip. The samples were included with another set of samples to use with PacBio RS II system based on the Real-Time (SMRT®) technology. The molecular data are archived at the Sequence Read Archive under accession number PRJNA309233 (www.ncbi.nlm.nih.gov/sra).

Quality control and bioinformatic analysis

Quality control, filtering and clustering was assessed with the SCATA pipeline (scata.mykopat.slu.se). Sequences were filtered removing data with an average quality score <20 and/or with a minimum sequence length of 100 bp, using the amplicon quality option. Sequences were also screened both for primers (using 0.9 as a minimum proportional primer match for both primers) and sample tags. We used 'usearch' as a search engine, with an established minimum match length of 85%. Homopolymers were collapsed to 3 bp before cluster analysis. Pairwise alignments were conducted using a mismatch penalty assigned at 1, gap open penalty of 0 and a gap extension penalty with value 1. Sequences were clustered in operational taxonomic units (OTUs) with single linkage clustering, using 1.5% as a threshold distance with the closest neighbour.

Taxonomic and functional identification

We assigned a putative taxonomy to the 559 most abundant OTUs, which

represented around 95% of the global number of reads. We selected the most abundant sequence from each OTU for taxonomical identification, using the option BLASTn massblaster in PlutoF from the UNITE (Abarenkov et al. 2010) and INSD database. Sequences from UNITE database were preferentially selected due to their accurate selection and revision (Kõljalg et al. 2005). When there was no consistency, we classified the OTU as unknown until the level in which we observed taxonomical consistency. In the case where two or more species showed the same or similar homology at 1.5% level, we included all the species names as potentially representing the OTU. We validated the taxonomical classification by constructing phylogenetic trees using the neighbor joining clustering method including reference sequences from the UNITE and INSD databases. Functional roles of the species were assigned as follows: a) ectomycorrhizal or ericoid fungi, b) moulds, c) yeast, d) saprotrophs or litter-decay fungi, e) pathogens, f) moss-associated fungi, g) root-associated fungi, h) Unknown function, based on the UNITE database (Abarenkov et al. 2010) and from DEEMY (www.deemy.de) or other published literature. OTUs classified as mould species belong mainly to the genera *Penicillium* sp., *Talaromyces* sp., *Trichoderma* sp., *Mortierella* sp., *Umbelopsis* sp. and *Mucor* sp. OTUs classified as "Not considered" were those with very low representation in the database (Table 4) or fungal species not relevant for this study such as animal pathogens.

Statistics

All statistical analyses were carried out using the R software environment (v3.0.2 version; R Core Team, 2013), using the vegan package (Oksanen 2013) for Hill's numbers and multivariate analysis, and the indicspecies package (De Caceres and Legendre 2009) for species indicator analysis.

The drying procedure effects on DNA yield of *L. vinosus* obtained by qPCR were tested using linear mixed-effects models (LME). Here, samples nested within plots were included as random factors to deal with the spatial structure of the data. The same test was carried out using the 260/230 DNA quality ratios in order to study whether these values affected the qPCR data from specific plots or drying procedures.

Hill's series of diversity indices were used to compare differences in diversity values between drying procedures (Hill 1973). Hill's series of diversity (Hill 1973) consists of three numbers: N0 is the richness (Number of OTUs per sample); N1 is the antilogarithm of Shannon's diversity index; and N2 is the inverse Simpson diversity. We did not rarefy the community due to the potential loss of information or incorrect

interpretation of results (McMurdie and Holmes 2014). However, an uneven read distribution across samples is very common due to the sequencing bias, which may affect these indices. Thus, as an alternative we used the square root transformation of read counts as an explaining variable when testing for the Hill's numbers (Bálint et al. 2015). LME models were used to test the effect of the drying procedures on Hill's numbers, with the samples nested within plots treated as random variables.

A general fungal community analysis was carried out using permutational multivariate analysis of variance based on distance matrices (using the function "adonis" and Bray-Curtis distances). Here, plots and drying procedures were included as factors, and count data were Hellinger transformed. Subsequently, Nonmetric Multidimensional Scaling (NMDS) was used to visualize community variation, and the effects of the drying treatments on the fungal community composition. Similarly, the effects of the drying procedures on the low abundant taxa were tested using adonis on fourth-root transformed data.

Changes in the relative abundance of functional groups were tested using ANOVAs. Also, we checked the possibility for a certain OTU to be identified as an indicator species for a certain type of drying treatment using Species Indicator Index (De Caceres and Legendre 2009). Here, we also considered the relative abundance of each OTU as an approach to take into account the bias caused by the different sequencing depths. We used the function multipatt together with the parameter IndVal.g to manage the unequal group size. In both analyses, relative abundances were arcsine transformed.

Results

Impact of drying procedures on the DNA yield of L. vinosus (Hypothesis 1)

Specific quantification of *L. vinosus* obtained by qPCR showed a wide range of values among soil samples, which ranged from 0.51 to 0.002 mg of *L. vinosus*$\cdot g^{-1}$ soil. In addition, from one plot we did not detect DNA of *L. vinosus* and the data from this plot were discarded from further analysis. Mean values obtained for DNA yield were 0.15± 0.03, 0.11± 0.02 and 0.09± 0.02 mg of *L. vinosus*$\cdot g^{-1}$ soil for the FD, O and RT procedures, respectively, with significant differences according to the drying treatment (df=10, F-value= 3.65, $p<0.05$). *L. vinosus* detection was greater from soils dried using the FD procedure in comparison with O and RT, with values 26% and 42% higher in FD. DNA quality ratios 260/230 of soil samples showed no significant differences among the plots (F-value=0.763, $p>0.05$) nor according to drying procedures (F-value=1.15, $p>0.05$) or

their interaction (F-value=2.14, p>0.05). DNA quality ratios 260/280 ranged from 1.8 to 2.

Impact of the drying procedure on diversity (Hypothesis 2)

After quality control and discarding singletons and sequences having two different tags (Sequences with tag jumps), we obtained 30,588 reads. None of the tags from the negative controls were detected in the database but one sample was discarded due to low sequence output. We recovered 1,139 OTUs and an average of 987 reads in each sample. Analysis of Hill's numbers showed that diversity indices were not affected by the drying procedure after accounting for the sequencing bias (df= 17, p>0.05, Table 2, Fig. 1). As expected, the effect of the sequencing bias was significant for richness (p<0.001, Table 2).

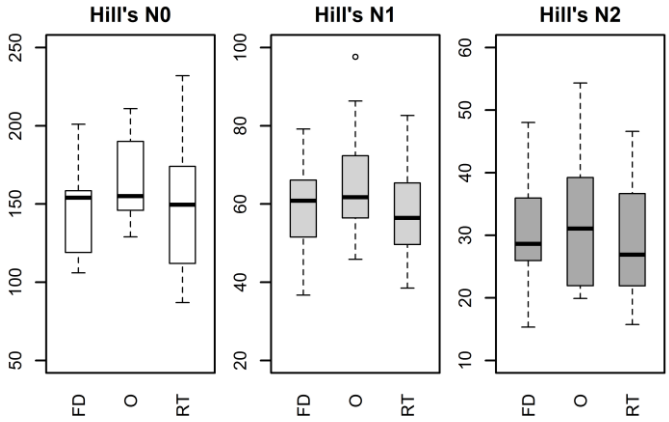

Fig. 1 The effect of the drying procedures represented by Hill's numbers. Boxplots of Hill's diversity numbers show diversity profile of the soil fungal communities in response to the soil drying procedures. No statistical differences in Hill's Numbers was observed between drying procedures (p>0.05). Values on Y axis indicate the Hill's diversity series numbers in absolute terms. Codes on X axis refers to the drying procedures (FD: Freeze-dried samples, O: Samples dried at the oven, RT: Samples dried at room temperature).

Table 2 Variation in Hill's diversity numbers obtained from the Linear-Mixed Models (LME) and their significance. At the LME, plot was considered a random factor and the bias caused by the

	DF	Hill's N0		Hill's N1		Hill's N2	
		F-value	Pr(>F)	F-value	Pr(>F)	F-value	Pr(>F)
Intercept	1	621.86	<.001	341.43	<.001	146.79	<.001
Sqrt (Read counts)	1	63.19	<.001	0.97	0.340	0.25	0.625
Drying procedure	2	2.59	0.105	1.69	0.215	0.37	0.702
Total	17						

different number or reads was included in the model. Hill's N0: Richness. Hill's N1: Antilogarithm of Shannon's diversity. Hill's N2: Inverse of Simpson's diversity.

Impact of the drying procedures on the fungal community (Hypothesis 3)

Permutational multivariate analysis of variance showed that there were no differences in the fungal community composition between the three drying procedures considered ($F=1.34$, $p>0.05$, $R^2=0.03$, Table 3). Here, most of the variation was observed among plots ($F=5.78$, $p<0.01$, $R^2=0.74$, Table 3), representing 74% of the total variation, whereas the drying procedures represented only 3%. Similarly, the drying procedures had no significant effect on the low-abundance taxa ($F=1.20$, $p>0.05$, $R^2=0.03$). The visual display of results is presented in the NMDS plot (Fig. 2), indicating that most of the observed variation in the fungal community was plot-related, whereas the three different drying procedures were mostly clustered together.

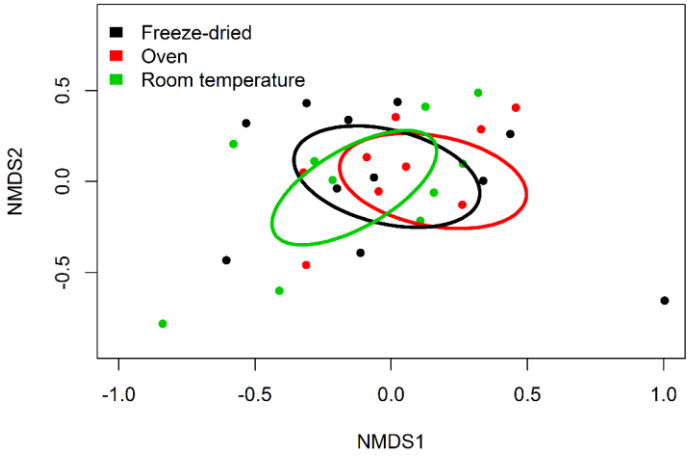

Fig. 2 Nonmetric multidimensional scaling (NMDS) of the community data showing the effect of the drying procedures on the fungal community composition. Each dot represents a soil sample with a given soil drying treatment for a given plot.

Table 3 Permutational multivariate analysis of variance test considering the plot as the main factor and the drying procedure as the second explanatory factor

	Df	SumsOfSqs	MeanSqs	F.Model	R^2	Pr(>F)
Plot	10	4.70	0.47	5.78	0.74	0.001
Drying procedure	2	0.22	0.11	1.34	0.03	0.060
Residuals	18	1.47	0.08		0.23	
Total	30	6.39			1	

The relative abundance of functional groups was similar among drying procedures analysed, with no significant differences ($p>0.05$). Ectomycorrhizal fungi in the FD

procedure represented 44.6% of the total fungal proportions, and soil from the O and RT treatments showed similar values of 41.2% and 42.0%, respectively (F-value= 0.148, $p>0.05$; Table 4). The second most abundant group was composed of OTUs that were not possible to identify or assign to a functional role, representing 25.6% of the read counts for the FD, 26.4% for the O and 24.2% for the RT procedures. Mould species were the third most abundant functional group, with percentages ranging from 10.3% for FD samples and 13.4% for RT samples, with no significant differences (F-value=0.117, $p>0.05$; Table 4).

Table 4 Average relative abundances of the functional groups obtained for each of the three drying procedures. Data is given in percentage of total OTUs. Ectomycorrhizal species include those classified as ectomycorrhizal and ericoid mycorrhizal. Unknown species were those that could not be assigned to a functional role. OTUs classified as "Not considered" were those with very low representation in the database or fungal species not relevant for this study.

Function	Freeze-dried	Oven	Room temp
Ectomycorrhizal	44.6±0.07	41.2±0.06	42.0±0.07
Unknown	25.6±0.04	26.5±0.05	24.2±0.04
Moulds	10.3±0.28	10.4±0.25	13.4±0.37
Saprotroph	3.6±0.02	3.7±0.02	3.7±0.02
Root-associated	2.4±0.16	3.1±0.22	3.3±0.29
Moss-associated	1.7±0.16	1.8±0.14	0.2±0.13
Yeasts	5.6±0.09	5.8±0.13	6.6±0.13
Plant pathogen	0.4±0.21	0.5±0.20	0.2±0.13
Not considered	5.8	7	6.4

Effect of the drying procedures to specific fungal species (Hypothesis 4)

From the 1,139 OTUs recovered, there were four and six OTUs that were significantly more frequent after the O and RT procedure, respectively (Table 5, $p<0.05$). The OTUs with increased frequency associated with the RT procedure, mostly belong to moulds or yeasts: (*Cryptococcus* sp., *Umbelopsis* sp., *Trichoderma spirale*, *Exophiala* sp. and *Mucor fragilis*), opportunistic or R-strategist fungal species (Table 5). In the case of samples from the O procedure, two OTUs were classified as moulds (*Mortierella* sp., *Penicillium* sp.) and two were of uncertain functional classification (Unknown Capnodiales, Unknown Helotiales) (Table 5). However, the magnitude of these changes was small for both drying procedures, with an overall increase of the indicator OTUs from the O samples at 0.4%, and the increase of the indicator OTUs from the RT samples at 1.84% (Table 5).

Table 5 Significant OTUs and their assigned identification for each of the drying procedures. To test the significance, Freeze-drying procedure was defined as a control. Component A shows the degree of specificity or the probable predictive value, and Component B shows the degree of fidelity or sensitivity, meaning the probability of finding the species in the treatment belonging to the treatment group. P.value is the statistical significance of each OTU. % total represents the total percentage of each significant OTU for a given drying procedure. In parenthesis it is shown the increase in percentage with respect to Freeze-drying procedure. % increase represents the overall increase of each significant OTU with respect to Freeze-drying procedure.

Method	Ecology	A	B	p.value	% total	% increase
Oven						
Unknown Capnodiales	Unknown	0.58	0.8	0.03	0.190 (52)	0.099
Mortierella sp.	Moulds	0.752	0.6	0.02	0.131 (81)	0.106
Unknown Helotiales	Unknown	0.718	0.6	0.01	0.183 (82)	0.151
Penicillium sp.	Moulds	0.692	0.6	0.04	0.102 (84)	0.086
						0.443
Room temperature						
Cryptococcus sp.	Yeast	0.566	1	0.025	0.767 (65)	0.499
Umbelopsis sp.	Moulds	0.921	0.6	0.005	0.185 (91)	0.169
Trichoderma spirale	Moulds	0.511	1	0.02	0.750 (39)	0.292
Exophiala sp.	Yeast	0.459	1	0.02	1.642 (45)	0.736
Odontia sp.	Unknown	0.892	0.4	0.045	0.073 (88)	0.064
Mucor fragilis	Moulds	0.831	0.4	0.02	0.097 (80)	0.077
						1.838

Discussion

Impact of drying procedures on the total DNA yield and the DNA quantification of L. vinosus (Hypothesis 1).

Our results have demonstrated that drying procedures had an effect on the DNA yield of the ectomycorrhizal fungus *L. vinosus*, with the greater yield resulting from the freeze-drying (FD) procedure. Similar to our findings, previous studies showed that drying at RT (or soil incubation) results in a decrease of specific DNA of known fungal species measured by Real-Time PCR such as the ectomycorrhizal *Tuber aestivum* (Gryndler et al. 2013) or the pathogenic *Gaeumannomyces graminis* (Herdina et al. 2004). Gryndler et al. (2013) suggested that DNA was degraded and only the DNA protected by humic acids was remaining after soil incubation. Similarly, Herdina et al. (2004) argued that dead mycelium was degraded after soil incubation, whereas live mycelium was much more stable. Our results, in line with the existing literature, suggest important effects of the drying procedure on the yield of specific fungal DNA, which will affect the estimated

ectomycorrhizal fungal biomass. In the case of studies quantifying fungi by Real-Time PCR, the use of a standard based on known concentrations of genomic DNA (Filion et al. 2003), hyphal biomass (Raidl et al. 2005) or mycelia biomass (Parladé et al. 2007) allows reliable comparison between runs. In addition, the use of this standard could also allow a comparison of absolute abundances of fungal biomass between studies if the same standard and sample handling are used. Finally, the drying procedure will also influence detection the limit. Thus, when targeting especially low-abundance fungal taxa in soils, freeze-drying would be the most appropriate drying approach.

Impact of the drying procedure on the richness and diversity indices (Hypothesis 2)

One of the hypothesized effects of drying procedures at community level was the alteration of diversity indices as a consequence of the colonization of soil samples by specific fungal species. Previous works have reported that a very abundant single OTU may distort the community composition and richness (Adams et al. 2013a). This very abundant single OTU could belong to abundantly sporulating fungi (Adams et al. 2013a), or could be a fast growing opportunistic species (Lindahl et al. 2010). However, our study did not reflect any drying procedure effect on diversity indices, which suggests that no colonization from other organisms occurred or that colonization occurred equally to all the drying procedures. Although it is not possible to determine the exact cause, we suggest that most likely there was no colonization in any of the drying procedures or this colonization only occurred to a limited extent by a few fungal species.

Impact of the drying procedure on the fungal community (Hypothesis 3)

There were no appreciable effects of drying procedures on the fungal community composition, where the plot factor was the main source of variation (74%). This variation among plots is probably produced by niche effects, which has been commonly reported for soil fungal communities (O'Hanlon and Harrington 2012). This variability was also observed for fruit body production in the studied plots since 2008 (Table 1), but also at a regional level (De Miguel et al. 2014). In any case, differences in fungal mycelia observed between plots (small geographical scales) can be attributed to biotic and abiotic factors (i.e. soil nutrients, aboveground vegetation) or stochastic processes such as dispersal limitation (Bahram et al. 2015). Also, discrete patches of organic matter or mineral nutrients in soil microcosms can also influence mycelia growth (Cairney 2005).

Certain structures from cell walls such as melanin can make the DNA much more resistant to degradation, especially in the short drying period used in this study. Fernandez et al. (2013) reported high stability and low degradation of *Cenoccocum geophilum* dead mycelia, most likely due to the presence of melanin, which prevents degradation of the cell walls. Similarly, Clemmensen et al. (2015) report impaired fungal degradation across fungal groups probably due to the presence of the same cell wall component. Although it seems that impaired degradation of fungal DNA may occur, we suggest that the drying procedures applied in this study may not be enough to induce any impaired fungal degradation with time. For instance, Bååth et al. (2004) observed decay in fungal biomarkers after incubation at 25ºC, but the effects after one month were very limited. Furthermore, the authors used the 18:2ω6.9 marker, which most likely is more sensitive to degradation than DNA (Wallander et al. 2013). Disconnection from host roots may cause the death of ectomycorrhizal mycelia (Bååth et al. 2004; Wallander et al. 2001), which may become available for other saprotrophs (Lindahl et al. 2010). However, according to our results, no important colonization at expenses of the dead mycelia occurred in the RT samples, most likely due to a fast decrease in moisture content during the drying treatments.

Effect of the drying procedures on specific fungal species (Hypothesis 4)

Finally, we observed four and six OTUs increasing in O and RT procedures, respectively. These increases were related to slight increases in read count proportions occurring for specific OTUs, mainly classified as moulds or yeasts and considered to be opportunistic fungal species. Thus, five out of the six OTUs that increased in RT samples were classified as moulds or yeasts, including *Mucor fragilis* or *Trichoderma spirale*, and genera represented by *Cryptococcus* sp., *Umbelopsis* sp. and *Exophiala* sp. However, the magnitude of the increase in their frequency was small for both drying procedures, with an overall increase in relative abundance of 0.4% and 1.8% for O and RT drying procedures, respectively. This small increase in their relative proportions suggests that the drying procedures had a limited impact on these taxa. Some of these species belong to the order Mucorales (i.e. *Umbelopsis* sp. *Mucor fragilis*), which has also been observed to increase in relative abundance after root severing in boreal ecosystems (Lindahl et al. 2010). Mould species, together with yeasts, contribute to increase the turnover of dead mycelium and other forest residues (Clemmensen et al. 2015) and might be considered fungal opportunists. However, these species are also an important component of the

airborne fungal community (Adams et al. 2013b) and could alter the samples dried at room temperature.

Conclusions

This study highlights the importance of choosing the appropriate drying method depending on the aims of the study. For Real-Time PCR studies, we suggest using the same drying technique for all the samples but also for the standard preparation to avoid DNA degradation. When aiming for the detection of low abundant species, we suggest freeze-drying soil samples before DNA extraction. For fungal community analysis, and contrary to our initial hypothesis, we would not expect a significant increase in relative abundance of opportunistic fungal species at the expenses of the ectomycorrhizal mycelia if the drying conditions are optimal, i.e. under low air moisture and temperatures around 20-25 ºC. Since we have observed that sample preparation has an effect on the DNA yield of specific fungal species, an open question is whether DNA from dried samples is stable over time. Thus, new studies should address the effect of the long-term storage of soil samples both at the individual species and at the community level.

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Conflict of interests

The authors declare no conflict of interests associated with this publication.

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CHAPTER II

Seasonal dynamics of the ectomycorrhizal fungus *Lactarius vinosus* are altered by changes in soil moisture and temperature.

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Seasonal dynamics of the ectomycorrhizal fungus *Lactarius vinosus* are altered by changes in soil moisture and temperature

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Abstract

Ectomycorrhizal fungi are important components of the carbon and nitrogen cycles in forest soils and improve the nutrient uptake of many plants. There have been few assessments of how soil fungal biomass is affected by climate; however, a good understanding of how soil mycelium is correlated with climate is essential to predict long-term responses to global warming. Soil extramatrical mycelia (EMM) of *Lactarius vinosus* Bataille, a highly valued, edible ectomycorrhizal species, was quantified by performing qPCR analyses of soil samples that were collected monthly from 28 long-term experimental plots. The belowground moisture and temperature of these plots were individually recorded every 2 h. *L. vinosus* soil EMM biomass fluctuated between 44 and 261 kg ha^{-1}, with the maximum biomass occurring during spring months and the minimum occurring during winter and summer months. Mean mycelial turnover was estimated to occur 7.0 times $year^{-1}$. Soil EMM was reduced under low soil temperatures and when there was a combination of high soil temperatures and low soil moisture, most likely caused by reduced C allocation from the host under both winter and summer conditions. Preliminary models using simulated reductions in soil moisture and increases in soil temperature showed that soil EMM increased significantly during winter–spring months, whereas EMM decreased significantly during summer months. These results suggest that warmer conditions would lengthen the period of *L. vinosus* biological activity during winter months, whereas increasing periods of drought would prevent the growth of this species during summer months. Our study shows significant soil EMM seasonality and highlights potential

climate-driven annual cycle shifts of soil mycelia.

Keywords: Extramatrical mycelia; Drought; *Lactarius*; Fungal biology; Temperature; Moisture.

1. Introduction

Mediterranean ecosystems are predicted to be very sensitive to changes in global climate, with expected negative effects on biodiversity, biomass and associated ecosystem services (Schröter et al., 2005; Thuiller et al., 2008). The predicted climate changes are expected to increase Mediterranean basin temperature ranges by between 1.4°C and 5.1°C by 2055 (Nogués Bravo et al., 2008), whereas total precipitation is predicted to be reduced by 10–15%, principally during the summer months (García-Ruiz et al., 2011; Giorgi and Lionello, 2008). Alterations in precipitation patterns are also expected in this region, with more irregularly distributed precipitation and an increasing number of extreme rainfall events (García-Ruiz et al., 2011). Recent studies of Mediterranean ecosystems indicate that climate change is currently altering ecosystem functioning by causing migrations, local extinctions and phenology changes in plants and animals (Menzel et al., 2006; Peñuelas et al., 2013). However, to date, no study has explicitly addressed climate-induced changes affecting belowground fungal dynamics in Mediterranean forest ecosystems because the majority of fungal ecology studies are focused on temperate or boreal ecosystems (Mohan et al., 2014).

Mycorrhizal species form a beneficial symbiotic association with the vast majority of trees and shrubs under natural conditions, providing their plant hosts with nitrogen and phosphorus in return for photosynthetically fixed carbon (Smith and Read, 2008; Van der Heijden et al., 2008). The extramatrical mycelia (EMM) is the main driver of this symbiotic interaction: the EMM colonizes the surrounding soil to forage for nutrients and to encounter new root tips to colonize (Cairney, 2012). Previous studies have estimated that between 2.5% and 20.0% of the photosynthetically assimilated carbon is used by the fungal symbiont to construct and maintain the fungal EMM (Ekblad et al., 2016; Hobbie, 2006). EMM accumulation in soils has been attributed to slow fungal and necromass turnover (Ekblad et al., 2016; Hagenbo et al., 2016) and has been recognized as an important soil organic matter (SOM) formation process in boreal soils (Fernandez et al., 2016). Standing soil EMM fluctuates between 700 and 5,800 kg ha^{-1} (Wallander et al., 2004, 2001), with an estimated yearly EMM production of 200–350 kg ha^{-1} in boreal ecosystems (Ekblad et al., 2013; Högberg and Högberg, 2002; Wallander et al., 2010). However, the yearly EMM production in Mediterranean ecosystems is unknown. Equally important to fungal production is the fungal turnover because standing biomass represents the balance between all these processes in forest soils (Ekblad et al., 2016; Hagenbo et al., 2016; Hendricks et al., 2016).

EMM biomass in soils has been studied by using biological indicators such as phospholipid-derived fatty acids (PFLA), ergosterol or DNA (Wallander et al., 2013). In general, the EMM biomass in soils shows seasonal fluctuations (De la Varga et al., 2013; Iotti et al., 2014; Wallander et al., 2001; 1997), with higher levels of EMM biomass occurring during spring and autumn in boreal forest ecosystems (Söderström, 1979; Wallander et al., 2001). However, the EMM of *Boletus edulis* or *Lactarius deliciosus* has been observed to increase during the late autumn and early winter (De la Varga et al., 2013) and the EMM of *Tuber magnatum* has been observed to increase during the spring (Iotti et al., 2014). These contrasting annual cycles of EMM biomass suggest that in addition to host–fungal interactions, climatic factors play an important but still unexplored role affecting EMM biomass belowground. In Mediterranean forests, although many plants have adaptive traits that make them resistant to drought, these communities often reduce their growth under drought stress (Sardans and Peñuelas, 2013), with potential negative effects on soil EMM biomass (McGuire et al., 2011; Staddon et al., 2003). However, to date, no consistent conclusions have been reached to predict how climate and, specifically, how drought affects soil fungal biomass in Mediterranean forests. Ectomycorrhizal fungi are key players in the alleviation of drought stress for host trees (Mohan et al., 2014) and their relevance is even more important under nutrient-limited conditions (Read and Perez-Moreno, 2003); however, it is not known to what extent different fungal species are resistant to the expected increase in drought conditions.

In this study, we investigate how soil temperature and soil moisture may be correlated with the annual cycle of the EMM biomass of *Lactarius vinosus*, an important edible ectomycorrhizal species. This species is commonly sold together with other related species of the *Lactarius* group *deliciosus* in markets in Europe, Asia and North Africa (Boa, 2004). The economic impact of *L.* group *deliciosus* trade, based on data from the three most important markets in Spain, was 5.3 M € $year^{-1}$ for the period 2002–2008, with on average almost 500 t of *L. deliciosus* sold every year (Voces et al., 2012). We selected this mushroom group as a target because it fruits from late autumn to mid-winter on different continents (Europe, America, Oceania and Africa) and forms mutualistic associations with conifer trees under a wide range of conditions. The frequent presence of this ectomycorrhizal species at the study site is well documented by fruiting body surveys (Bonet et al., 2012).

We first describe the annual cycle of *L. vinosus* EMM over 12 months. Given that the Mediterranean climate pattern is characterized by high temperatures and low rainfall during summer, low water availability is an important constraint for tree growth (Sardans and Peñuelas, 2013), which most likely will negatively affect fungal biomass. By contrast, mild temperatures and rainfall events during spring and autumn will result in greater EMM biomass during the

growing season, as observed in other ecosystems (Söderström, 1979; Voříšková et al., 2013; Wallander et al., 2001). Moreover, low levels of biomass are expected during winter as a result of tree dormancy resulting from colder temperatures. Second, we model EMM biomass using belowground climate data (soil temperature and moisture) recorded every 2 h in each of the sampled plots to test whether fungal mycelial biomass is correlated with belowground moisture and temperature. Finally, increases and decreases in soil EMM biomass are predicted by simulating increases in soil temperature and decreases in soil moisture.

2. Material and Methods

2.1 Site selection

The study was carried out between June 2013 and May 2014 in the Natural Park of Poblet (Northeast Spain, 41° 21' 6.4728" latitude and 1° 2' 25.7496" longitude), which has been used previously as an experimental area to study the effects of climate change in Mediterranean ecosystems (Ogaya et al., 2014). The average annual temperature at the study site is 11.8°C. The average annual rainfall is 666.5 mm, with the average minimum and maximum rainfall occurring in July (16 mm) and October (79.8 mm), respectively, with two peaks occurring in spring and autumn and a pronounced summer drought that usually lasts for three months. Mushroom production has been studied in 28 plots at the study site since 2008 as part of a long-term experimental set-up. Sporocarp surveys during these years have revealed that sporocarps predominantly belonged to the *Lactarius* group *deliciosus* and were primarily *Lactarius vinosus* (Bonet et al., 2012).

Soil samples were collected from all 28 long-term plots. The plots are composed of even-aged (60-year-old) *Pinus pinaster* plantations, with isolated *Quercus ilex* trees sometimes forming scrub. The plots are characterized by having a range of altitudes (from 594 to 1013 m.a.s.l.), slopes (3–23%), stand densities (350 to 2,657 trees ha^{-1}) and basal areas (16.5 to 81.7 m^2 ha^{-1}) (Table S1). This range of plot conditions was specifically selected to capture as much variability as possible. The soils are siliceous with franc-sandy textures, pHs ranging from 6.1 to 6.6 and organic matter contents ranging from 2.95 to 10.51 (Table S1). On average, the study plots produce approximately 15 kg ha^{-1} yr^{-1} of *Lactarius vinosus* fruiting bodies (unpublished results), which indicates that this species is well established at the plots.

2.2 Soil sampling

Soil samples were systematically collected from the 28 plots, which were each 100 m^2 (10 × 10 m) (Fig. S1a, FigS1b). Eight soil cores (12 cm deep and 5 cm in diameter), two cores from each side of the plot (Fig. S1b) and at least 10 m apart to avoid spatial autocorrelation,

were extracted from each plot every month. Because the litter material is most likely inhabited by saprotrophic fungi (Voříšková et al., 2013), we discarded the litter but included humus and mineral soil to obtain soil profiles with a depth of 12 cm and a composite sample of approximately 500 g for each plot (Fig. S1c). This sampling scheme was repeated monthly; in total, 2,688 soil cores were extracted between June 2013 and May 2014. Soil samples were stored at 4°C for <24 h and then sieved through a 3-mm mesh. Sieved soil samples were freeze-dried and pooled to obtain a composite sample for each plot. Each composite soil sample was homogenized using a pestle and mortar, resulting in a very fine powder, and an aliquot of 500 mg was stored at –20°C before DNA extraction.

2.3 DNA quantification of Lactarius vinosus

DNA was extracted from the soil samples and negative controls (DNA extractions without soil addition to control for contamination) using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol, with a few modifications: the volume of lysis buffer (SL1) was increased to 900 µl and DNA was eluted in 100 µl of elution buffer.

For DNA quantification of *Lactarius vinosus*, we used the previously validated TaqMan® probe designed to quantify this ectomycorrhizal fungal species (Castaño et al., 2016). Standard curves for quantification of the mycelium were constructed according to the procedure previously described (Parladé et al., 2007). DNA extractions to build the standard curve were obtained from 0.48 g of a soil nearby to the study area, previously checked for *L. vinosus* absence by Real-Time PCR using the probe and primers described in the previous section, mixed with 0.02 g of fungal tissue obtained from a dried *L. vinosus* fruit body. Several tenfold dilutions to 10^{-4} of the original DNA extraction were prepared.

Real-time PCR reactions were prepared using 2X Premix Ex Taq™ (Takara Bio Europe SAS, France) according to the manufacturer's instructions, with 5 µl of DNA template, 400 nM of each oligo, 200 nM of TaqMan probe, 0.8 µl ROX and HPLC water to adjust the mixture to a final reaction volume of 20 µl. Cycling conditions in the StepOnePlus instrument (Applied Biosystems) were 30 s at 95°C, followed by 40 cycles at 95°C for 5 s and 34 s at 60°C. Three replicates of each sample and of the standards were included in the analysis, as well as a negative control. Only amplification efficiencies ranging from 95% to 105% were accepted and considered for analysis. We followed the standard recommendations for studies using Real-Time PCR (Bustin et al., 2009). Quantification cycle (C_T) values from the standard preparation were plotted against the logarithm of the corresponding amount of added mycelium to generate a standard curve. Quantification of *L. vinosus* mycelia, expressed as mg of mycelium g soil^{-1}, in each composite sample was determined by interpolation of the C_T value on the standard

curve. The estimated soil mycelia in 1 g of soil was converted to kg ha^{-1} using the soil density calculated for each of the soil cores. The soil volume (V) of the core was first derived by $V = \pi \times r^2 \times L$, where the radius is r (2.5 cm) and the longitude of the core is L (12 cm). From these soil samples we also recorded the total weight (including soil particles > 3 mm) and the sieved soil weight. This method allowed us to calculate the estimated soil mycelia in 1 g of soil, assuming that: (i) particles > 3 mm do not have soil mycelia and (ii) all DNA from *L. vinosus* can be extrapolated to soil mycelia, either dead or active.

2.4 Estimation of fungal production and turnover

Annual fungal production was estimated using the soil biomass data obtained in each plot for each month, and fungal turnover was calculated following Hendricks et al. (2016). Fungal production was determined for each plot by calculating the difference between the observed standing biomass produced over one month with that produced the preceding month. First, the difference between the fungal biomass produced during two preceding months was calculated as:

$$\Delta B = B_{t(n)} - B_{t(n-1)}, \quad (1)$$

where B = *L. vinosus* biomass (mg g $soil^{-1}$), from where production (P_t) was obtained.

$$\text{Thus, if } \Delta B > 0, \text{ then } P_t = B_{t(n)} - B_{t(n-1)}, \quad (2)$$

$$\text{or if } \Delta B < 0, \text{ then } P_t = 0. \quad (3)$$

If the difference in fungal biomass between two months was positive, we considered that there was fungal production for a given plot and month, whereas negative values were considered 0. Finally, turnover (T) in each plot was defined as:

$$T = P_t / SB_{average} \quad (4)$$

where $SB_{average}$ is the annual fungal biomass averaged for each plot.

2.5 Climate data

Aboveground relative humidity and temperature and belowground volumetric soil water content and temperature were measured using Decagon 5 TM probes (Decagon devices Inc., USA) throughout the sampling period. Soil sensors were placed in the middle of each of the 28 plots (Fig. S1d), buried 10 cm belowground. The same soil from the plots was used to bury the sensors. Temperature and relative humidity sensors were placed 1.30 m aboveground in the middle of the plot. All the climate measurements were recorded every minute and averaged every 2 h on a data logger (EM50, Decagon Devices Inc., USA). Data were downloaded and processed with DATATRAC® III software (Alpharetta, USA) and converted to average, minimum and maximum monthly values.

2.6 Data analysis

All statistical analyses were implemented in the R software environment (version 2.15.3;

R Development Core Team 2013) using the "nlme" package for Linear Mixed Models (LMM; Pinheiro et al., 2016) and the "mgcv" package for General Additive Mixed Models (GAMM; Wood et al., 2015).

The seasonal dynamics of EMM biomass was tested by GLMM using square-root transformed fungal biomass data. In these analyses, the plot was considered as a random factor due to the high stochastic effects shaping soil fungal communities, whereas month was a fixed factor. Plots with less than 0.05 mg of *L. vinosus* g soil^{-1} year^{-1} or a single signal across all the months were discarded in order to ensure that the EMM annual cycle was only studied in plots where *L. vinosus* was effectively present (n = 17). Models were tested with and without temporal autocorrelation structure (q = 1) and the most parsimonious model was selected based on the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). Finally, the differences in monthly EMM biomass were checked by performing post-hoc Tukey comparisons.

The effects of soil temperature and soil moisture on EMM biomass were tested using General Additive Mixed Models (GAMM). This approach was chosen because it makes no *a priori* assumption about the functional relationship between variables. As a result, the model examines the yearly variation of EMM biomass and the extent to which it is related to soil moisture and temperature. Multicollinearity between climatic variables was evaluated based on the variance inflation factor (VIF) and checked by simple regression analysis. Because there was a strong correlation (r^2>0.9) between soil and air climatic parameters, we selected only soil climate data for further analysis. Soil moisture, temperature (i.e. averaged monthly soil moisture and soil temperature) and months were included as explanatory variables and plots were included as random factors. In this analysis, model simplification started from the full model (all interactions included), non-significant terms were then removed sequentially to derive the Minimum Adequate Model (MAM; Lee et al., 2013). Simultaneously, temporal autocorrelation in the residuals was accounted for by adding a first-order autoregressive process structure (Pinheiro et al., 2016). The selection of the more parsimonious model was explained in the previous section and also guided by the goodness of fit statistic. The interaction surface between soil temperature and moisture was visualized from the GAMM values obtained.

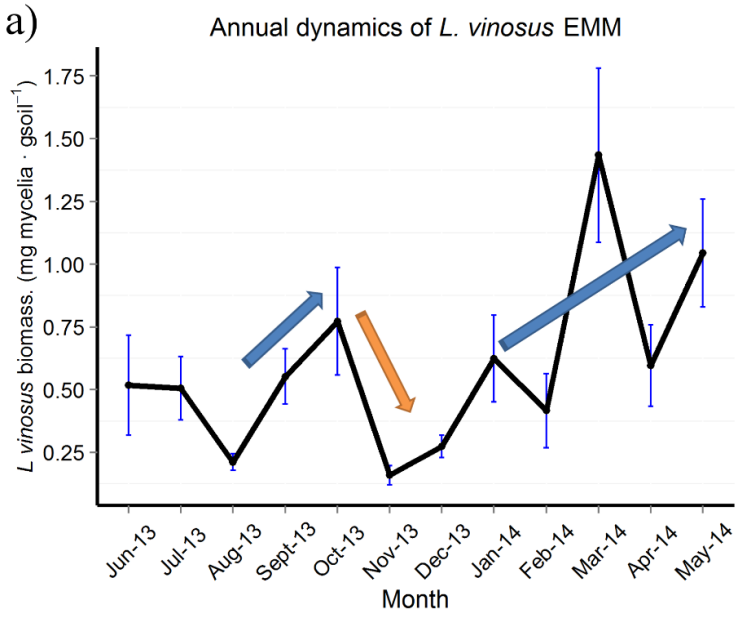
Changes in the future annual cycle of mycelia were projected based on the gradient of changing soil moisture and soil temperature conditions. Based on the forecasted scenarios, a temperature increase of 1.4°C– 5.1°C by 2055 has been predicted (Nogués Bravo et al., 2008; Giorgi and Lionello, 2008). By 2040–2070, an overall reduction in precipitation of approximately 10–15% has been predicted for the Mediterranean basin (García-Ruiz et al., 2011), which is similar to the future predicted reduction in soil moisture (Dai, 2013). However,

depending on the model used, precipitation during the summer months in the Mediterranean basin is also predicted to be reduced by up to 40% by 2100 (Giorgi and Lionello, 2008). Thus, the predicted changes in soil fungal biomass were obtained using a set of models considering serial increases in temperature ranging from +0.5°C coupled with –5% soil volumetric water content, until a maximum of +5°C and –50% of soil volumetric content. Mycelial changes were built using GAMM, including the monthly plot changes in soil moisture and temperature. In these models the mycelial biomass was previously square-root transformed.

3. Results

3.1 Annual dynamics of L. vinosus EMM biomass

L. vinosus soil mycelial biomass was significantly variable across months ($F_{[1,11]}$ value = 4.53, ***P <0.001; Fig. 1, Table S2). Biomass values were stable during early summer (June, July) but decreased slightly during August, when the second lowest EMM biomass value was recorded (0.21 mg of *L. vinosus* g $soil^{-1}$ $plot^{-1}$). Following this decline in the measureable EMM biomass, we observed a significant increasing tendency during late-summer and early autumn (September to October, **P =0.01; Fig. 1). This tendency dramatically changed in November, when a significant decreasing trend was observed, producing the lowest annual value. Finally, the maximum EMM biomass was reached during spring months (March and May; Fig. 1). Thus, *L. vinosus* biomass was twice as abundant in spring months than in the coldest and hottest months (summer vs spring; z value = –3.53, **P = 0.002; winter vs spring; z value = –3.079, **P =0.011).



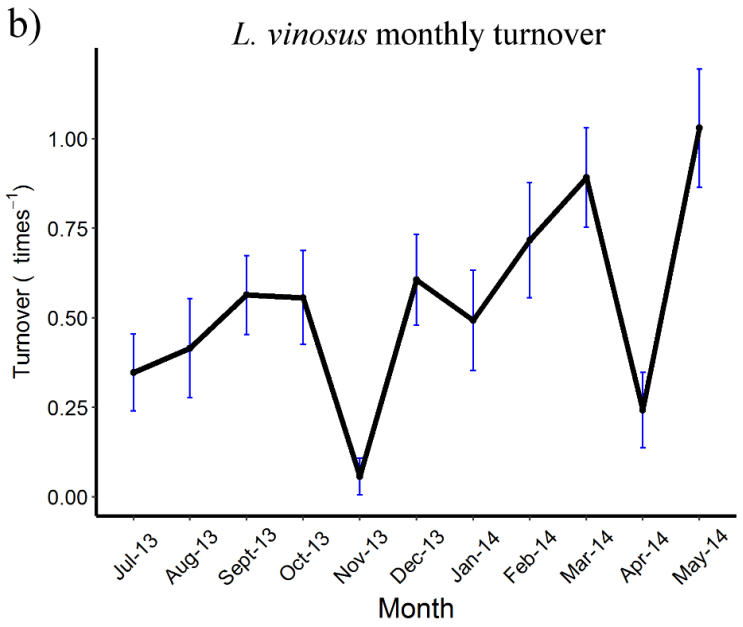


Fig. 1. *L. vinosus* soil biomass (mg mycelia g soil^{-1}) (a) and *L. vinosus* turnover (times year^{-1}) pattern (b) observed during the annual cycle at the study site. Mean values are shown with their standard error. The orange arrow in (a) indicates significant decreasing trends, whereas blue arrows indicate significant increasing trends (*P <0.05).

Taking into account the mean soil density of the study area and assuming no EMM of *L. vinosus* in soil particles >3 mm, the estimated global average of *L. vinosus* EMM biomass from these plots is approximately 108 kg ha^{-1}, with a minimum of 44 kg ha^{-1} in November and a maximum of 261 kg ha^{-1} in March (values refer to the first 12 cm, including humus, the E horizon and the first 12 cm belowground of the mineral horizon).

3.2 Correlation of L. vinosus EMM biomass with climatic parameters

GAMM revealed that *L. vinosus* EMM biomass was significantly correlated with the month and the interaction of soil moisture and soil temperature (Table S2). Biomass showed a non-linear monthly variation (t-value = 5.64, ***P <0.001; Table S3, Fig. S2), which was correlated with the soil moisture and temperature interaction (t-value = 5.14, ***P <0.001; Table S2). The interactive correlations predicted higher EMM biomass under high temperatures and high soil moisture (top right corner of Fig. 2, attributed to spring months). By contrast, EMM biomass was predicted to decrease under high temperatures (an average temperature higher than 20°C) but low soil moisture (below 0.10 m^3 water m^3 soil^{-1} month^{-1}), mostly coinciding with worsening drought conditions during the summer (July–August) (top left corner of Fig. 2, attributed to summer months). Low temperatures during the winter months were also negatively correlated with EMM biomass (bottom right corner of Fig. 2).

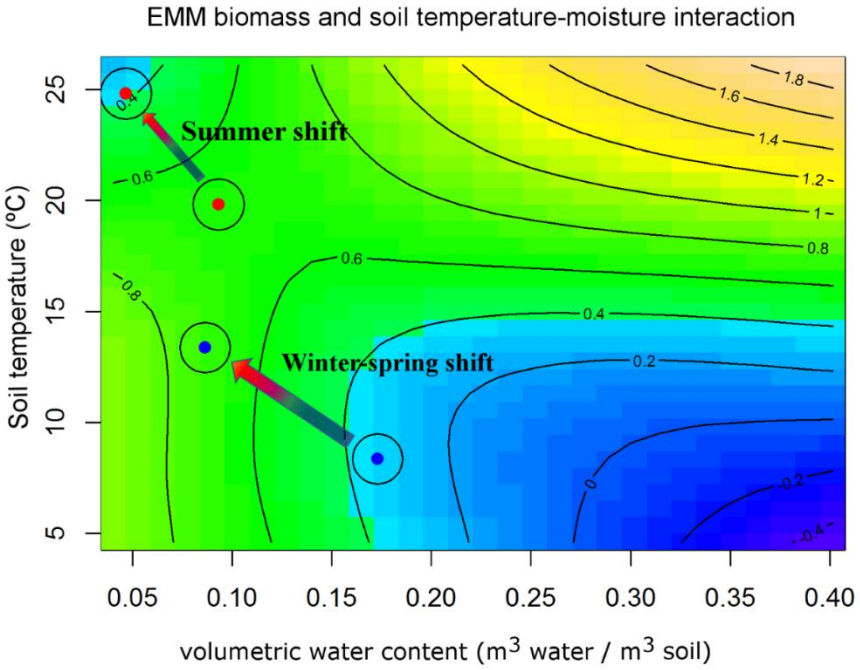


Fig. 2. Predicted EMM biomass and different temperatures and soil moisture using GAM models. Soil moisture (soil volumetric water content) is represented on the X-axis, whereas soil temperature (°C) is represented on the Y-axis. The predictor response (*L. vinosus* EMM biomass) is expressed in mg of *L. vinosus* g soil^{-1}. Colours indicate the gradient of EMM biomass: blue colours represent low EMM biomass values, whereas yellow to orange colours represent high EMM values. Althought only soil temperature and soil moisture is shown, the observed correlation between EMM biomass and soil temperature and moisture is most likely also influenced by the host tree response.

3.3 Estimation of fungal production and turnover

In total, the mean annual mycelial production for the 28 plots was estimated to be 2.4 mg g soil^{-1}. The monthly mean mycelial production was estimated to be 0.22 mg g soil^{-1}, representing 65 kg ha^{-1}. Mean fungal turnover in the plots was estimated to occur 7.0 ± 2.1 times year^{-1}. The estimated monthly turnover pattern was very similar to the seasonal fluctuation in fungal biomass observed over a year (Fig. 1a, 1b).

3.4 Sensibility of EMM biomass to changes in soil moisture and temperature

Based on a set of 11 serial models obtained from simulating increasing soil temperatures and decreasing moisture content, two significant changes in *L. vinosus* EMM biomass were observed within a year (Fig. 3). First, an increase in drought conditions during summer would result in decreasing EMM biomass (from June to September; reddish lines, Fig. 3). Thus, lower water availability together with higher temperatures would result in lower EMM biomass (Fig. 2; summer–autumn shift). Considering the greatest changes in soil temperature and soil

moisture (+5°C and –50%, respectively), our models predict a 34% decrease of EMM biomass during the summer (compared with the models assuming no change in soil moisture and temperature) from 122 to 80 kg of *L. vinosus* mycelium ha^{-1}.

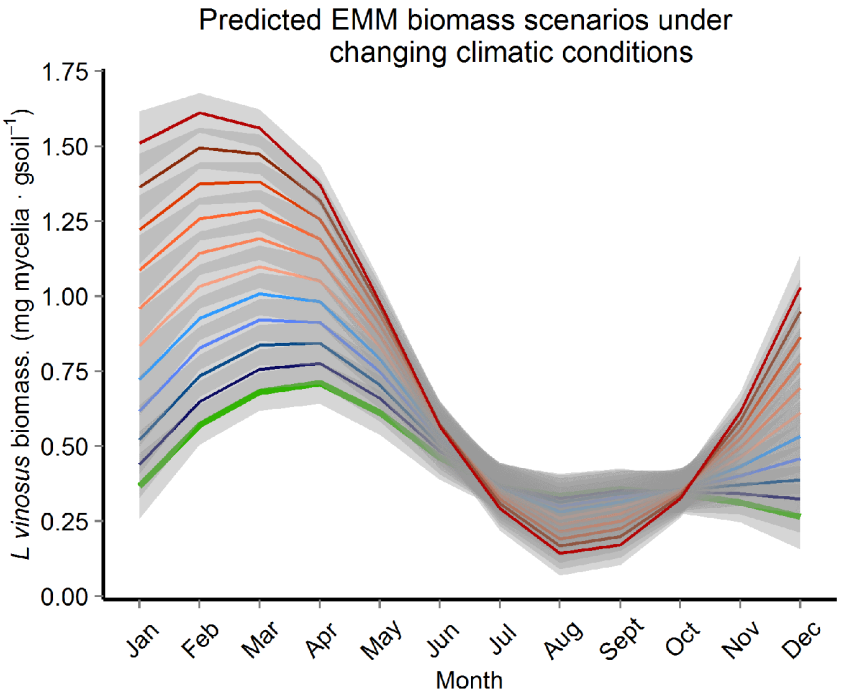


Fig. 3. Predicted *L. vinosus* soil biomass using serial increases in temperature and reductions in soil moisture. The green line represents the predicted *L. vinosus* biomass assuming no change, whereas the blue to red colours represent a gradient of changing climatic conditions, from minimal changes at +0.5°C and –5% volumetric soil water content (blue colours) to large changes at +5°C and –50% volumetric soil water content (reddish colours). Two main changes and shifts are observed under rising temperatures and decreasing soil moisture: first, the winter–spring EMM production would be enhanced earlier (from November to May) and the mycelial biomass would be higher. Second, the summer mycelial biomass would decrease during the summer months (July to September).

Second, there would be an increase in *L. vinosus* EMM biomass from winter to spring months (October to May, blue lines, Fig. 3). Thus, the development of EMM biomass could be favoured during winter months (Fig. 2; winter–spring shift), especially under increasing temperatures. During this period, the model considering the greatest changes in temperature and soil moisture (+5.0°C and –50%, respectively) predicted an increase of 97% in soil EMM biomass during spring–winter (compared with the models assuming no change in soil moisture and temperature) from 140 to 275 kg of *L. vinosus* mycelium ha^{-1}.

4. Discussion

In this study, we show that the soil EMM of *L. vinosus* is correlated with soil moisture

and temperature and that the direction of changes in soil EMM will be dependent on the season. Despite we have monitored the soil EMM of *L. vinosus* in detail from a temporal perspective, our data do not consider the hyphal dynamics of the entire fungal community, thus other species should be considered to fully understand the fungal dynamics of the whole community. Our results, despite focussed on a single species, suggest that soil moisture and temperature may better estimate fungal responses to climate than precipitation, since water availability of the fungi is likely dependant on soil type and soil biochemistry.

To date, most investigations of soil EMM biomass have been carried out in temperate and boreal ecosystems (Ekblad et al., 2013; Mohan et al., 2014) and, hence, there is a lack of data relating to Mediterranean forests. We estimate that the *L. vinosus* soil EMM biomass at our study sites fluctuates between 44 kg ha^{-1} and 261 kg ha^{-1}. Similarly, the EMM biomass values of *L. vinosus* obtained in this study ranged from 0.16 to 1.4 mg g $soil^{-1}$, which is in the range reported for *Lactarius deliciosus* in a study conducted in an area with a more continental climate than our study (De la Varga et al., 2013). In boreal ecosystems, it is estimated that the total ectomycorrhizal biomass in soils ranges from 700 kg ha^{-1} to 5,800 kg ha^{-1} (Wallander et al., 2004, 2001), with a mean production of approximately 160 kg ha^{-1} $year^{-1}$ (Ekblad et al., 2013). Our estimated mycelial turnover values are similar to other recent estimates (Ekblad et al., 2016; Hagenbo et al., 2016; Hendricks et al., 2016). Hagenbo et al. (2016) estimated turnover rates ranging from 0 to 8 times $year^{-1}$, depending on forest age. Our estimated turnover values (approximately 7 times $year^{-1}$) for a 60-year-old conifer forest are very similar to the observed values in younger boreal forests (Hagenbo et al., 2016). Differences in ecosystem type and climate from our study (warmer and drier) may explain these slight differences in turnover rates; thus it seems that a warmer climate may favour the turnover of mycelia. In addition, the turnover rates observed for *L. vinosus* are probably different to those of other species because these values are influenced by the quality of the fungal necromass, mycelial architecture, or other extrinsic factors such as the soil protection or saprotrophic community inhabiting the soil (Fernandez et al., 2016).

L. vinosus EMM biomass varied over the 12-month period, with a greater abundance during the spring and autumn seasons. This increasing abundance during the growing seasons has been observed in other studies (Söderström, 1979; Voříšková et al., 2013; Wallander et al., 2001). In contrast, little or no seasonal variation has been observed among the ectomycorrhizas found in *Q. ilex* trees (Queralt et al., 2017; de Román and de Miguel, 2005), except for specific taxa (Richard et al., 2011). A potential lower seasonal fluctuation in ectomycorrhizas compared with soil EMM suggests that soil EMM may be more sensible than ectomycorrhizas to changing climate conditions. For example, we observed low biomass during the summer, especially in

August, when the lowest soil volumetric water content was recorded together with high temperatures (8% of volumetric water content or soil moisture; Fig. S3). This decay of soil EMM biomass during the summer reflects a contrasting annual cycle of ectomycorrhizal species in Mediterranean ecosystems to those reported in temperate or boreal ecosystems, which show increasing ectomycorrhizal biomass during the summer (Nilsson et al., 2007; Voříšková et al., 2013).

We provide evidence that soil temperature and moisture are correlated with the annual cycle of *L. vinosus* EMM biomass in soils. These results show that local topsoil variations in environmental conditions could explain why EMM biomass and fungal communities are more dynamic in the topsoil layer than in the lower horizons (Andreetta et al., 2011; Voříšková et al., 2013). First, as deduced from the annual cycle, summer drought (low soil moisture and high temperatures) was negatively correlated with the EMM biomass of *L. vinosus*. Even though soil fungal biomass can be high all year in warmer climates (Sims et al., 2007), our study shows that low water availability and extremely high temperatures during the summer months in Mediterranean forest ecosystems could be a significant limiting factor for EMM production and survival. Our results support the low *Tuber magnatum* soil EMM found during summer months in Mediterranean systems (Iotti et al., 2014). Similarly, Queralt et al., (2017) reported positive correlation between precipitation and fungal mycelia of *Tuber melanosporum* in a Mediterranean *Q. ilex* plot. In contrast, Parladé et al., (2017) found that *Boletus edulis* soil EMM was negatively correlated with precipitation in a more humid and continental climate-type ecosystem, which highlights the importance of considering other species but also other ecosystems to predict fungal responses to climate.

We suggest that the decrease in fungal production and biomass of *L. vinosus* in our ecosystem is most likely due to the combination of: i) challenging soil conditions (temperature and water) for mycelial survival and growth and ii) decreasing tree activity and lower C allocation to the fungal symbiont under these conditions (Sardans and Peñuelas, 2013). These results also confirm that EMM biomass may be highly vulnerable to drought (McGuire et al., 2011; Staddon et al., 2003) but less vulnerable than fruiting body production (Agreda et al., 2016; Alday et al., 2017a; Bonet et al., 2012; Taye et al., 2016). The severity of drought and, hence, the impact on soil EMM biomass might also depend on whether the tree is able to obtain water from lower horizons through hydraulic lift (Cairney, 2012, Querejeta et al., 2009).

The soil EMM biomass of *L. vinosus* reacted relatively rapidly to moisture pulses at the end of summer (first precipitation events), with similar responses found for fruiting body communities (Agreda et al., 2016; Alday et al., 2017b). Thus, the EMM biomass increased during the same month as these precipitation events occurred (September–October). During this

season, soil moisture pulses can enhance soil fungal growth, supported by a shift in tree physiology status relocating more carbon belowground (Högberg et al., 2010). This fungal biomass growth enhancement after the summer drought also confirms previous studies reporting a rapid response in fungal growth after precipitation events in arid ecosystems (Vishnevetsky and Steinberger, 1997). Laboratory microcosm studies showed that growth rates of EM hyphae are variable depending on the species, ranging from 2 to 4 mm day^{-1} and can even achieve 8 mm day^{-1} (Ekblad et al., 2013), indicating that fungal biomass can grow at relatively high rates under optimum conditions.

In winter, EMM biomass was especially reduced, most likely because it coincided with low soil temperature conditions. Thus, the disappearance of EMM biomass was particularly pronounced at the end of November, when trees are most likely already inactive. This decay of EMM biomass over the course of a single month supports the view that EMM soil biomass turnover can be very rapid (Ekblad et al., 2016; Hendricks et al., 2016; Smith and Read, 2008). The sudden decrease in soil mycelia during November might be a consequence of carbon relocation for fruit body emergence or the growth of other saprotrophic species that feed on fungal mycelia. We have previously shown that incubating soil for one week at room temperature resulted in a large decrease in *L. vinosus* mycelia, which was associated with an increase of specific mould species (Castaño et al., 2016).

Our predictive models showed potential changes in the annual cycle of *L. vinosus* EMM biomass in response to changing climatic conditions. These models represent a first preliminary estimation of the direction of the effects on EMM biomass under changing climatic conditions; further research and additional years of data collection will be needed for accurate estimations. Based on these models, a theoretical framework for EMM biomass under changing climatic conditions is presented, with two important shifts discussed (Fig. 4). First, predicted mild winters and warmer early springs may result in greater EMM production during winter–spring months (Fig. 4; summer–autumn shift), which means that the biological activity of these soil organisms could be increased during early spring and during winter, as already observed and predicted for other organisms in a climate change context (Peñuelas et al., 2002). Second, predicted increases in temperature and decreases in precipitation during summer may result in a greater EMM biomass decrease as a result of worsening summer droughts (Fig. 4; winter–spring shift). As observed, a lack of moisture and high temperatures prevent fungal growth during this season, dramatically reducing the amount of surviving EMM biomass. Fungal mycelia growing under drought conditions may rely more on water transferred through hydraulic lift (Cairney, 2012). However, this transfer is not enough to maintain or preserve the spring EMM biomass throughout the predicted climatic conditions of future summer periods in

Mediterranean forest ecosystems. Finally, how the predicted EMM annual cycle shifts will affect tree productivity, tree resistance to drought, and fruiting body production and emergence remain open questions (Fig. 4).

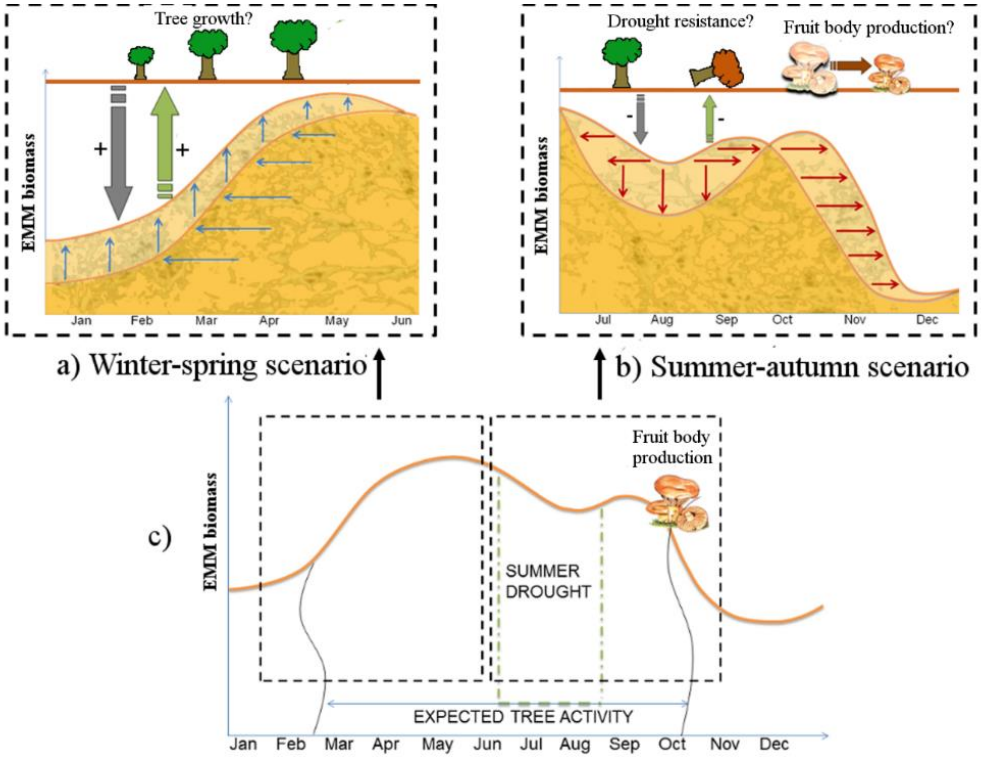


Fig. 4. Theoretical framework of the annual cycle of EMM biomass under a climate change context. (a) Trends in mycelial biomass are represented together with the observed averaged soil temperature and soil moisture values obtained in this study. Two important shifts are predicted to occur within a year (the winter–spring scenario and the summer–autumn scenario). Under higher temperatures predicted for winter, the winter–spring scenario (b) depicts increases in EMM production during the winter–spring months and a shift towards earlier increases in EMM production, which coincide with the lengthening of the biological activity of the host tree. Approximations of the observed values (dark-orange colour) are lower than the predicted values under changing climate conditions (light-orange colour). Under lower soil moisture and higher temperatures, the summer–autumn scenario (c) predicts a decrease in EMM biomass during summer and the lengthening of this decay. Approximations of the observed values (dark-orange colour) are higher than the predicted values under changing climate conditions (light-orange colour). EMM biomass is shown on the Y-axis and months are shown on the X-axis. The role of host is emphasized in this figure and most likely very important in explaining the changes in soil EMM observed.

5. Conclusions

Our results indicate that the annual EMM cycle of *L. vinosus* is characterized by two peaks, occurring during the autumn and spring months. During summer, the EMM biomass of *L. vinosus* remains stable or decreases slightly, especially during August, and dramatically

decreases during late autumn. Soil moisture and soil temperature were significantly correlated with EMM biomass; high EMM *L. vinosus* biomass is observed under high temperatures and high moisture, but low EMM biomass is observed with high temperatures if soil moisture is low. In parallel, our preliminary models simulating changing climatic conditions predict a lengthening of the biological activity of fungal biomass during winter–spring months, but a more intense decrease during summer months.

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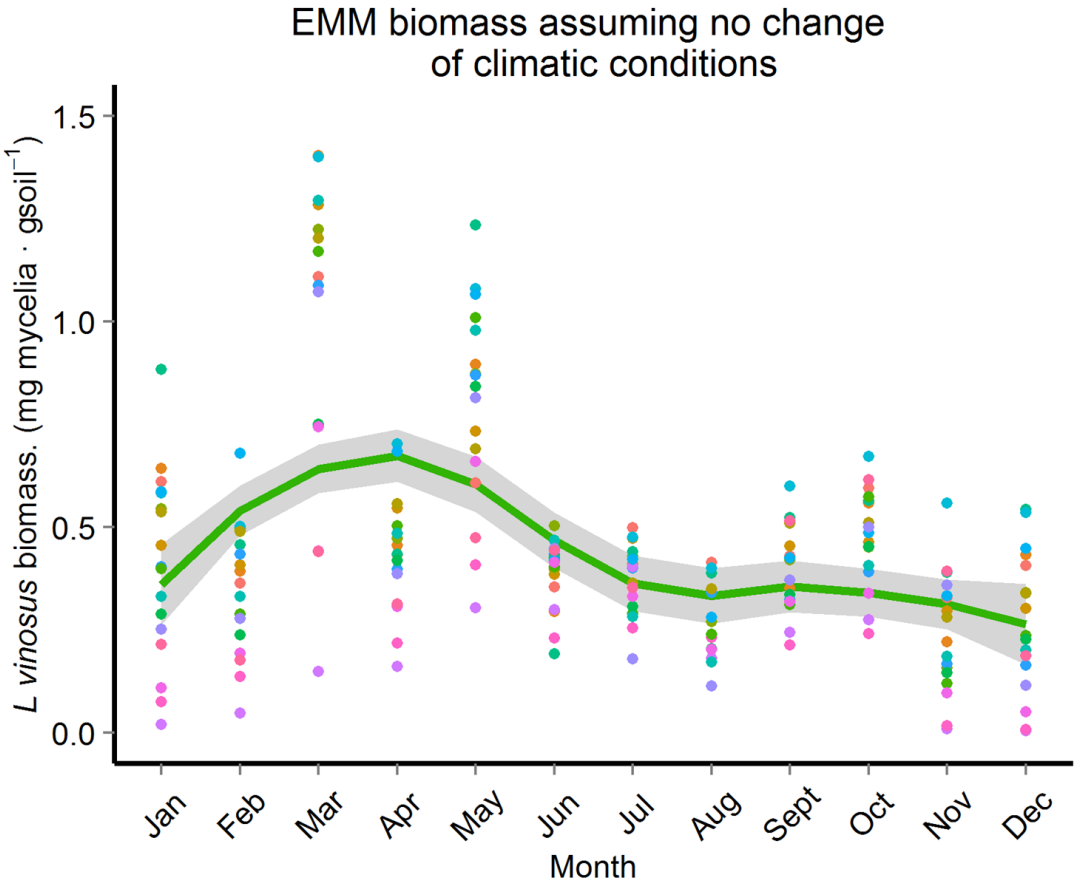
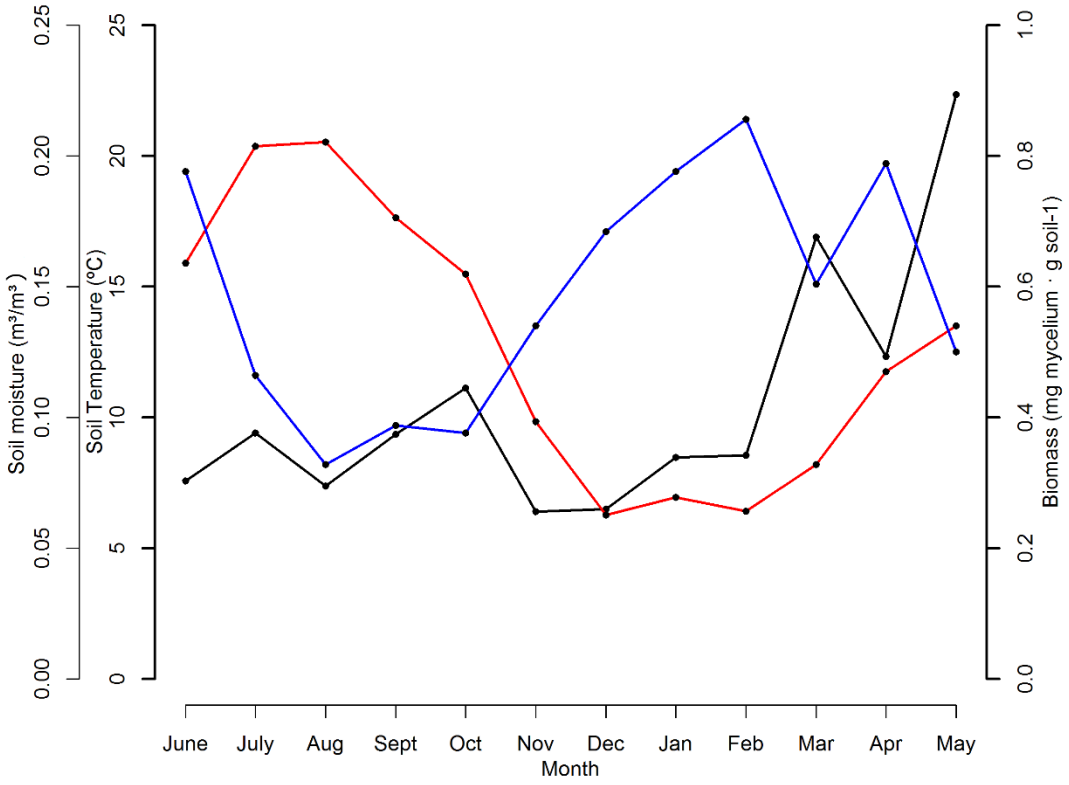
Supplementary material



Supplementary Figure 1. Predicted biomass of *L. vinosus* for each of the plots considered in this study (coloured dots), assuming no change in climatic conditions. The green line represents the smoothed modelled biomass for each month.



Supplementary Figure 2. Averaged soil moisture (Blue line) and soil temperature (Red line) recorded in each of the 28 plots considered in this study. Black line represents the predicted averaged *L. vinosus* soil biomass values predicted by the model used in this study.

Supplementary Table 1. Main sampling plot characteristics. OM: Organic matter (%). N: Nitrogen (%). BA: Basal area (m^2). N: Number of tress · ha^{-1}.

Plot	OM	N	pH	Altitude	Aspect	BA	Slope	Nactual
301	6.67	0.29	6.8	1010	110	78.1	19	2451
302	5.17	0.22	6.6	1013	135	81.7	22	2552
303	5.9	0.25	6.8	903	20	49.8	19	1169
304	10.53	0.31	6.6	879	360	35.3	22	717
305	3.61	0.16	6.8	744	90	61.1	18	2657
306	4.13	0.18	6.7	759	40	59.8	23	1808
307	6.36	0.31	7.3	796	60	36.1	18	1149
308	3.91	0.22	6.4	835	65	32.4	15	776
309	4.49	0.22	7	852	20	31.5	13	861
311	3.91	0.17	6.7	594	360	21.1	3	621
312	4.08	0.13	6.6	633	10	31.1	23	1123
313	4.34	0.14	6.9	609	340	42.7	5	957
314	5.23	0.16	6	612	10	30	8	446
315	3.69	0.17	6.1	626	260	39.8	23	835
316	7.04	0.25	6.5	644	30	34	3	688
301C	9.48	0.31	6.9	1010	110	28.1	19	732
302C	8.07	0.29	6.9	1013	135	39.8	22	1273
303C	7.54	0.24	6.8	903	20	32.5	19	764
304C	7.79	0.26	6.9	879	360	26.6	22	573
305C	3.97	0.13	7	744	90	41.7	18	1528
306C	5.06	0.16	6.8	759	40	25.5	23	700
307C	5.79	0.25	6.7	796	60	21.2	18	668
308C	3.03	0.2	6.1	835	65	21.4	15	668
309C	5.31	0.21	6.9	852	20	16.5	13	350
312C	2.98	0.11	6.5	633	10	36.1	23	1146
313C	4.1	0.16	6.8	609	340	29.3	5	668
315C	5.56	0.19	6.5	626	260	47.2	23	1114
316C	2.95	0.15	6.8	644	30	31.4	3	509

Supplementary Table 2. Contrast test showing statistically different monthly values of EMM biomass, obtained at the LME models and analyzed by Tukey (α=0.05).

Season	Constrats	Estimate±SE	z value	Pr(>|z|)
Summer	August -October	0.27±0.08	3.15	0.072
Summer	August - March	0.32±0.09	3.46	0.026
Summer	August - May	0.36±0.09	4.07	<0.01
Early Autumn	September - November	-0.32±0.08	-3.81	<0.01
Early Autumn	October - November	-0.34±0.08	-4.05	<0.01
Late Autumn	November - January	0.29±0.08	3.45	0.027
Late Autumn	November - March	0.40±0.09	4.29	<0.01
Late Autumn	November – May	0.44±0.09	4.94	<0.01
Early Winter	December – May	0.31±0.09	3.47	0.026
Winter	February - May	0.30±0.09	3.34	0.039

Supplementary Table 3. Statistical significance of the fixed factors considered at the GAMM model. Month and climatic parameters were fixed factors, whereas plot was defined as a random factor.

	edF	F	p-value
te(soil_H,soil_T)	6.405	7.150	<0.001
s(Month)	5.115	5.548	<0.001

CHAPTER III

Temporal and spatial changes in soil fungal communities across moisture and temperature gradients in a Mediterranean pine forest

Under review in New Phytologist: Second revision

Temporal and spatial changes in soil fungal communities across moisture and temperature gradients in a Mediterranean pine forest

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Summary

- Soil microclimate, a potential regulator of local fungal community composition, is likely to be particularly important in climates with significant drought periods. Here, we investigated spatio-temporal dynamics of soil fungal communities in Mediterranean *Pinus pinaster* forest across gradients in soil moisture and temperature.
- Fungal communities in 336 soil samples collected monthly during a year from 28 long-term experimental plots were assessed by PacBio sequencing of ITS2 amplicons. Total fungal biomass was estimated by analysing ergosterol. Fungal community changes were analysed in the context of functional traits.
- Soil fungal biomass was lowest during summer and late winter and highest during autumn, concurrent with a greater relative abundance of mycorrhizal species. Intra-annual spatio-temporal changes in community composition correlated significantly with soil moisture and temperature. Mycorrhizal fungi were less affected than free-living fungi by summer droughts. In particular, mycorrhizal species with mycelial growth concentrated tightly around the roots increased in relative abundance under dry conditions, whereas species with more extensive mycelia were favoured by wetter conditions.

- Our observations demonstrate a potential for compositional and functional shifts in fungal communities in response to changing climatic conditions. Free-living fungi and mycorrhizal species with extensive mycelia may be negatively affected by increasing drought periods in Mediterranean forest ecosystems.

Keywords: climate, drought, ergosterol, fungal biomass, fungal community, mycorrhizal.

Introduction

Soil fungi are essential drivers of organic matter dynamics and of nutrient release and uptake in coniferous forest ecosystems (Clemmensen *et al.*, 2013; 2015; Averill *et al.*, 2014). However, climate changes such as warming or increased drought may alter the composition of soil fungal communities (Fernandez *et al.*, 2016; Solly *et al.*, 2017; Hartmann *et al.*, 2017). Mediterranean ecosystems are among the most sensitive to climate changes, with predicted negative effects on biodiversity, biomass and associated ecosystem services (Thuiller *et al.*, 2008). In these ecosystems, drought stress and low nutrient availability are important determinants of functional and structural traits of plants (e.g. sclerophylly and low growth rate; Sardans & Peñuelas, 2013). However, expected increases in temperature and the unprecedented duration and intensity of drought events could exceed the tolerance of plant communities (Collins *et al.*, 2013). Ectomycorrhizal (ECM) fungi are key players in the alleviation of drought stress for host trees, and increasingly so as the frequency of drought events increases (Mohan *et al.*, 2014). Thus, mycorrhizal fungi may contribute to plant water acquisition, both directly by increasing access to soil water (Allen, 2007), and indirectly by providing their host plants with nitrogen and phosphorus (Smith & Read, 2008) and improving soil structure and porosity through the formation and stabilisation of soil aggregates and organic matter (Querejeta, 2017). This relationship may also be reciprocal given that the plant host may also improve water access of its associated mycorrhizal fungi through hydraulic lift, especially during summer (Querejeta *et al*., 2003; Unestam & Sun, 1995; Querejeta, 2017).

Generally, soil fungal communities are affected by soil chemistry, such as nitrogen (N) forms and availability (Lilleskov *et al.*, 2002; Allison *et al.,* 2007; Sterkenburg *et al.,* 2015) and pH (Rincón *et al.*, 2015; Romanowicz *et al.*, 2016), as well as by stand age (Bonet *et al.,* 2004), forest management practices (Kyaschenko *et al.*, 2017a), fire (Taudière *et al.*, 2017), tree genotype (Pérez-Izquierdo *et al.,* 2017), global atmospheric climate patterns (Tedersoo *et al.*, 2014) and local alterations in soil temperature (Solly *et al.*, 2017). In addition, several studies have shown that changes in climate conditions may

directly or indirectly affect soil fungal communities (Hartmann *et al.,* 2017; Fernandez *et al.,* 2016; Solly *et al.,* 2017). Among these fungal communities, mycorrhizal species have shown contrasting responses to changes in climate, likely because these responses are also modulated by nutrient availability (Clemmensen *et al.*, 2006; Solly *et al.*, 2017) and by differential host tree responses (Fernandez *et al.*, 2016; Hartmann et al., 2017). Overall, warming has generally been observed to increase mycorrhizal biomass in boreal or arctic ecosystems (Clemmensen *et al.*, 2006; Mohan *et al.*, 2014). By contrast, recent studies of Mediterranean ecosystems have suggested that the biomass of some mycorrhizal species is dynamic and that mycelial production is often arrested during summer and winter months (Iotti *et al.*, 2014; Castaño *et al.*, 2017; Queralt *et al.*, 2017). For example, by simulating future increases in summer drought in Mediterranean areas, we have recently predicted sharp decreases in *Lactarius vinosus* mycelial biomass during summer but increases during the winter–spring months (Castaño *et al.*, 2017). In temperate or boreal ecosystems, ectomycorrhizal fungi have been found to increase in relative abundance during summer or autumn (Wallander *et al.*, 2001; Jumpponen *et al.*, 2010; Voříšková *et al.*, 2014; Santalahti *et al.*, 2016), probably due to a higher below-ground allocation of host sugars during the growth season (Högberg *et al.*, 2010; Žifčáková et al., 2017). However, there is a lack of information relating to intra-annual patterns in fungal community composition and biomass and the effects of climate drivers in Mediterranean forests soils.

In addition to mycorrhizal species, other soil fungal species such as litter saprotrophs and moulds may be affected by changes in climatic conditions and exhibit seasonal changes in community composition (Jumpponen *et al.*, 2010; Andreetta *et al.*, 2011; Voříšková *et al.*, 2014; Santalahti *et al.*, 2016). For instance, literature addressing seasonality in fungi suggests a general pattern with saprotrophs and opportunistic moulds increasing under colder conditions (Jumpponen *et al.*, 2010; Voříšková *et al.*, 2014; Santalahti *et al.*, 2016). In addition to the influence of microclimatic parameters, these species may also be indirectly influenced by changes in soil properties or ground cover (Vašutová *et al.*, 2016). Climate may also select for specific groups of fungal traits (Fernandez *et al.*, 2016; Treseder & Lennon, 2015). Among these traits, ectomycorrhizal (ECM) mycelial exploration types (i.e. long exploration, short exploration, contact or mat formation) have been proposed to represent several important fungal traits that affect water and nutrient acquisition (Agerer, 2001, 2006). It has been proposed that species with short- or contact-exploration types, i.e. with mycelial biomass largely concentrated

around the roots and their immediate surroundings (Agerer, 2001, 2006; Deslippe *et al.*, 2011; Fernandez *et al.*, 2016), might impose a lower carbon cost on the host plant, which could be advantageous for the host plant under stressful conditions (Fernandez *et al.*, 2016). By contrast, species with more extensive mycelium (i.e. medium–long distance exploration) may be more demanding in terms of host C (Agerer, 2001; Lehto & Zwiazek, 2011; Fernandez et al., 2016). The limited water availability in Mediterranean forests often results in a reduction of tree growth or even tree dormancy during summer (Sardans & Peñuelas, 2013), which likely has a negative effect on mycorrhizal fungi (Shi *et al.,* 2002). Even though both direct climate effects and indirect and host tree effects may select for specific fungal communities, this question has not been addressed from an intra-annual perspective in drier forest ecosystems.

Here we studied monthly changes in soil fungal community composition by performing high-throughput sequencing of amplified fungal markers (Lindahl *et al.*, 2013) across 28 long-term experimental plots over the course of a year. Our main objective was to determine whether temporal fluctuations in fungal community composition were correlated with intra-annual changes in soil moisture and temperature. We also investigated micro-climatic effects on soil fungal communities by studying spatial variation across the whole set of plots. The results were interpreted in the context of fungal functional guilds and traits. Responses of the total fungal biomass were also assessed by analysing the fungus-specific biochemical marker ergosterol (Wallander *et al.*, 2013). Due to the pronounced summer drought typically occurring in Mediterranean areas, often resulting in decreased tree activity, we hypothesised that (i) total fungal biomass in soils will be lower during drier conditions. However, mycorrhizal fungi may be more resistant to drought because they may use water provided by their host tree. Thus we expected (ii) an increase in the relative proportions of mycorrhizal species during drier conditions and (iii) that free-living fungi would increase their relative proportions under cooler and wetter conditions. Finally, we hypothesised (iv) that responses to climate across mycorrhizal species would be related to exploration types, with drier conditions favouring species with mycelial biomass largely focused around roots and their immediate surroundings, whereas higher soil moisture would favour species with more extensive mycelial proliferation away from the roots.

Materials and Methods

Site selection

The study site was located in the Natural Park of Poblet (Northeast Spain, 41° 21' 6.4728'' E, 1° 2' 25.7496'' N), where 28 previously established 10 × 10 m long-term monitoring plots were selected for the study. Plots consisted of even-aged (60-years-old) reforested *Pinus pinaster* (Aiton) forest, with isolated *Quercus ilex* (L.) trees, sometimes forming scrub, together with other understory plant species, mostly *Erica arborea* (L.), *Arbutus unedo* (L.) and *Calluna vulgaris* (L.) Hull. The plots were distributed across a range of different altitudes (from 594 to 1013 m above sea level) and slopes (3–23%). Variation in microclimatic conditions across plots did not follow a clear altitudinal pattern (Supporting Information Fig. S1), but depended on a combination of aspect, slope, altitude and stand density. The soils in these plots are characterised by siliceous minerals with franc-sandy textures, pHs ranging from 6.1 to 6.6, and organic matter contents in the upper 12 cm ranging from 3.0% to 10.5%. The mean annual temperature is 11.8°C and the mean annual rainfall is 667 mm, with summer droughts usually lasting for three months (July–August–September). During the study period, the summer drought lasted three months, with 29 mm of precipitation recorded between July and September, whereas 210 mm of precipitation were recorded in November alone. The average temperature during the study period was 12.3°C and the total precipitation was 655.9 mm.

Soil sampling

All 28 plots were sampled monthly from June 2013 until May 2014. Each month, eight soil cores (12 cm deep and 5 cm in diameter) were collected systematically in each plot (two cores were extracted from each 10-m-side of the plot). We focused primarily on mycorrhizal fungi, and given that the fungal community composition in the needle material diverges from that of the soil (Lindahl *et al.*, 2007), we discarded intact and partially decomposed needles and sampled well-decomposed organic layers and mineral soil. Soil cores were stored at 4°C for <24 h and then sieved through a 3-mm mesh. Sieved soil samples were freeze-dried and pooled to obtain a composite soil sample for each plot and month, totalling 336 samples. Composite soil samples were ground to a fine powder using a mortar and pestle.

Ergosterol analyses

The total fungal biomass present in the soil samples was estimated by quantifying the fungal-specific biomarker ergosterol. Ergosterol was extracted as described by Nylund & Wallander (1992) and chromatographically analysed as described by Hagenbo

et al. (2017). Ergosterol data were converted to fungal biomass using a conversion factor of 3 µg ergosterol mg^{-1} dry matter (Salmanowicz & Nylund, 1988) and a correction factor (1.62) was applied to compensate for unextracted ergosterol (Montgomery *et al.*, 2000).

Fungal community analysis

Genomic fungal DNA was extracted from 500 mg aliquots of each soil sample using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol, but with 900 µl of lysis buffer.

Each subsample was subjected to PCR-amplification of 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) and ITS4 (White *et al.*, 1990), both of which were fitted with unique 8-bp tags, differing in at least three positions. The number of PCR cycles was optimised for individual samples, with most of the samples amplifying well at 21–24 cycles. The final concentrations in the 50-µl PCR reaction mixtures were: 25 ng template, 200 µM of each nucleotide, 2.75 mM $MgCl_2$, primers at 200 nM and 0.025 U μl^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA) in 1X buffer. PCR cycling conditions were as follows: 5 min at 95°C, followed by 24–30 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 before storage at 4°C. Samples were amplified in triplicates with negative extraction and PCR controls included. PCR products were purified using the 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 further purified using the EZNA Cycle Pure kit (Omega Bio-Tek). Quality control of purified amplicons was carried out using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and a 7500 DNA chip. Samples were sequenced at SciLifeLab NGI, Uppsala, Sweden on a PacBio RS II system (Pacific Biosciences, Menlo park, CA, USA) using 28 SMRT cells.

Quality control and bioinformatic analysis

Sequences were quality filtered and clustered using the SCATA pipeline (https://scata.mykopat.slu.se/). Sequences with an average quality score of <20, individual bases with a quality score of <10 or a length of <200 bp were removed, after which remaining sequences were screened both for primers (requiring 90% primer match) and sample tags. After the collapse of homopolymers to 3 bp, sequences were pair-wise

compared using 'usearch' (Edgar, 2011). Pairwise alignments were scored using a mismatch penalty of 1, gap open penalty of 0 and a gap extension penalty of 1. 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. Sequence data are archived at NCBI's Sequence Read Archive under accession number PRJNA309233 (www.ncbi.nlm.nih.gov/sra).

Taxonomic and functional identification

We assigned putative taxonomy to the 550 most abundant Species Hypothesis (SHs), which represented 93% of the total sequences (Table S1). We selected the most abundant sequence from each operational taxonomic unit for taxonomic identification, using the massBLASTer in PlutoF against the UNITE (Abarenkov *et al.,* 2010) and INSD databases. Taxonomic identities were assigned based on > 98.5% similarity with database references, or based on supported monophyletic neighbour-joining clades that included reference sequences (Supporting Information Fig. S2). Species hypotheses were assigned to the following functional guilds: a) ectomycorrhizal, b) moulds, c) yeasts, d) black yeasts, e) litter saprotrophs, f) soil saprotrophs (saprotrophic taxa commonly found in N-rich mineral soils), g) pathogens, h) moss-associated fungi, i) root-associated ascomycetes, and j) unknown function, based on the UNITE database and DEEMY (www.deemy.de) or other published literature. Assignment of ectomycorrhizal fungi to exploration types was based on DEEMY and other published literature.

Climate data

Volumetric soil water content and soil temperature were measured using Decagon 5 TM probes (Decagon devices Inc., Pullman, WA, USA) during the entire sampling period. Soil sensors were placed in the middle of each of the 28 plots, buried 10 cm below ground. All climate measurements were recorded every minute and averaged across 2-h intervals on a data logger (EM50, Decagon Devices Inc., Pullman, WA, USA). Data were downloaded and processed using the DATATRAC® III software (Pullman, WA, USA) and aggregated as monthly averages, minima and maxima.

Data analysis

Fungal community data were subjected to analyses using CANOCO version 5.0 (Biometris Plant Research International, Wageningen, Netherlands) for ordinations and the 'nlme' R package for linear mixed effect models (LME; R version 3.0.2, R

Development Core Team 2015).

Differences in soil fungal biomass were analysed using LME models after square-root transformation. Two different independent analyses were carried out to test temporal and spatial relationships between fungal biomass and soil conditions. To analyse temporal variation in fungal biomass in relation to soil moisture and temperature, as well as their interaction, 'plot identity' was considered a random factor, whereas 'month' was defined as a fixed factor together with the climatic variables. To analyse spatial variation in fungal biomass in relation to soil temperature and moisture, as well as their interactions, 'month' was considered a random factor, whereas 'plot identity' was defined as a fixed factor together with the climatic variables. In addition, models were tested with and without accounting for 1-week temporal autocorrelation among observations (AR1), and the most parsimonious model was selected based on the Akaike Information Criterion (AIC).

SH abundance data were Hellinger transformed before all multivariate analyses. Detrended correspondence analysis (DCA) was used to obtain graphical representations of fungal community similarity between different plots and months. Temporal variation in fungal community composition was related to soil moisture and temperature by performing canonical correspondence analysis (CCA). Here, 'plot identity' was defined as a covariate, and months were randomly permuted using Monte Carlo permutation tests (999 permutations), without permutation between spatial replicates within single months (i.e. effective N = 12) treating 'month' as a repeated measure. Three independent tests were performed, considering: (i) SH level community composition, (ii) relative abundances of functional guilds and (iii) relative abundances of exploration types within the ECM community. Changes in the relative abundance of individual functional guilds and exploration types in response to monthly changes of soil temperature and soil moisture were assessed *post-hoc*, using LME models of square-root transformed relative proportions, in which 'plot' identity was defined as a random factor and soil temperature and moisture were defined as fixed terms.

CCA was also used to relate spatial variation in fungal community composition to soil moisture and temperature. The significance of the explanatory variables was established using Monte Carlo permutation tests (9999 permutations under the full model), without permutation of repeated observations from single plots (i.e. effective N = 28), and forward selection of explanatory variables. The same analysis was carried out using the relative proportion data of each functional guild and ECM exploration type. These effects were analysed *post-hoc* using LME models of square-root transformed data,

with ‘month’ defined as a random factor and soil temperature and moisture as fixed terms.

Results

The average soil fungal biomass (dry matter), as based on ergosterol measurements, was 4.9 ± 0.1 mg g soil^{-1} (145 g m^2 or 1,45 kg ha^{-1}) but varied significantly between months (F = 5.04, P < 0.001). Fungal biomass was lowest during the summer months, especially in August (109 g m^2), but increased during late summer–autumn to a maximum in October (171 g m^2). Biomass then decreased again progressively during the winter months to another minimum in February (118 g m^2) and increased again during the spring (Fig. 1a). Fungal biomass was significantly related to the interaction between soil temperature and moisture (F = 5.28, P = 0.022) (Fig. 1b), with mycelial biomass having a negative correlation with moisture under cold conditions, a positive correlation with temperature under wet conditions, and a negative correlation with temperature under dry conditions (Fig. 1b).

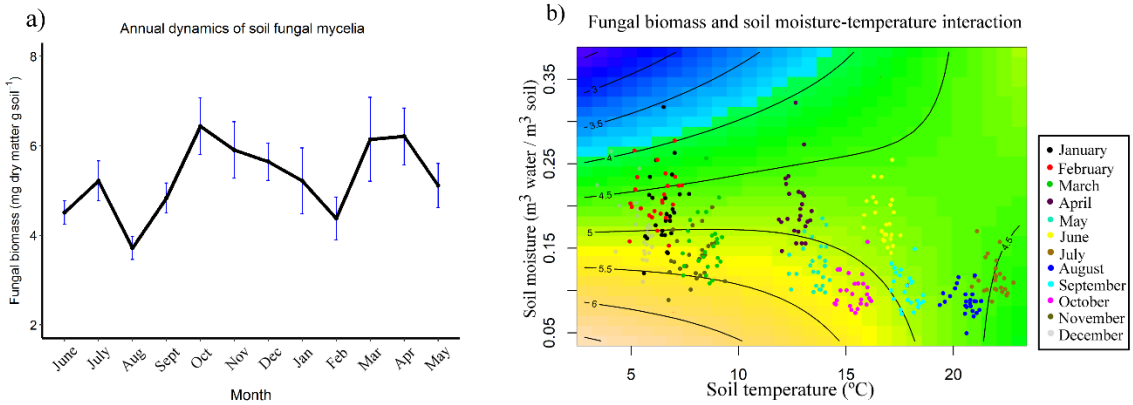

Fig. 1 (a) Estimated soil fungal biomass production (mg mycelia g soil^{-1} month^{-1}) over a year, based on ergosterol analysis, in a Mediterranean *Pinus pinaster* forest, and (b) correlations between soil fungal biomass and volumetric soil water content (m^3 water m^3 soil^{-1}). Mean values ± 1 SE are shown in (a). Background colours in (b) indicate soil fungal biomass (mg mycelia g soil^{-1}): yellow background colours represent high levels of fungal biomass, whereas blue background colours represent lower levels of fungal biomass. Coloured dots represent data points for each month.

A total of 408,788 out of 791,099 sequences (52%) passed quality filtering. Single-linkage clustering resulted in 3,063 SHs, of which 550 (93% of the high-quality sequences) were assessed for identification to species level, functional guild and exploration strategy (for ectomycorrhizal fungi). Overall, basidiomycota dominated the

fungal community (56 ± 1% of the identified sequences), followed by ascomycota (30.1 ± 0.6%). Regarding functional guilds, mycorrhizal species were by far the most abundant (53 ± 5%), followed by moulds (7.2 ± 0.6%) and other functional groups such as yeasts or saprotrophs (12.2%). Taxa with unknown or non-determined function accounted for 27.4 ± 0.6% of the reads. The three most abundant mycorrhizal genera were *Inocybe* spp. (30.2 ± 0.6% of the mycorrhizal sequences), *Russula* spp. (23.3 ± 0.8%) and *Tricholoma* spp. (7.9 ± 0.4%). Among ECM exploration strategies, the short exploration type was most abundant (35.9 ± 0.3% of the mycorrhizal sequences), followed by the contact type (22.1 ± 0.5%) and the medium-distance fringe type (20.4 ± 0.5%).

Fungal species composition varied systematically across months according to soil moisture and temperature (total monthly inertia = 0.29, adjusted explained variation = 4.4%, Fig. 2a). The largest temporal differences in fungal community composition were observed between the cold and moist months (positive first-axis values, corresponding to winter months; Fig 2a) and the warm and dry months (negative first-axis values, corresponding to summer months; Fig. 2a).

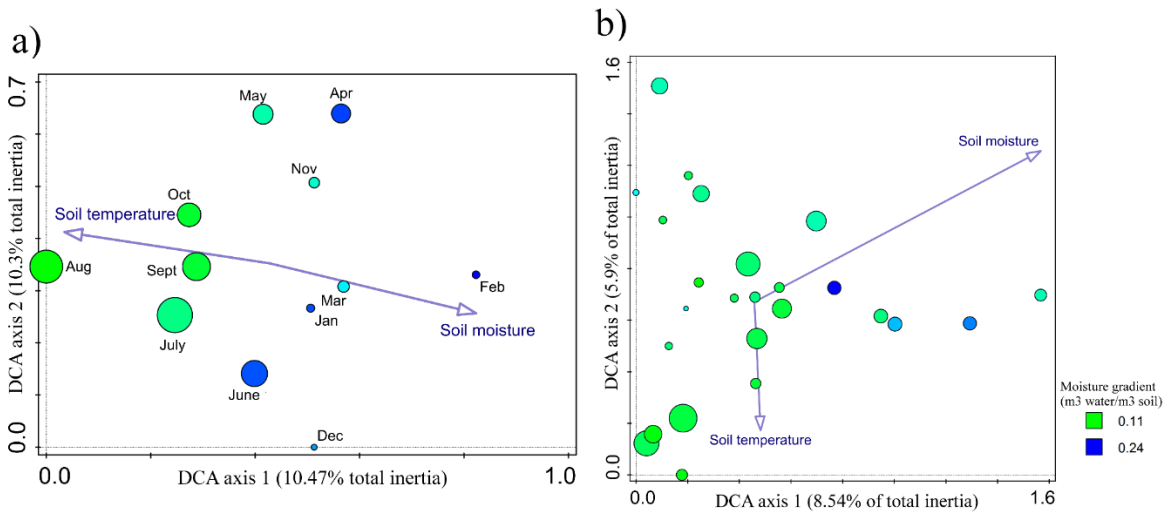

Fig. 2 Detrended correspondence analyses (DCA) of the species level community composition of soil fungi in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons. The figures illustrate the variation in community composition across months (a) and across plots (b). The shift from green to blue colours represents the shift in gradient from low to high soil moisture. The increasing size of the circles represents the increasing soil temperature. Soil moisture and temperature are shown as supplementary variables.

Forward selection indicated that soil moisture was more important in terms of explaining temporal variations in community composition (59.7% of fitted variation, P = 0.002) than soil temperature (40.3%, P = 0.001). Relative abundances of certain species,

especially moulds (e.g. *Mortierella* and *Umbelopsis*) and yeasts (e.g. *Cryptococcus*), were higher during wetter and colder months (positive first-axis values in Fig. 3), whereas other species, especially mycorrhizal taxa (e.g. *Russula* and *Inocybe*), were relatively more abundant during drier and warmer months (negative first-axis values in Fig. 3).

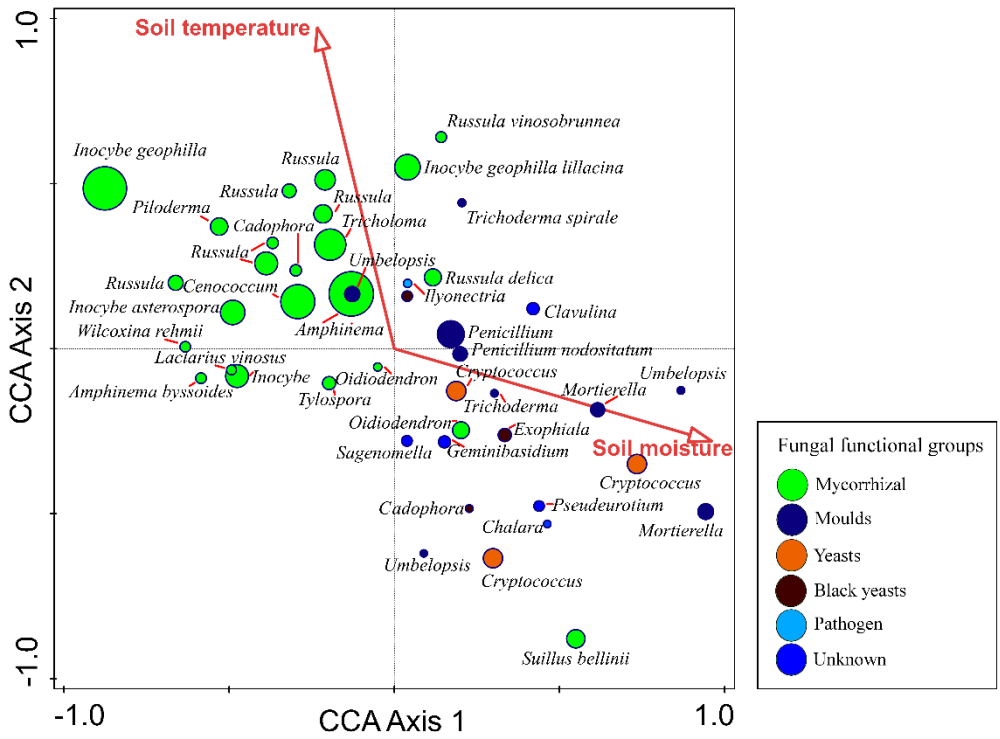

Fig. 3 Canonical correspondence analysis (CCA) species plot showing the variation in the community composition of soil fungi in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons related to temporal changes in soil temperature and moisture. Species symbols are coloured according to functional guilds, and symbol sizes are proportional to the average relative abundance. The figure only shows the 45 most abundant species hypotheses.

Accordingly, many functional guilds correlated with soil temperature and moisture across months (pseudo-$F = 9.7$, $P < 0.001$), accounting for 6.7 % of the total inertia (Table 1, Fig. 4a). Forward selection of the explanatory variables identified that soil moisture was more important (85% of fitted variation) than soil temperature (15% of fitted variation) in terms of explaining changes in guild composition across months.

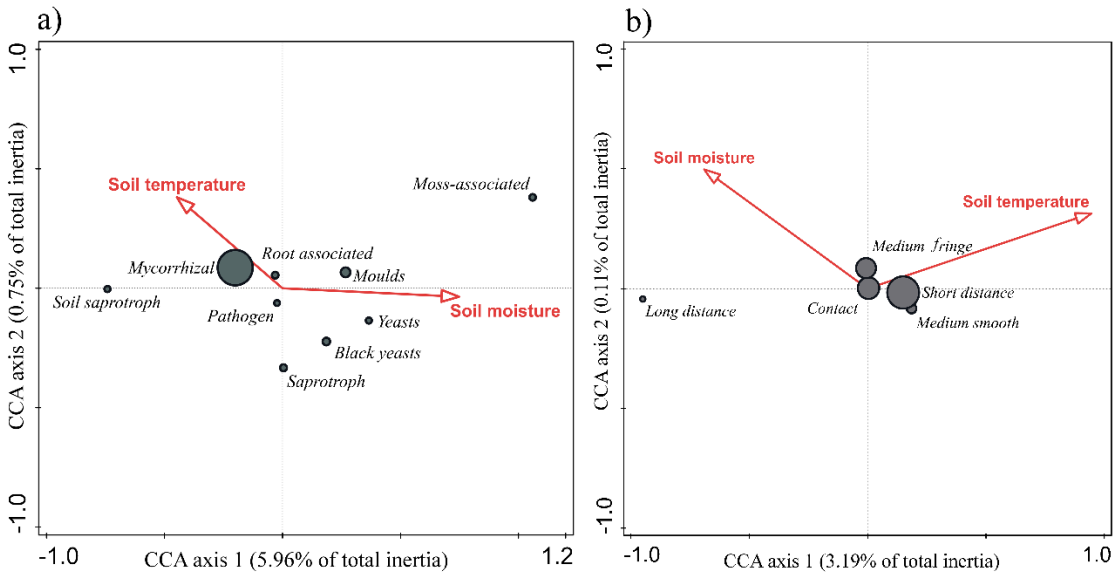

Fig. 4 Canonical correspondence analysis (CCA) plots of relative proportions of (a) functional guilds of soil fungi and (b) exploration types of ectomycorrhizal fungi in relation to temporal variation in soil temperature and moisture in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons.

Specifically, the overall proportion of amplicons attributed to mycorrhizal species correlated negatively with soil moisture across months (temporal effects in Table 1), with a significant decrease from 75% in the driest month (August) to 58% in the wettest month (February) (Table 1, Fig. 5a). By contrast, the proportion of amplicons attributed to yeasts, black yeasts, moulds and moss-associated fungi correlated positively with soil moisture across months (temporal effects in Table 1). The relative proportions of moulds increased from 6% during the summer months to *c*. 15% during the wetter winter months (Fig. 5b), whereas moss-associated fungi almost disappeared during the summer months, but accounted for more than 3% of the amplicons during spring (Fig. 5c). The overall proportion of amplicons attributed to black yeasts and litter saprotrophs correlated negatively with soil temperature (Fig. 4a).

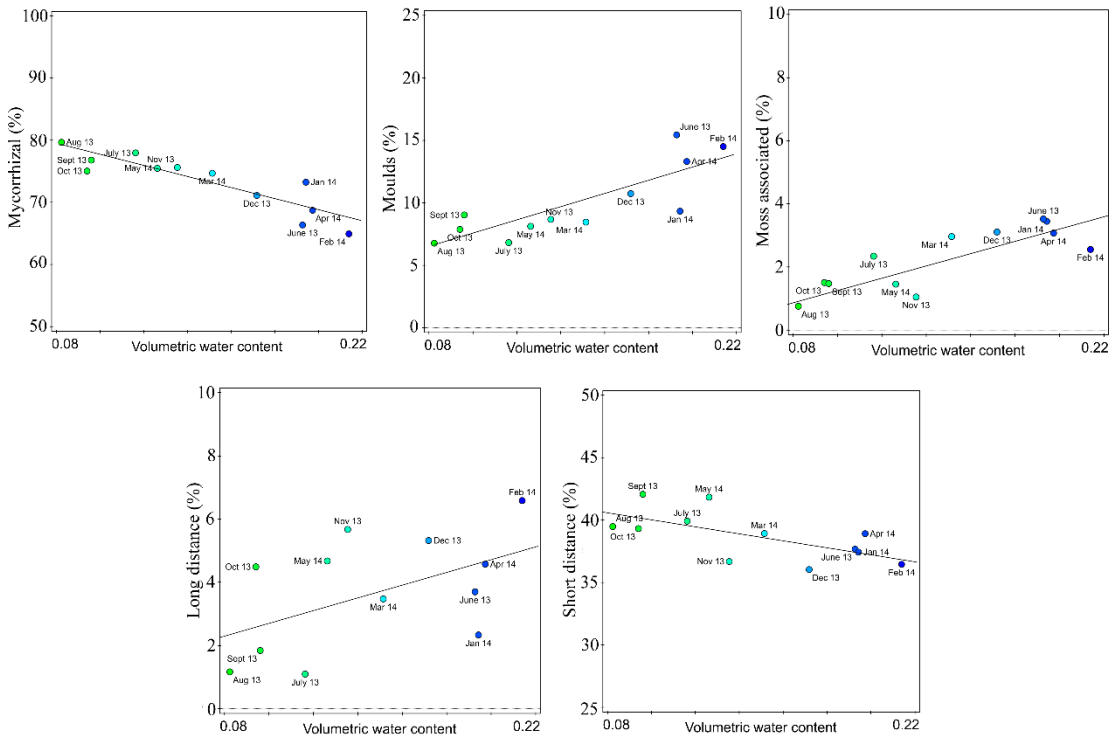

Fig. 5 Mean relative abundances of relevant and significantly affected functional guilds of soil fungi and exploration types of ectomycorrhizal fungi in relation to soil moisture content in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons. Data points represent monthly averages across 28 plots.

Exploration types of mycorrhizal species also correlated significantly with soil temperature and moisture across months (pseudo-$F = 4.7$, $P = 0.004$, Fig. 4b), accounting for 3.3% of the total inertia (Table 1, Fig. 4b). Forward selection of explanatory variables indicated that soil temperature (87% of fitted variation) was more important than soil moisture (13% of fitted variation) in terms of explaining the temporal variation in exploration types. These effects were particularly driven by species belonging to the long-distance exploration type, which were more abundant during wetter and colder months (temporal effects in Table 1, Fig. 5d), whereas the short-distance exploration type was more abundant during drier months (temporal effects in Table 1, Fig. 5e).

Spatially, fungal community composition changes across plots correlated significantly with both soil moisture ($F = 3.1$, $P = 0.035$) and soil temperature ($F = 2.7$, $P = 0.008$), accounting for 2.7% of the total inertia (Fig. 6a). Changes in the distribution of functional guilds between plots were also correlated with soil moisture ($F = 14.2$, $P = 0.006$, Fig. 6b), accounting for 5.0% of the total inertia. The overall proportion of amplicons attributed to mycorrhizal species correlated negatively with soil moisture,

whereas amplicons attributed to moss-associated fungi and root-associated ascomycetes correlated positively (spatial effects in Table 1). Spatial variation in soil moisture, but not temperature, correlated significantly with the relative distribution of exploration types (CCA: Pseudo-F = 7.4, P = 0.044, Fig. 6c), accounting for 2.7% of the total inertia. The medium-distance mat type, which was represented by only a single species (*Phellodon niger*), was marginally affected by temperature (spatial effects in Table. 1, positive Y-axis in Fig. 6c), whereas the short-distance exploration type was more abundant in drier plots, and species representing the long-distance exploration type were more abundant in wetter plots (spatial effects in Table 1, positive first-axis in Fig. 6c).

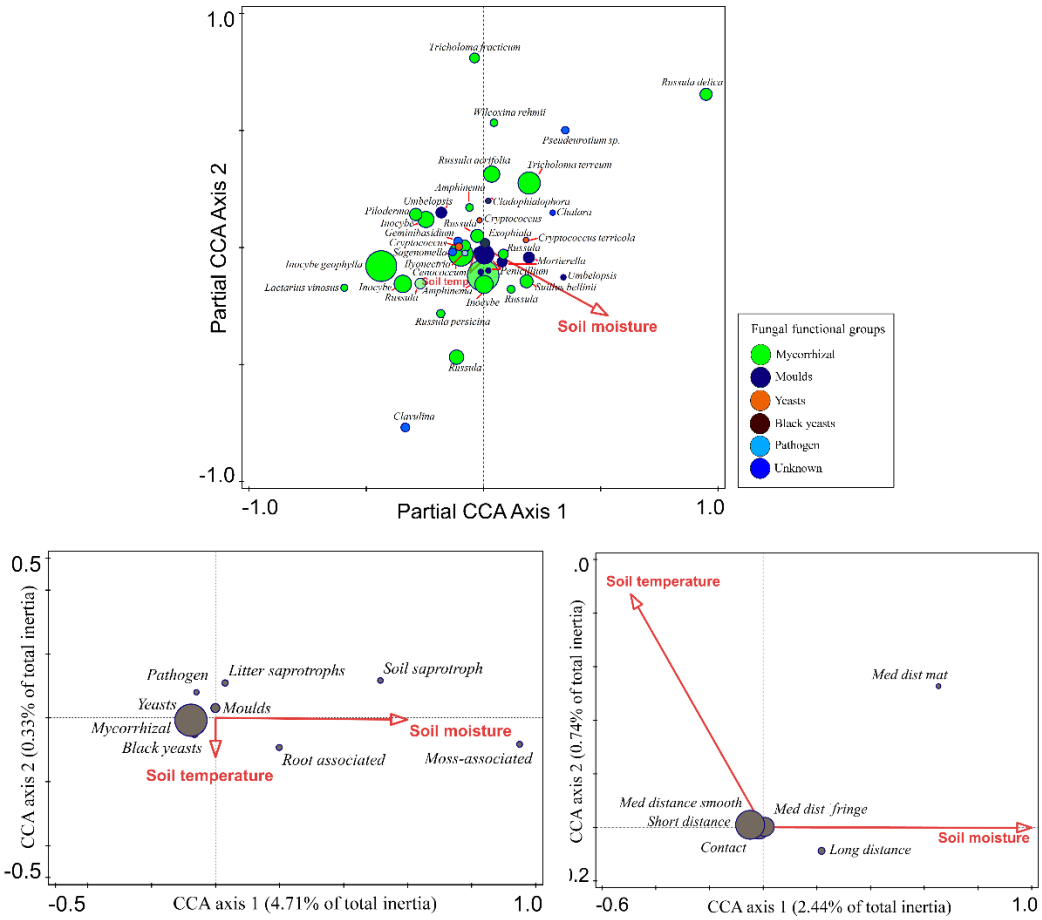

Fig. 6 Canonical correspondence analyses (CCAs) of the community composition of soil fungi, as analysed by sequencing internal transcribed spacer 2 amplicons, in relation to spatial variation in soil temperature and moisture in a Mediterranean *Pinus pinaster* forest. The analyses were based on (a) species composition (total inertia = 2.7%), (b) functional guilds (total inertia = 5.04%) and (c) exploration types of ectomycorrhizal fungi (total inertia = 3.2%). In (a), species symbols are coloured according to functional groups, and symbol sizes are proportional to average relative abundances.

Table 1 Significance of linear mixed effect models of temporal and spatial correlations between relative proportions of specific functional guilds of soil fungi and exploration types of mycorrhizal fungi and climatic variables. To test temporal effects, 'plot' was included as a random factor. To test spatial effects, 'month' was included as a random factor. The symbol (–) indicates a negative correlation.

		Temporal effects (Across months)						**Spatial effects (Across plots)**					
		Soil moisture			**Soil temperature**			**Soil moisture**			**Soil temperature**		
Groups	**Response**	**R^2[%]**	**F**	**p**	**R^2[%]**	**F**	**p**	**R^2[%]**	**F**	**p**	**R^2[%]**	**F**	**p**
Functional guilds	**Black yeast**	73.7	34.58	**<0.001**	45.3(–)	7.04	**0.008**	9(–)	0.12	0.728	2	7.23	**0.007**
	Mycorrhizal	83.6(–)	44.56	**<0.001**	34.8	1.08	0.298	22(–)	23.94	**<0.001**	0.1	0.83	0.363
	Moss associated	63.2	18.41	**<0.001**	13.9(–)	1.4	0.238	18	11.5	**<0.001**	1.6	0.45	0.502
	Moulds	63.0	84.56	**<0.001**	7.8(–)	1.9	0.169	0.3(–)	0.467	0.494	0.1(–)	0.59	0.442
	Pathogen	9.2	0.1	0.75	2.3(–)	0.37	0.541	3	0.49	0.484	0	1.46	0.228
	Root associated	13.2(–)	0.26	0.61	11	0.29	0.589	10	0.76	0.382	0.5	2.06	0.152
	Saprotroph	0	0.99	0.32	19.4(–)	5.32	**0.022**	1.6	0.37	0.54	0.2(–)	4.92	**0.027**
	Soil saprotroph	38.2(–)	1.31	0.252	35.3	0.39	0.531	25.8	0.13	0.715	2.4	2.81	0.094
	Yeast	75.7	40.56	**<0.001**	24.2(–)	0.58	0.45	20.9	1.6	0.207	0.5	3.61	0.059
Exploration types	**Contact**	0.8(–)	0.34	0.556	5.1(–)	0.04	0.838	0	0.01	0.919	1.3	0.14	0.705
	Long distance	26.3	16.65	**<0.001**	49.8(–)	4.31	**0.039**	26.1	10.54	**0.001**	3(–)	3.37	0.067
	Fringe	10.5	1.41	0.236	0.1(–)	0.49	0.486	0.4	0.5	0.479	2.2(–)	0.56	0.813
	Mat	15.3	4.92	**0.027**	4.5	3.08	0.081	15	4.64	**0.032**	2	4.17	**0.042**
	Smooth	32.5(–)	2.75	0.098	13.3	0.13	0.718	4.6	0.29	0.589	9.9(–)	0.19	0.66
	Short distance	42.5(–)	3.85	**0.05**	45.2	1.04	0.309	6(–)	7.87	**0.005**	7.7	0.23	0.628

Discussion

We found that intra-annual variation in fungal biomass and fungal community compositional correlated with changes in soil moisture and temperature. Intra-annual changes were also found among guilds and among specific mycorrhizal exploration types. Correspondingly, spatial fungal community patterns across local plots also reflected different microclimatic features. Shifting balances between fungal functional guilds together with changes in fungal biomass suggest potential alteration in ecosystem functioning with respect to plant nutrition, soil organic matter decomposition and carbon storage (Averill *et al.,* 2014; Clemmensen *et al.,* 2015; Kyaschenko et al. 2017b).

Drought conditions during the summer (July–September) affected the soil fungal biomass in our plots negatively, supporting our first hypothesis that summer drought would reduce soil fungal biomass. Surprisingly, we found that high levels of fungal biomass can occur also under conditions of draught, such as those recorded in October, provided that soil temperatures are not too high (Fig. 1b). In our study, the increase in fungal biomass during autumn was associated with an increase in the relative abundance of ECM species. Increase in ECM fungal abundance in the autumn despite low precipitation supports that ECM fungi can access water from their hosts under low potentials (Allen, 2007). Furthermore, tree allocation of carbon to mycorrhizal fungi may be larger during the autumn months than during the summer months (Högberg *et al.*, 2010). In other ecosystems, such as boreal or temperate forests, increasing relative abundance of ECM species has also been observed during the late growing season (Jumpponen *et al.*, 2010; Voříšková *et al.*, 2014; Santalahti *et al.*, 2016), concurrent with increases in total fungal biomass in soils (Wallander *et al.*, 2001; Nilsson *et al.*, 2007; Högberg *et al.*, 2010).

The lower fungal biomass observed during the drier and hotter summer months (June–August) but the higher relative abundance of ECM species suggests that all functional groups decreased during summer, but that mycorrhizal species seemed to be less adversely affected by summer drought than free-living fungi. The increase in ECM fungal biomass during autumn (September–October) along with a higher relative representation of ECM species supports the theory that the increasing reallocation of carbon from the tree host to the roots during the autumn enhances the growth of mycorrhizal fungi (Wallander *et al.*, 2001; Högberg & Högberg, 2002; Högberg *et al.*, 2010). Moreover, during the winter months (November–February) the decreasing trend

in fungal biomass, together with the decreasing relative representation of mycorrhizal species, suggests that mycorrhizal fungi decline when the carbon allocation from the host is reduced (Jumpponen *et al.*, 2010; Voříšková *et al.*, 2014), whereas the growth of free-living fungi appears to be favoured under the colder and wetter conditions (Jumpponen *et al.,* 2010; Santalahti *et al.*, 2016). Thus, it seems likely that the wetter and colder conditions during late autumn and winter may favour some free-living fungi, such as moulds (Castaño *et al.,* 2016), or other biota that feed on fungal mycelia, such as collembola (Högberg *et al.,* 2010).

A marked seasonality of soil fungal communities has been reported previously (Jumpponen *et al.*, 2010; Andreetta *et al.*, 2011; Voříšková *et al.*, 2014), with seasonal changes being more pronounced in the topsoil layer than in deeper horizons (Andreetta *et al.*, 2011; Voříšková *et al.*, 2014). Our results indicate that the soil fungal communities of Mediterranean forest ecosystems also show marked seasonality. Soil moisture may be more important than temperature in driving the variation in fungal community composition in these ecosystems. In contrast, studies of boreal or temperate ecosystems have reported that warming may cause important fungal community shifts and changes in soil fungal biomass because higher temperatures may stimulate nutrient cycling and plant production (Clemmensen *et al.*, 2006; Solly *et al.*, 2017). Here, soil fungal biomass was lower during summer than during autumn and spring, potentially reflecting differences between Mediterranean and boreal forest ecosystems. This result also supports previous literature reporting decreases in the biomass of specific mycorrhizal fungal species during the summer (Iotti *et al.*, 2014; Castaño *et al.*, 2017; Queralt *et al.*, 2017).

In Mediterranean forests, trees may shift their primary water source from the surface soil to groundwater during the summer and other drought-stress periods (Barbeta *et al.*, 2015). The capacity of deeper tree roots to access groundwater under dry conditions could help to maintain fungal symbionts via hydraulic lift (Unestam & Sun, 1995; Allen, 2007; Querejeta *et al.*, 2003; Querejeta, 2017). Such a strategy could explain why fungi have been observed to be more resistant than bacteria to the increasing frequency and duration of drought conditions in Mediterranean forests (Yuste *et al.*, 2011). Even though mycorrhizal fungi improve plant survival during drought (Barea *et al.*, 2011), severe drought conditions may cause a decrease in mycorrhizal colonisation (Kennedy & Peay, 2007). However, when soil becomes extremely dry, hydraulic lift may help to maintain fungal symbionts (Querejeta *et al.*, 2003). This adaptation to drought may represent an

advantage for root-associated symbionts versus free-living fungi (i.e. saprotrophs, moulds, yeasts and moss-associated fungi). Our results support this hypothesis, with a greater abundance of mycorrhizal fungi relative to free-living fungi during the summer period, as well as a higher relative proportion of mycorrhizal fungi in drier plots. It is well known that drought hinders the function of hydrolytic enzymes (Sardans & Peñuelas, 2013), which may imply that saprotrophs fail to establish and grow during summer months.

The increasing relative abundance of yeasts (*Cryptococcus*, *Rhodotorula*), litter saprotrophs (*Mycena*) and moulds (*Mortierella*) that we observed during winter has been observed previously in both boreal and temperate forests (Jumpponen *et al.*, 2010; Voříšková *et al.*, 2014; Santalahti *et al.*, 2016). Litter saprotrophs are mainly found in the uppermost organic soil horizons (Lindahl *et al.*, 2007) and include genera such as *Mycena* and *Gymnopus*. Other saprotrophs, such as mould species (i.e. *Mortierella*), can be found throughout all the soil horizons (Lindahl *et al.*, 2007; Santalahti *et al.*, 2016). In our study, moulds (particularly *Mortierella* spp.) exhibited strong monthly fluctuations. These species can use a variety of carbohydrates, such as cellulose, pectin and starch (Thormann *et al.*, 2001), and have been observed to increase under increased water availability (Hartmann *et al.*, 2017). It is likely that these free-living fungi contribute to the turnover of dead mycorrhizal mycelium (Lindahl *et al.*, 2010; Jumpponen *et al.*, 2010), together with other soil biota such as collembola (Högberg *et al.,* 2010).

The mycelial architecture of fungal species has been used to differentiate species traits (Agerer, 2001; 2006), with clear implications for nutrient and water mobilisation capacity, as well as for the demand for host carbon. Following this line, Fernandez *et al.* (2016) attributed observed decreases in medium-distance fringe exploration types (high biomass) under warming treatments to poor host performance. This was in line with the findings of Rygiewicz & Andersen (1994), who proposed that 'high biomass' fungal species may represent a high C cost to the host tree, whereas 'low biomass' species with contact or short-distance exploration types (Agerer, 2001, 2006; Deslippe *et al.,* 2011; Fernandez *et al*., 2016), as well as specific stress-resistant mycorrhizal ascomycetes (Gordon & Gehring, 2011), could be less demanding in terms of host carbon and, thus, favoured under drought or other stresses. In this study, the relative abundance of mycorrhizal species with long-distance exploration types decreased under drier conditions, whereas short-distance and contact species increased. This pattern was observed temporally but also spatially across plots with varying micro-climatic

conditions. Similarly, ECM fungi with extensive extramatrical mycelia may 'parasitise' roots for water under drought conditions (Unestam & Sun, 1995; Querejeta *et al.*, 2003), but an association with such fungi may imply a higher C demand (Agerer, 2001; 2006) and may also reduce tree fitness. From a fungal perspective, drought conditions imply a moisture gradient away from the roots, with the non-suberized root tips of deeply rooted trees constituting "moisture hot spots" in the otherwise dry surface soil. This situation is likely to favour fungi that focus their biomass to the interior and surface of tree roots, as well as the closer rhizosphere, over both non-symbiotic fungi and ECM fungi with extensive extramatrical mycelial proliferation. Thus, when soil becomes extremely dry, it is likely that fungi that mainly reside close to roots such as short-distance exploration types or other low biomass ascomycetes typically found in xeric environments (Smith *et al.*, 2007) have a comparative advantage over fungi with a large proportion of their mycelium in the drier bulk soil (e.g. long exploration types).

Here, we found that below-ground fungal biomass and community composition changed in relation to intra-annual temporal and spatial climatic variation, with potential functional implications. Variable climate effects on different mycorrhizal species suggest that species may be selected or disfavoured under a climate change scenario depending on their ecological traits. Free-living fungi and mycorrhizal species with extensive mycelia could be negatively affected under increasing periods of drought. Under drought stress conditions, mycorrhizal species with mycelia concentrated more tightly around the roots may be favoured due to better water availability and lower C demand. Warmer and drier summers will most likely affect soil fungal biomass negatively, with decreasing saprotrophic activity potentially having a negative effect on soil organic matter decomposition. Whether warmer conditions during winter may compensate for summer droughts is uncertain, underlining the need for further research on the effects of induced climate changes on fungal communities and their functionality. Further research is needed to predict how shifts in dominance between fungal guilds and ECM exploration types in drier and warmer scenarios may affect host tree performance, drought resistance and nutrient cycling in Mediterranean forests ecosystems.

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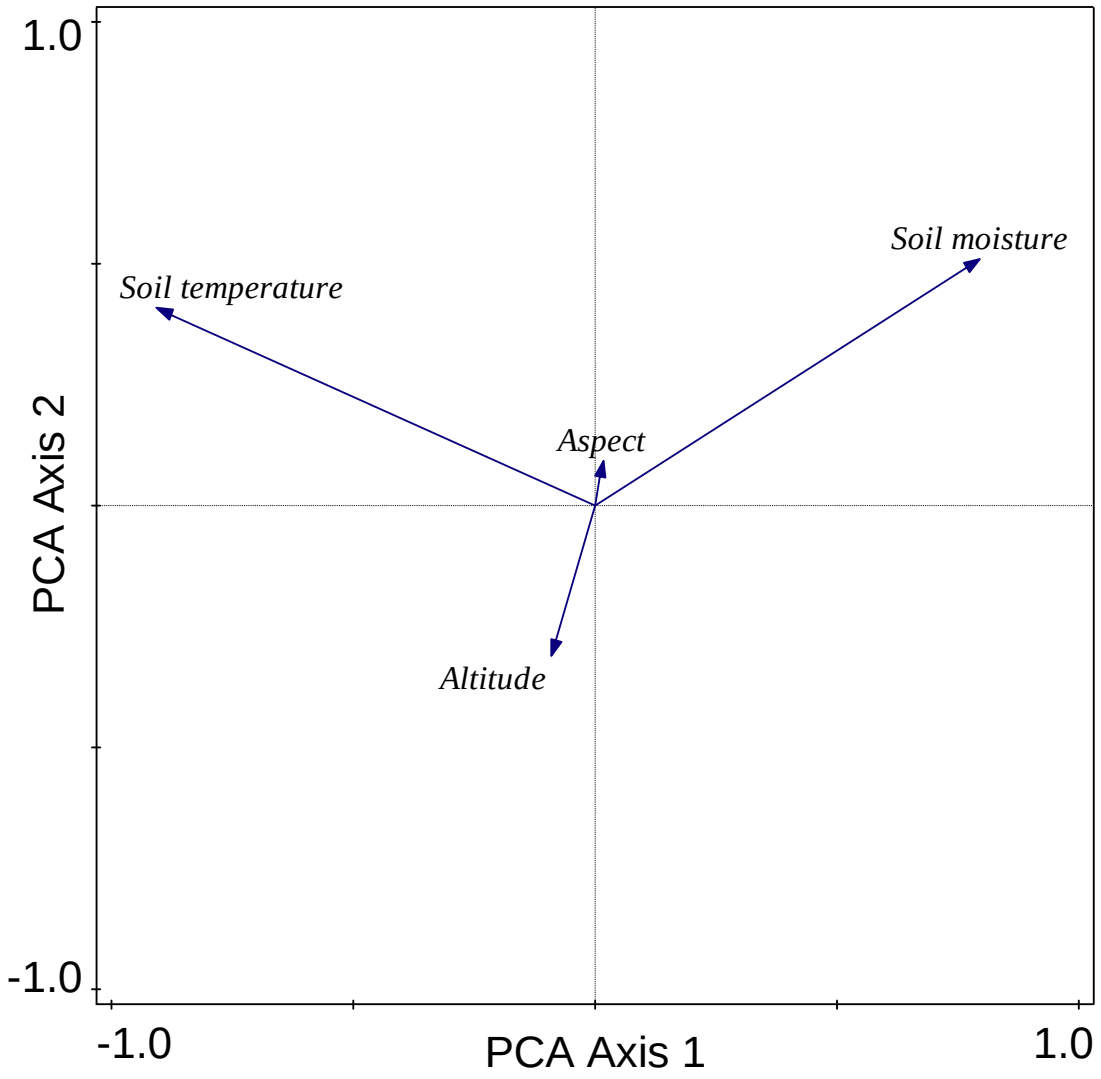
Supplementary material

Supplementary figure 1. Principal component analysis of the climate-related parameters (soil moisture, soil temperature, aspect and altitude).



Supplementary table 1. OTU representative identities and guild assignments.

Abundance order	OTU	Identity	Family	Order	Class	Phyllum	Guild
1	**OTU_0**	Uncultured Amphinema	Atheliaceae	Atheliales	Agaricomycetes	Basidiomycota	ectomycorrhizal
2	**OTU_1**	Inocybe geophylla (var. Geophylla)	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
3	**OTU_6**	Cenococcum geophilum	Gloniaceae	Hysteriales	Dothideomycetes	Ascomycota	Ectomycorrhizal
4	**OTU_13**	Penicillium restrictum/meridianum	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota	Moulds
5	**OTU_2**	Tricholoma terreum/gausapatum	Tricholomataceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
6	**OTU_11**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
7	**OTU_7**	Inocybe asterospora	Gloniaceae	Hysteriales	Agaricomycetes	Ascomycota	Ectomycorrhizal
8	**OTU_10**	Inocybe geophilla (var lillacina)	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
9	**OTU_9**	Suillus bellinii	Suillaceae	Boletales	Agaricomycetes	Basidiomycota	ectomycorrhizal
10	**OTU_4**	Russula acrifolia/anthracina/dissimulans	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
11	**OTU_5**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	ectomycorrhizal
12	**OTU_3**	Mortierella sp.	Mortierellaceae	Mortierellales	Incertae sedis	Zygomycota	Moulds
13	**OTU_18**	Oidiodendron sp.	Myxotrichaceae	Incertae sedis	Leotiomycetes	Ascomycota	ericoid mycorrhizal
14	**OTU_8**	Russula sp.	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
15	**OTU_12**	Mortierella elongata	Mortierellaceae	Mortierellales	Incertae sedis	Zygomycota	Moulds
16	**OTU_14**	Russula sp.	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
17	**OTU_22**	Umbelopsis sp.	Umbelopsidaceae	Mucorales	Incertae sedis	Zygomycota	Moulds
18	**OTU_17**	Unknown	Mycosphaerellaceae	Capnodiales	Dothideomycetes	Ascomycota	Unknown
19	**OTU_16**	Piloderma sp.	Atheliaceae	Atheliales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
20	**OTU_20**	Tricholoma batschii/subannulatum/fracticum	Tricholomataceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
21	**OTU_15**	Russula delica/chloroides	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
22	**OTU_38**	Penicillium nodositatum	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota	Moulds
23	**OTU_29**	Russula sp.	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
24	**OTU_24**	Exophiala sp.	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Black yeast
25	**OTU_19**	Geminibasidium sp.	Geminibasidiaceae	Geminibasidiales	Wallemiomycetes	Basidiomycota	Saprotroph

26	**OTU_23**	Tylospora sp.	Atheliaceae	Atheliales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
27	**OTU_33**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
28	**OTU_27**	Unknown	Unknown	Unknown	Dothideomycetes	Ascomycota	Unknown
29	**OTU_21**	Russula sp.	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
30	**OTU_56**	Archaerorhizomyces sp.	Archaeorhizomycetaceae	Archaeorhizomycetales	Archaeorhizomycetes	Ascomycota	Root associated
31	**OTU_25**	Clavulina sp.	Clavulinaceae	Cantharellales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
32	**OTU_26**	Unknown	Unknown	Saccharomycetales	Saccharomycetes	Ascomycota	Unknown
33	**OTU_30**	Unknown	Unknown	Saccharomycetales	Saccharomycetes	Ascomycota	Unknown
34	**OTU_36**	Unknown	Unknown	Agaricales	Agaricomycetes	Basidiomycota	Unknown
35	**OTU_68**	Unknown	Dermateaceae	Helotiales	Leotiomycetes	Ascomycota	Unknown
36	**OTU_49**	Sagenomella diversispora	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota	Saprotroph
37	**OTU_37**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
38	**OTU_35**	Unknown	Unknown	Agaricales	Agaricomycetes	Basidiomycota	Unknown
39	**OTU_32**	Inocybe obsoleta/rimosa/melliolens	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
40	**OTU_45**	Unknown	Herpotrichiellaceae	chaetothyriales	Eurotiomycetes	Ascomycota	Unknown
41	**OTU_34**	Cryptococcus sp.	Incertae sedis	Tremellales	Tremellomycetes	Basidiomycota	yeast
42	**OTU_51**	Pseudeurotium sp.	Pseudeurotiaceae	Incertae sedis	Incertae sedis	Ascomycota	Unknown
43	**OTU_31**	Russula persicina/solaris	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
44	**OTU_41**	Cadophora finlandica	Incertae sedis	Helotiales	Leotiomycetes	Ascomycota	ectomycorrhizal/ericoid
45	**OTU_28**	Russula sp.	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
46	**OTU_53**	Pseudogymnoascus pannorum	Myxotrichaceae	Incertae sedis	Leotiomycetes	Ascomycota	Pathogen
47	**OTU_59**	Unknown	Venturiaceae	Venturiales	Dothideomycetes	Ascomycota	Unknown
48	**OTU_42**	Hygrocybe chlorophana/persistens	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	Moss associated basidiomycetes
49	**OTU_47**	amphinema byssoides	Atheliaceae	Atheliales	Agaricomycetes	Basidiomycota	ectomycorrhizal
50	**OTU_48**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Black yeast
51	**OTU_72**	Wilcoxina rehmii	Pyronemataceae	Pezizales	Pezizomycetes	Ascomycota	ectomycorrhizal
52	**OTU_40**	Russula vinosobrunnea	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
53	**OTU_39**	Lactarius vinosus	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal

54	**OTU_64**	Mycena pura	Mycenaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
55	**OTU_52**	Cryptococcus terricola	Incertae sedis	Tremellales	Tremellomycetes	Basidiomycota	yeast
56	**OTU_66**	Inocybe posterula/subnudipes/sindonia	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
57	**OTU_43**	Russula sp.	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
58	**OTU_62**	Inocybe maculata f fulva/cookei/flavella/rimosa	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
59	**OTU_63**	Humicola nigrescens	Chaetomiaceae	Sordariales	Sordariomycetes	Ascomycota	Saprotroph
60	**OTU_130**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
61	**OTU_50**	Russula praetervisa	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
62	**OTU_44**	Unknown	Unknown	Unknown	Microbotryomycetes	Basidiomycota	Unknown
63	**OTU_46**	Rhizopogon rubescens	Rhizopogonaceae	Boletales	Agaricomycetes	Basidiomycota	ectomycorrhizal
64	**OTU_54**	Clavulina sp.	Clavulinaceae	Cantharellales	Agaricomycetes	Basidiomycota	ectomycorrhizal
65	**OTU_65**	Unknown	Unknown	Trechisporales	Agaricomycetes	Basidiomycota	Unknown
66	**OTU_91**	Unknown	Dermateaceae	Helotiales	Leotiomycetes	Ascomycota	Unknown
67	**OTU_58**	Hygrophorus quercetorum/cossus	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	ectomycorrhizal
68	**OTU_55**	Clavulina sp.	Clavulinaceae	Cantharellales	Agaricomycetes	Basidiomycota	ectomycorrhizal
69	**OTU_75**	Unknown	Atheliaceae	Atheliales	Agaricomycetes	Basidiomycota	Unknown
70	**OTU_61**	Clavulina cinerea	Clavulinaceae	Cantharellales	Agaricomycetes	Basidiomycota	ectomycorrhizal
71	**OTU_60**	Mortierella sp.	Mortierellaceae	Mortierellales	Incertae sedis	Zygomycota	Moulds
72	**OTU_70**	Cortinarius diasemospermus	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	ectomycorrhizal
73	**OTU_81**	Oidiodendron griseum	Myxotrichaceae	Incertae sedis	Leotiomycetes	Ascomycota	ericoid mycorrhizal
74	**OTU_57**	Unknown	Unknown	Unknown	Agaricomycetes	Basidiomycota	Unknown
75	**OTU_90**	Unknown	Unknown	Chaetothyriales	Eurotiomycetes	Ascomycota	Unknown
76	**OTU_67**	Russula decipiens	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
77	**OTU_83**	Leucopaxillus gentianeus	Tricholomataceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
78	**OTU_93**	Trechispora sp.	Hydnodontaceae	Trechisporales	Agaricomycetes	Basidiomycota	Saprotroph
79	**OTU_88**	Unknown	Dermateaceae	Helotiales	Leotiomycetes	Ascomycota	Unknown
80	**OTU_71**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
81	**OTU_89**	Unknown	Unknown	Chaetothyriales	Eurotiomycetes	Ascomycota	Unknown

82	**OTU_73**	Cuphophyllus virgineus	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	Moss associated basidiomycetes
83	**OTU_77**	Tricholoma squarrulosum/atrosquamosum	Lyophyllaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
84	**OTU_108**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
85	**OTU_84**	Macrolepiota procera	Agaricaceae	Agaricales	Agaricomycetes	Basidiomycota	Soil saprotroph
86	**OTU_96**	Trichoderma spirale	Hypocreaceae	Hypocreales	Sordariomycetes	Ascomycota	Moulds
87	**OTU_76**	Cryptococcus sp.	Incertae sedis	Tremellales	Tremellomycetes	Basidiomycota	yeast
88	**OTU_105**	Trichocladium sp.	Chaetomiaceae	Sordariales	Sordariomycetes	Ascomycota	Moulds
89	**OTU_112**	Cortinarius obtusus	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	ectomycorrhizal
90	**OTU_79**	Rhodocollybia butyracea f. Asema	Marasmiaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
91	**OTU_80**	Russula sp.	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
92	**OTU_100**	Ilyonectria sp.	Incertae sedis	Hypocreales	Sordariomycetes	Ascomycota	Pathogen
93	**OTU_87**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
94	**OTU_69**	Umbelopsis sp.	Umbelopsidaceae	Mucorales	Incertae sedis	Zygomycota	Moulds
95	**OTU_82**	Inocybe quietiodor	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
96	**OTU_92**	Archaeorhizomyces sp.	Archaeorhizomycetaceae	Archaeorhizomycetales	Archaeorhizomycetes	Ascomycota	Root associated
97	**OTU_114**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
98	**OTU_99**	Agaricus impudicus	Agaricaceae	Agaricales	Agaricomycetes	Basidiomycota	Soil saprotroph
99	**OTU_78**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
100	**OTU_115**	Hygrophorus arbustivus	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	ectomycorrhizal
101	**OTU_95**	Inocybe geophylla	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
102	**OTU_132**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
103	**OTU_106**	Pustularia sp.	Pyronemataceae	Pezizales	Pezizomycetes	Ascomycota	Unknown
104	**OTU_98**	Archaeorhizomyces sp.	Archaeorhizomycetaceae	Archaeorhizomycetales	Archaeorhizomycetes	Ascomycota	Root associated
105	**OTU_86**	Tomentella sp.	Thelephoraceae	Thelephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
106	**OTU_135**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
107	**OTU_97**	Umbelopsis sp.	Umbelopsidaceae	Mucorales	Incertae sedis	Zygomycota	Moulds
108	**OTU_152**	Archaeorhizomyces sp.	Archaeorhizomycetaceae	Archaeorhizomycetales	Archaeorhizomycetes	Ascomycota	Root associated
109	**OTU_104**	Helvellosebacina helvelloides	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown

110	**OTU_74**	Cuphophyllus sp.	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	Moss associated basidiomycetes
111	**OTU_129**	Chalara sp.	Unknown	Unknown	Unknown	Ascomycota	saprotroph
112	**OTU_94**	Cladophialophora sp.	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Black yeast
113	**OTU_85**	Sistotrema sp.	Hydnaceae	Cantharellales	Agaricomycetes	Basidiomycota	Unknown
114	**OTU_124**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Unknown
115	**OTU_102**	Hydnum ovoideisporum	Hydnaceae	Cantharellales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
116	**OTU_118**	Hebeloma sordidum/mesophaeum	Strophariaceae	Agaricales	Agaricomycetes	Basidiomycota	ectomycorrhizal
117	**OTU_101**	Inocybe subbrunnea	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
118	**OTU_141**	Thelebolus microsporus/elipsoideus	Thelebolaceae	Thelebolales	Leotiomycetes	Ascomycota	Soil saprotroph
119	**OTU_128**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
120	**OTU_119**	Unknown	Unknown	Agaricales	Agaricomycetes	Basidiomycota	Unknown
121	**OTU_103**	Lactarius decipiens	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
122	**OTU_131**	Russula vesca	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
123	**OTU_125**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
124	**OTU_175**	Tomentella sp.	Thelephoraceae	Thelephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
125	**OTU_109**	Lactarius deliciosus	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
126	**OTU_110**	Hygrocybe sp.	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	Moss associated basidiomycetes
127	**OTU_116**	Russula sp.	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
128	**OTU_127**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
129	**OTU_206**	Penicillium arenicola	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota	Moulds
130	**OTU_122**	Clavulina sp.	Clavulinaceae	Cantharellales	Agaricomycetes	Basidiomycota	Unknown
131	**OTU_123**	Hygrocybe conica	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	Moss associated basidiomycetes
132	**OTU_153**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
133	**OTU_133**	Laccaria laccata/bicolor	Hydnangiaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
134	**OTU_107**	Clavulina sp.	Clavulinaceae	Cantharellales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
135	**OTU_137**	Mucor sp.	Mucoraceae	Mucorales	Incertae sedis	Zygomycota	Moulds
136	**OTU_120**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal

137	**OTU_111**	Hymenogaster griseus/knappii/boozeri	Strophariaceae	Agaricales	Agaricomycetes	Basidiomycota	ectomycorrhizal
138	**OTU_113**	Phellodon niger	Bankeraceae	Thelephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
139	**OTU_117**	Tricholoma viridilutescens/sejunctum	Tricholomataceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
140	**OTU_121**	Craterellus cornucopioides	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
141	**OTU_147**	Unknown	Unknown	Unknown	Eurotiomycetes	Ascomycota	Unknown
142	**OTU_134**	Tomentella sp.	Thelephoraceae	Thelephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
143	**OTU_139**	Unknown	Unknown	Cantharellales	Agaricomycetes	Basidiomycota	Unknown
144	**OTU_148**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
145	**OTU_146**	Tomentella sp.	Thelephoraceae	Thelephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
146	**OTU_126**	Tomentella sp.	Thelephoraceae	Thelephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
147	**OTU_145**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
148	**OTU_151**	Inocybe amblyspora	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
149	**OTU_144**	Tuber borchii	Tuberaceae	Pezizales	Pezizomycetes	Ascomycota	Ectomycorrhizal
150	**OTU_196**	Archaeorhizomyces sp.	Archaeorhizomycetaceae	Archaeorhizomycetales	Archaeorhizomycetes	Ascomycota	Root associated
151	**OTU_156**	Tricholoma scalpturatum	Tricholomataceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
152	**OTU_140**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
153	**OTU_142**	Russula sp.	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
154	**OTU_149**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
155	**OTU_173**	Unknown	Unknown	Unknown	Unknown	Protista	Unknown
156	**OTU_143**	Tricholoma saponaceum	Tricholomataceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
157	**OTU_161**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
158	**OTU_155**	Unknown	Unknown	Agaricales	Agaricomycetes	Basidiomycota	Unknown
159	**OTU_150**	Mycena galopus/leptocephala	Mycenaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
160	**OTU_188**	Cadophora sp.	Incertae sedis	Helotiales	Leotiomycetes	Ascomycota	Endophyte
161	**OTU_154**	Sistotrema sp.	Hydnaceae	Cantharellales	Agaricomycetes	Basidiomycota	Unknown
162	**OTU_138**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
163	**OTU_136**	Mycena olivaceomarginata	Mycenaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
164	**OTU_157**	Guehomyces pullulans	Cystofilobasidiaceae	Cystofilobasidiales	Tremellomycetes	Basidiomycota	yeast

165	**OTU_166**	Suillus luteus	Suillaceae	Boletales	Agaricomycetes	Basidiomycota	ectomycorrhizal
166	**OTU_178**	Unknown	Unknown	Unknown	Eurotiomycetes	Ascomycota	Unknown
167	**OTU_164**	Cuphophyllus sp.	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	Moss associated basidiomycetes
168	**OTU_159**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
169	**OTU_185**	Trichophaea woolhopeia	Pyronemataceae	Pezizales	Pezizomycetes	Ascomycota	Ectomycorrhizal
170	**OTU_186**	Venturia sp.	Venturiaceae	Venturiales	Dothideomycetes	Ascomycota	Unknown
171	**OTU_189**	Unknown	Unknown	Unknown	Unknown	Plantae	Unknown
172	**OTU_160**	Tomentella sp.	Thelephoraceae	Thelephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
173	**OTU_182**	Phialocephala sp.	Vibrisseaceae	Helotiales	Leotiomycetes	Ascomycota	Root associated
174	**OTU_158**	Mucor plumbeus	Mucoraceae	Mucorales	Incertae sedis	Zygomycota	Moulds
175	**OTU_170**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Unknown
176	**OTU_168**	Hygrocybe conica	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	Moss associated basidiomycetes
177	**OTU_190**	Inocybe subnudipes	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
178	**OTU_187**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
179	**OTU_199**	Unknown	Myxotrichaceae	Incertae sedis	Leotiomycetes	Ascomycota	Unknown
180	**OTU_195**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Unknown
181	**OTU_200**	Unknown	Unknown	Eurotiales	Eurotiomycetes	Ascomycota	Unknown
182	**OTU_169**	Cortinarius sp.	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
183	**OTU_167**	Inocybe tenebrosa	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
184	**OTU_179**	Terfezia sp.	Pezizaceae	Pezizales	Pezizomycetes	Ascomycota	Ectomycorrhizal
185	**OTU_163**	Clavulina sp.	Clavulinaceae	Cantharellales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
186	**OTU_181**	Rhizopogon sp.	Rhizopogonaceae	Boletales	Agaricomycetes	Basidiomycota	ectomycorrhizal
187	**OTU_184**	Tomentella sp.	Thelephoraceae	Thelephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
188	**OTU_226**	Trichoderma sp.	Hypocreaceae	Hypocreales	Sordariomycetes	Ascomycota	moulds
189	**OTU_174**	Agaricus sp.	Agaricaceae	Agaricales	Agaricomycetes	Basidiomycota	Soil saprotroph
190	**OTU_203**	Cortinarius favrei/trivialis/septentrionalis	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	ectomycorrhizal
191	**OTU_177**	Sebacina epigaea	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	ectomycorrhizal

192	**OTU_180**	Russula pallidospora	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
193	**OTU_172**	Cortinarius hinnuleus	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	ectomycorrhizal
194	**OTU_198**	Trechispora sp.	Hydnodontaceae	Trechisporales	Agaricomycetes	Basidiomycota	Saprotroph
195	**OTU_209**	Archaeorhizomyces	Archaeorhizomycetaceae	Archaeorhizomycetales	Archaeorhizomycetes	Ascomycota	Root associated
196	**OTU_165**	Unknown	Clavariaceae	Agaricales	Agaricomycetes	Basidiomycota	Unknown
197	**OTU_171**	Unknown	Unknown	Trechisporales	Agaricomycetes	Basidiomycota	Unknown
198	**OTU_183**	Russula livescens/insignis	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
199	**OTU_191**	Russula romellii	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
200	**OTU_213**	Cortinarius sp.	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	ectomycorrhizal
201	**OTU_162**	Russula odorata/cessans	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
202	**OTU_204**	Ramaria sp.	Gomphaceae	Gomphales	Agaricomycetes	Basidiomycota	Unknown
203	**OTU_210**	Agaricus sp.	Agaricaceae	Agaricales	Agaricomycetes	Basidiomycota	Soil saprotroph
204	**OTU_205**	Lycoperdon mammiforme	Agaricaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
205	**OTU_194**	Russula sp.	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
206	**OTU_289**	Archaeorhizomyces sp.	Archaeorhizomycetaceae	Archaeorhizomycetales	Archaeorhizomycetes	Ascomycota	Root associated
207	**OTU_192**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
208	**OTU_176**	Russula laricinoaffinis	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	ectomycorrhizal
209	**OTU_216**	Unknown	Unknown	Pezizales	Pezizomycetes	Ascomycota	Unknown
210	**OTU_201**	Mycenella sp.	Tricholomataceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
211	**OTU_214**	Cladophialophora	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Black yeast
212	**OTU_208**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
213	**OTU_219**	Unknown	Unknown	Hypocreales	Sordariomycetes	Ascomycota	Unknown
214	**OTU_212**	Phoma herbarum	Incertae sedis	Pleosporales	Dothideomycetes	Ascomycota	Pathogen
215	**OTU_202**	Unknown	Unknown	Unknown	Agaricomycetes	Basidiomycota	Unknown
216	**OTU_238**	Trechispora sp.	Hydnodontaceae	Trechisporales	Agaricomycetes	Basidiomycota	Saprotroph
217	**OTU_242**	Unknown	Unknown	Sordariales	Sordariomycetes	Ascomycota	Unknown
218	**OTU_266**	Unknown	Unknown	Hypocreales	Sordariomycetes	Ascomycota	Unknown
219	**OTU_197**	Russula sp.	Russulaceae	Russulales	Agaricomycetes	Basidiomycota	Ectomycorrhizal

220	**OTU_224**	Sistotrema sp.	Hydnaceae	Cantharellales	Agaricomycetes	Basidiomycota	Unknown
221	**OTU_193**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
222	**OTU_236**	Unknown	Unknown	Capnodiales	Dothideomycetes	Ascomycota	Unknown
223	**OTU_245**	Unknown	Unknown	chaetothyriales	Eurotiomycetes	Ascomycota	Unknown
224	**OTU_228**	Xenochalara juniperi	Unknown	Helotiales	leotiomycetes	Ascomycota	saprotroph
225	**OTU_217**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
226	**OTU_240**	Unknown	geoglassaceae	geoglossales	Geoglomycetes	Ascomycota	Unknown
227	**OTU_218**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
228	**OTU_235**	Mycena rosea	Mycenaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
229	**OTU_234**	Unknown	Unknown	Capnodiales	Dothideomycetes	ascomycota	Unknown
230	**OTU_282**	Archaerorhizomyces sp.	Archaeorhizomycetaceae	Archaeorhizomycetales	Archaeorhizomycetes	Ascomycota	Root associated
231	**OTU_336**	Oidiodendron sp.	Myxotrichaceae	Unknown	leotiomycetes	ascomycota	ericoid mycorrhizal
232	**OTU_207**	Russula olivacea/alutacea	Russulaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
233	**OTU_232**	Inocybe cincinnatula	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
234	**OTU_222**	Unknown	Agaricaceae	Agaricales	Agaricomycetes	Basidiomycota	Unknown
235	**OTU_223**	Cortinarius sp.	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
236	**OTU_225**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
237	**OTU_230**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
238	**OTU_271**	Cladosporium sp.	Davidiellaceae	Capnodiales	Dothideomycetes	Ascomycota	Endophyte
239	**OTU_270**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Black yeast
240	**OTU_244**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
241	**OTU_256**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
242	**OTU_237**	Cryptococcus sp.	Unknown	Tremellales	Tremellomycetes	Basidiomycota	yeast
243	**OTU_243**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
244	**OTU_255**	Helvellosebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	saprotroph
245	**OTU_262**	Inocybe tenebrosa	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
246	**OTU_298**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
247	**OTU_246**	Agaricus excellens/essettei/silvicola	Agaricaceae	Agaricales	Agaricomycetes	Basidiomycota	Soil saprotroph

248	**OTU_273**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Unknown
249	**OTU_229**	Lactarius vellereus	Russulaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
250	**OTU_252**	Leptosporomyces fruscostatus	Atheliaceae	Atheliales	Agaricomycetes	Basidiomycota	saprotroph
251	**OTU_272**	Cortinarius sp.	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
252	**OTU_221**	Unknown	Unknown	Unknown	Unknown	Ascomycota	Unknown
253	**OTU_259**	Unknown	Unknown	Pezizales	Pezizomycetes	Ascomycota	Unknown
254	**OTU_247**	Tuber sp.	Tuberaceae	Pezizales	Pezizomycetes	Ascomycota	Ectomycorrhizal
255	**OTU_211**	Gymnopus erythropus/sejunctum	Tricholomataceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
256	**OTU_283**	Unknown	Unknown	Unknown	Dothideomycetes	ascomycota	Unknown
257	**OTU_227**	Unknown	Unknown	Unknown	Microbotryomycetes	Basidiomycota	Unknown
258	**OTU_258**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Unknown
259	**OTU_241**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
260	**OTU_280**	Talaromyces sp.	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota	Moulds
261	**OTU_215**	Cantharellus sp.	Cantharellaceae	Cantharellales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
262	**OTU_250**	Unknown	Unknown	Trechisporales	Agaricomycetes	Basidiomycota	Unknown
263	**OTU_231**	Mortierella spp.	Mortierellaceae	Mortierellales	Incertae sedis	Zygomycota	Moulds
264	**OTU_251**	Thelonectria veuillotiana	Nectriaceae	Hypocreales	Sordariomycetes	Ascomycota	Saprotroph
265	**OTU_303**	Inocybe ochroalba/splendens/amblyspora	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
266	**OTU_274**	Cortinarius sp.	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
267	**OTU_253**	Tomentella coerulea	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
268	**OTU_260**	Ramaria sp.	Gomphaceae	Gomphales	Agaricomycetes	Basidiomycota	Unknown
269	**OTU_261**	Piloderma sp.	Atheliaceae	Atheliales	agaricomycetes	basidiomycota	Ectomycorrhizal
270	**OTU_233**		Unknown	Unknown	Unknown	Plantae	Unknown
271	**OTU_248**	Unknown	Unknown	Helotiales	Leotiomycetes	ascomycota	Unknown
272	**OTU_286**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
273	**OTU_305**	Odiodendron sp.	mycotrichaceae	Unknown	Leotiomycetes	Ascomycota	ericoid mycorrhizal
274	**OTU_322**	Penicillium sp.	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota	Moulds
275	**OTU_220**	Gymnopus aquosus	Marasmiaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph

276	**OTU_323**	Unknown	Dermateaceae	Helotiales	leotiomycetes	ascomycota	Unknown
277	**OTU_263**	Clavaria sp.	Clavariaceae	agaricales	Agaricomycetes	Basidiomycota	saprotroph
278	**OTU_278**	Bovista aestivalis/promontorii	Agaricaceae	Agaricales	Agaricomycetes	Basidiomycota	Soil saprotroph
279	**OTU_264**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
280	**OTU_294**	Archaerorhizomyces sp.	Archaeorhizomycetaceae	Archaeorhizomycetales	Archaeorhizomycetes	Ascomycota	Root associated
281	**OTU_302**	Hygrophorus persoonii	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	Moss associated basidiomycetes
282	**OTU_304**	Unknown	Unknown	agaricales	agaricomycetes	basidiomycota	Unknown
283	**OTU_254**	Rhodotorula cresolica	Unknown	Sporidiobolales	Microbotryomycetes	basidiomycota	yeast
284	**OTU_239**	Mortierella exigua	Mortierellaceae	Mortierellales	Incertae sedis	Zygomycota	Moulds
285	**OTU_249**	Unknown	Unknown	Unknown	Agaricomycetes	Basidiomycota	Unknown
286	**OTU_299**	Phomopsis columnaris	Diaporthaceae	Diaporthales	Sordariomycetes	Ascomycota	Pathogen
287	**OTU_257**	Umbelopsis sp.	Umbelopsidaceae	Mucorales	Incertae sedis	Zygomycota	Moulds
288	**OTU_281**	Trechispora sp.	Hydnodontaceae	Trechisporales	Agaricomycetes	Basidiomycota	Saprotroph
289	**OTU_277**	Unknown	Pyronemataceae	Pezizales	Pezizomycetes	Ascomycota	Unknown
290	**OTU_327**	Geoglossum sp.	geoglassaceae	geoglossales	Geoglomycetes	Ascomycota	Unknown
291	**OTU_297**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
292	**OTU_344**		Unknown	Unknown	Unknown	Protozoo	Unknown
293	**OTU_267**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
294	**OTU_301**	Unknown	Unknown	Pleosporales	Dothideomycetes	ascomycota	Unknown
295	**OTU_288**	Cortinarius sp.	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
296	**OTU_335**	Metarhizium sp.	Clavicipitaceae	Hypocreales	sordariomycetes	ascomycota	saprotroph
297	**OTU_279**	Trichoglossum sp.	geoglassaceae	geoglossales	Geoglomycetes	Ascomycota	Unknown
298	**OTU_268**	Russula risigallina	Russulaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
299	**OTU_291**	Tuber aestivum/uncinatum	Tuberaceae	Pezizales	Pezizomycetes	Ascomycota	Ectomycorrhizal
300	**OTU_387**	Unknown	Unknown	Pleosporales	Dothideomycetes	Ascomycota	Unknown
301	**OTU_284**	Rhizopogon roseolus	Rhizopogonaceae	Boletales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
302	**OTU_330**	Inocybe umbrinella	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
303	**OTU_275**	Russula sp.	Russulaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal

304	**OTU_318**	Cortinarius sp.	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
305	**OTU_321**	Tomentella punicea	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
306	**OTU_292**	Tomentella cinereoumbrina	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
307	**OTU_308**	Inocybe ochroalba	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
308	**OTU_347**	Unknown	Unknown	Unknown	Dothideomycetes	ascomycota	Unknown
309	**OTU_276**	Russula sp.	Russulaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
310	**OTU_285**	Russula sp.	Russulaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
311	**OTU_287**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Black yeast
312	**OTU_312**	Russula albonigra	Russulaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
313	**OTU_325**	Unknown	Unknown	Unknown	Dothideomycetes	ascomycota	Unknown
314	**OTU_265**	Unknown	Unknown	Unknown	Unknown	ascomycota	Unknown
315	**OTU_296**	Unknown	Unknown	Unknown	Leotiomycetes	ascomycota	Unknown
316	**OTU_306**	Unknown	Unknown	Capnodiales	Dothideomycetes	ascomycota	Unknown
317	**OTU_295**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
318	**OTU_317**	Amanita spissa/franchettii	Amanitaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
319	**OTU_339**	Unknown	Unknown	Helotiales	Leotiomycetes	ascomycota	Unknown
320	**OTU_326**	Lepiota forquignonii	Agaricaceae	Agaricales	Agaricomycetes	Basidiomycota	Soil saprotroph
321	**OTU_310**	Mycenella lasiosperma	Tricholomataceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
322	**OTU_328**	Unknown	Unknown	Sordariales	Sordariomycetes	ascomycota	Unknown
323	**OTU_340**	Umbelopsis sp.	Umbelopsidaceae	Mucorales	Incertae sedis	Zygomycota	Moulds
324	**OTU_375**	Cortinarus sp.	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
325	**OTU_320**	Cortinarius sp.	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
326	**OTU_293**	Sistotrema sp.	Hydnaceae	Cantharellales	Agaricomycetes	Basidiomycota	Unknown
327	**OTU_329**	Inocybe sp.	Inocybaceae	agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
328	**OTU_346**	Lophium sp.	Mytilinidiaceae	Mytilinidiales	Dothideomycetes	Ascomycota	Saprotroph
329	**OTU_353**	Unknown	Unknown	Unknown	Eurotiomycetes	Ascomycota	Unknown
330	**OTU_345**	Unknown	Unknown	Unknown	Unknown	Ascomycota	Unknown
331	**OTU_311**	Penicillium	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota	Moulds

332	**OTU_351**	Unknown	Myxotrichaceae	Unknown	Leotiomycetes	Ascomycota	Unknown
333	**OTU_300**	Mycena amicta	Mycenaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
334	**OTU_290**	Tomentella stuposa	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
335	**OTU_403**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
336	**OTU_338**	Unknown	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Unknown
337	**OTU_313**	Amanita pantherina	Amanitaceae	agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
338	**OTU_314**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
339	**OTU_315**	Unknown	Unknown	Unknown	agaricomycetes	basidiomycota	Unknown
340	**OTU_309**	Mycena sp.	Mycenaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
341	**OTU_324**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
342	**OTU_333**		Unknown	Unknown	Unknown	Plantae	Unknown
343	**OTU_362**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
344	**OTU_386**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
345	**OTU_331**	Unknown	Unknown	Unknown	Unknown	ascomycota	Unknown
346	**OTU_364**	Unknown	Trichocomaceae	Eurotiales	Eurotiomycetes	ascomycota	Unknown
347	**OTU_341**	Trichoderma sp.	Hypocreaceae	Hypocreales	Sordariomycetes	Ascomycota	Moulds
348	**OTU_352**	Tomentella ellisii	Thelephoraceae	Thelephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
349	**OTU_391**	Unknown	Nectriaceae	Hypocreales	Sordariomycetes	Ascomycota	Unknown
350	**OTU_269**	Unknown	Kickxellaceae	Kickxellales	Incertae sedis	Zygomycota	Unknown
351	**OTU_349**	Unknown	Unknown	Unknown	Unknown	ascomycota	Unknown
352	**OTU_316**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
353	**OTU_357**	Unknown	Clavariaceae	agaricales	Agaricomycetes	Basidiomycota	Unknown
354	**OTU_373**	Tuber rufum	Tuberaceae	Pezizales	Pezizomycetes	Ascomycota	Ectomycorrhizal
355	**OTU_343**	Hygrocybe pratensis	Hygrophoraceae	Agaricales	Agaricomycetes	Basidiomycota	Moss associated basidiomycetes
356	**OTU_368**	Clavulinopsis sp.	clavariaceae	Agaricales	Agaricomycetes	Basidiomycota	Unknown
357	**OTU_334**	Odontia sp.	Meruliaceae	Agaricales	Agaricomycetes	basidiomycota	saprotroph
358	**OTU_337**		Unknown	Unknown	Unknown	Plantae	Unknown
359	**OTU_384**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal

360	**OTU_428**	Coniochaeta sp./Lecythophora sp.	Coniochaetaceae	coniochaetales	Sordariomycetes	ascomycota	saprotroph
361	**OTU_385**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
362	**OTU_359**	Chalara sp.	Unknown	Unknown	Unknown	ascomycota	saprotroph
363	**OTU_354**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
364	**OTU_415**	Mucor saturninus	Mucoraceae	Mucorales	Incertae sedis	Zygomycota	Moulds
365	**OTU_348**	Unknown	Unknown	Trechisporales	Agaricomycetes	Basidiomycota	Unknown
366	**OTU_407**	Infundichalara sp.	Unknown	Helotiales	Leotiomycetes	ascomycota	saprotroph
367	**OTU_419**	Unknown	Dermateaceae	Helotiales	Leotiomycetes	ascomycota	Unknown
368	**OTU_342**	Unknown	Unknown	Unknown	Unknown	Basidiomycota	Unknown
369	**OTU_365**	Naemacyclus/Cyclaneusma minus	Unknown	Unknown	Leotiomycetes	ascomycota	saprotroph
370	**OTU_390**	Pseudoomphalina kalchbrenneri	Tricholomataceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
371	**OTU_404**	Unknown	Unknown	Unknown	Leotiomycetes	ascomycota	Unknown
372	**OTU_307**	Gymnopus ocior	Marasmiaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
373	**OTU_360**	Mycena aurantiomarginata	Mycenaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
374	**OTU_367**	Russula postiana	Russulaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
375	**OTU_389**	Hypholoma fasciculare	Strophariaceae	Agaricales	Agaricomycetes	Basidiomycota	Soil saprotroph
376	**OTU_319**	Unknown	Marasmiaceae	Agaricales	Agaricomycetes	Basidiomycota	Unknown
377	**OTU_381**	Unknown	geoglassaceae	geoglossales	Geoglomycetes	Ascomycota	Unknown
378	**OTU_416**	Helicodendron sp./Xenopolyscytalum sp.	Unknown	Helotiales	Leotiomycetes	ascomycota	Unknown
379	**OTU_350**	Inocybe cincinnata	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
380	**OTU_376**	Hydnobolites cerebriformis	Pezizaceae	Pezizales	Pezizomycetes	Ascomycota	Ectomycorrhizal
381	**OTU_388**	Unknown	Unknown	Helotiales	Leotiomycetes	Basidiomycota	Unknown
382	**OTU_395**	Unknown	Unknown	Pleosporales	Dothideomycetes	Ascomycota	Unknown
383	**OTU_417**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
384	**OTU_361**	Russula aurea	Russulaceae	agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
385	**OTU_380**	Lycoperdon perlatum	Agaricaceae	agaricales	Agaricomycetes	Basidiomycota	saprotroph
386	**OTU_394**	Clavaria sp.	Clavariaceae	agaricales	Agaricomycetes	Basidiomycota	saprotroph
387	**OTU_374**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown

388	**OTU_379**	Unknown	Unknown	Saccharomycetales	Saccharomycetes	ascomycota	Unknown
389	**OTU_473**	Archaerorhizomyces sp.	Archaeorhizomycetaceae	Archaeorhizomycetales	Archaeorhizomycetes	Ascomycota	Root associated
390	**OTU_371**	Unknown	Unknown	agaricales	Agaricomycetes	Basidiomycota	Unknown
391	**OTU_363**	Clitocybe sp.	Tricholomataceae	agaricales	Agaricomycetes	Basidiomycota	saprotroph
392	**OTU_392**	Unknown	Unknown	Helotiales	Leotiomycetes	ascomycota	Unknown
393	**OTU_355**		Unknown	Unknown	Unknown	Plantae	Unknown
394	**OTU_370**	Unknown	Unknown	agaricales	agaricomycetes	basidiomycota	Unknown
395	**OTU_372**	Hygrocybe sp.	Hygrophoraceae	agaricales	Agaricomycetes	Basidiomycota	Moss associated basidiomycetes
396	**OTU_410**	Clonostachys/Bionectria sp.	Bionectriaceae	Hypocreales	Sordariomycetes	Ascomycota	Pathogen
397	**OTU_456**	Unknown	Unknown	Unknown	Eurotiomycetes	Ascomycota	Moulds
398	**OTU_332**	Xerocomus sp.	Boletaceae	Boletales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
399	**OTU_408**	Unknown	Unknown	Unknown	Agaricomycetes	Basidiomycota	Unknown
400	**OTU_422**	Inocybe sp.	Inocybaceae	agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
401	**OTU_435**	Inocybe sp.	Inocybaceae	agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
402	**OTU_438**	Cortinarius sp.	Cortinariaceae	agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
403	**OTU_455**	Cortinarius saturninus	Cortinariaceae	agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
404	**OTU_459**	Pochonia suchlasporia	Clavicipitaceae	Hypocreales	Sordariomycetes	Ascomycota	saprotroph
405	**OTU_378**	Tricholoma sp.	Tricholomataceae	Agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
406	**OTU_399**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Agaricomycetes	Unknown
407	**OTU_412**	Cuphophyllus sp.	Hygrophoraceae	Agaricales	Agaricomycetes	basidiomycota	Unknown
408	**OTU_420**	Candida sp.	Unknown	Saccharomycetales	Saccharomycetes	ascomycota	Moulds
409	**OTU_369**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
410	**OTU_382**	Inocybe griseolilacina	Inocybaceae	Agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
411	**OTU_426**	Cortinarius sp.	Cortinariaceae	Agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
412	**OTU_445**	Acremonium sp.	Unknown	Hypocreales	Sordariomycetes	Ascomycota	saprotroph
413	**OTU_396**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
414	**OTU_397**	Fusarium sp.	Nectriaceae	Hypocreales	Sordariomycetes	Ascomycota	Unknown
415	**OTU_442**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal

416	**OTU_401**	Laccaria lacacata	Hydnangiaceae	Agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
417	**OTU_463**	Paecilomyces marquandii	Trichocomaceae	Hypocreales	Sordariomycetes	Ascomycota	saprotroph
418	**OTU_501**	Chalara sp.	Unknown	Unknown	Unknown	ascomycota	saprotroph
419	**OTU_594**	Unknown	geoglassaceae	geoglossales	Leotiomycetes	ascomycota	Unknown
420	**OTU_444**	Preussia funiculata/fleischhakii/flanaganii	Sporomiaceae	pleosporales	Dothideomycetes	ascomycota	saprotroph
421	**OTU_472**	Beauveria bassiana	Cordycipitaceae	Hypocreales	Sordariomycetes	Ascomycota	saprotroph
422	**OTU_377**	Russula sp.	Russulaceae	Agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
423	**OTU_464**	Cortinarius sp.	Cortinariaceae	Agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
424	**OTU_471**	Unknown	Hygrophoraceae	Agaricales	Agaricomycetes	basidiomycota	Unknown
425	**OTU_356**	Unknown	Unknown	Unknown	Agaricomycetes	basidiomycota	Unknown
426	**OTU_448**	Unknown	Gloniaceae	Hysteriales	Dothideomycetes	Ascomycota	Unknown
427	**OTU_452**	Unknown	Unknown	Sporidiobolales	Microbotryomycetes	Basidiomycota	Unknown
428	**OTU_418**	Unknown	Unknown	Unknown	Eurotiomycetes	Ascomycota	Unknown
429	**OTU_468**	Volutella sp.	Nectriaceae	Hypocreales	Sordariomycetes	Ascomycota	saprotroph
430	**OTU_358**	Clavulina rugosa	Clavulinaceae	Cantharellales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
431	**OTU_411**	Unknown	Unknown	Helotiales	Leotiomycetes	Ascomycota	Unknown
432	**OTU_437**	Unknown	Unknown	Unknown	Unknown	Ascomycota	Unknown
433	**OTU_519**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
434	**OTU_528**	Cenococcum sp.	Gloniaceae	Hysteriales	Dothideomycetes	ascomycota	Ectomycorrhizal
435	**OTU_366**	Hygrocybe insipida	Hygrophoraceae	agaricales	Agaricomycetes	basidiomycota	Moss associated basidiomycetes
436	**OTU_487**	Aureobasidium pullulans	xilariaceae	Unknown	Sordariomycetes	ascomycota	saprotroph
437	**OTU_434**	Unknown	Unknown	Unknown	Sordariomycetes	ascomycota	Unknown
438	**OTU_443**	Inocybe sp.	Inocybaceae	agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
439	**OTU_514**	Unknown	Unknown	Unknown	Unknown	ascomycota	Unknown
440	**OTU_432**	Unknown	Botryobasidiaceae	Cantharellales	Agaricomycetes	basidiomycota	Unknown
441	**OTU_498**	Odiodendron periconioides	Myxotrichaceae	Unknown	Leotiomycetes	ascomycota	ericoid mycorrhizal
442	**OTU_383**	Unknown	Spizellomycetaceae	Spizellomycetales	Chytridiomycetes	basidiomycota	Unknown
443	**OTU_439**	Geopora sp.	Pyronemataceae	Pezizales	Pezizomycetes	Ascomycota	Unknown

444	**OTU_457**	Unknown	Atheliaceae	atheliales	agaricomycetes	Basidiomycota	Unknown
445	**OTU_466**	Coniophora arida	coniophoraceae	Boletales	Agaricomycetes	Basidiomycota	saprotroph
446	**OTU_483**	Dothidea sp.	Dothioraceae	Dothideales	Dothideomycetes	Ascomycota	saprotroph
447	**OTU_441**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	basidiomycota	Ectomycorrhizal
448	**OTU_512**	Unknown	Dermateaceae	Helotiales	Leotiomycetes	Ascomycota	Unknown
449	**OTU_530**	Otidea sp.	Pyronemataceae	Pezizales	Pezizomycetes	Ascomycota	Ectomycorrhizal
450	**OTU_453**	Unknown	Unknown	Unknown	Unknown	ascomycota	Unknown
451	**OTU_589**	Tomentella lilacinogrisea	Telephoraceae	Telephorales	Agaricomycetes	basidiomycota	Ectomycorrhizal
452	**OTU_610**	Lepista nuda/glaucocana/sordida	Tricholomataceae	Agaricales	Agaricomycetes	basidiomycota	saprotroph
453	**OTU_497**	Unknown	Unknown	Unknown	Orbiliomycetes	Ascomycota	Unknown
454	**OTU_502**		Unknown	Unknown	Unknown	Unknown	Unknown
455	**OTU_566**	Unknown	Gloniaceae	Hysteriales	Dothideomycetes	Unknown	Unknown
456	**OTU_398**		Unknown	Unknown	Unknown	No match	Unknown
457	**OTU_414**	Unknown	Unknown	Pezizales	Pezizomycetes	ascomycota	Unknown
458	**OTU_504**	Unknown	Telephoraceae	Telephorales	Agaricomycetes	basidiomycota	Unknown
459	**OTU_520**	Lophiostoma cynaroidis	Lophiostomaceae	pleosporales	Dothideomycetes	Ascomycota	saprotroph
460	**OTU_554**	Lecanicillium psalliotae	Cordycipitaceae	Hypocreales	Sordariomycetes	Ascomycota	saprotroph
461	**OTU_583**	Archaerorhizomyces sp.	Archaeorhizomycetaceae	Archaeorhizomycetales	Archaeorhizomycetes	Ascomycota	Root associated
462	**OTU_393**	Mycena angusta/rebandeugoi	Mycenaceae	agaricales	Agaricomycetes	Basidiomycota	saprotroph
463	**OTU_499**	Unknown	Venturiaceae	Venturiales	Dothideomycetes	Ascomycota	Unknown
464	**OTU_500**	Unknown	Atheliaceae	atheliales	Agaricomycetes	basidiomycota	Unknown
465	**OTU_517**	Lepiota pseudolilacea	Agaricaceae	Agaricales	Agaricomycetes	basidiomycota	Soil saprotroph
466	**OTU_446**	Sydowia polyspora	Dothioraceae	Dothideales	Dothideomycetes	Ascomycota	saprotroph
467	**OTU_460**	Geminibasidium sp.	Geminibasidiaceae	Geminibasidiales	Wallemiomycetes	basidiomycota	saprotroph
468	**OTU_476**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Black yeast
469	**OTU_529**	Unknown	Unknown	pleosporales	Dothideomycetes	ascomycota	Unknown
470	**OTU_652**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Black yeast
471	**OTU_402**	Mortierella sp.	Mortierellaceae	Mortierellales	Incertae sedis	Zygomycota	Moulds

472	**OTU_424**	Mycena calvicularis/amicta	Mycenaceae	agaricales	Agaricomycetes	basidiomycota	saprotroph
473	**OTU_425**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
474	**OTU_440**	Tomentella terrestris	Telephoraceae	Telephorales	Agaricomycetes	basidomycota	Ectomycorrhizal
475	**OTU_431**	Unknown	Unknown	agaricales	Agaricomycetes	Basidiomycota	Unknown
476	**OTU_451**	Sebacina incrustans	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	ectomycorrhizal
477	**OTU_458**	Mucor sp.	Mucoraceae	Mucorales	Incertae sedis	Zygomycota	Moulds
478	**OTU_496**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	basidiomycota	Ectomycorrhizal
479	**OTU_538**	Spizellomyces sp.	Spizellomycetaceae	Spizellomycetales	Chytridiomycetes	basidiomycota	Pathogen
480	**OTU_406**	Russula sp.	Russulaceae	agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
481	**OTU_474**	Lepiota echinella	Agaricaceae	Agaricales	Agaricomycetes	basidiomycota	Soil saprotroph
482	**OTU_409**	Unknown	Unknown	Unknown	Agaricomycetes	basidiomycota	Unknown
483	**OTU_421**	Unknown	Unknown	Unknown	agaricomycetes	basidiomycota	Unknown
484	**OTU_465**	Clavulinopsis spp.	Clavariaceae	Agaricales	Agaricomycetes	Basidiomycota	Unknown
485	**OTU_490**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	ascomycota	Unknown
486	**OTU_712**	Unknown	Unknown	Unknown	Unknown	ascomycota	Unknown
487	**OTU_433**	Hygrocybe sp.	Hygrophoraceae	agaricales	Agaricomycetes	basidiomycota	Moss associated basidiomycetes
488	**OTU_477**	Terfezia sp.	Pezizaceae	Pezizales	Pezizomycetes	ascomycota	Ectomycorrhizal
489	**OTU_479**	Unknown	Unknown	Unknown	Unknown	Strange	Unknown
490	**OTU_511**	Inocybe sp.	Inocybaceae	Agaricales	agaricomycetes	basidiomycota	Ectomycorrhizal
491	**OTU_576**	Unknown	geoglassaceae	geoglossales	Geoglomycetes	ascomycota	Unknown
492	**OTU_603**	Unknown	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Unknown
493	**OTU_605**	Unknown	Unknown	Unknown	Dothideomycetes	Ascomycota	Unknown
494	**OTU_482**	Tomentella sp.	Telephoraceae	Telephorales	agaricomycetes	basidiomycota	Ectomycorrhizal
495	**OTU_489**	Unknown	Unknown	Capnodiales	Dothideomycetes	ascomycota	Unknown
496	**OTU_523**	Unknown	Unknown	agaricales	agaricomycetes	basidiomycetes	Unknown
497	**OTU_612**	Unknown	Unknown	Unknown	Leotiomycetes	Ascomycota	Unknown
498	**OTU_481**		Unknown	Unknown	Unknown	Strange	Unknown
499	**OTU_491**	Tomentella sp.	Telephoraceae	Telephorales	Agaricomycetes	basidiomycota	Ectomycorrhizal

500	**OTU_524**	Rhodotorula sp.	Unknown	Sporidiobolales	Microbotryomycetes	basidiomycetes	yeast
501	**OTU_537**	Trichothecium crotocinigenum	Unknown	hypocreales	Sordariomycetes	ascomycota	Pathogen
502	**OTU_544**	Glutinoglossum sp.	Geoglossaceae	Geoglossales	Geoglossomycetes	Ascomycota	saprotroph
503	**OTU_561**	Alternaria alternata	Pleosporaceae	pleosporales	Dothideomycetes	Ascomycota	Moulds
504	**OTU_588**	Inocybe sp.	Inocybaceae	Agaricales	agaricomycetes	basidiomycota	Ectomycorrhizal
505	**OTU_676**	Tetracladium furcatum/serigeum	Unknown	Helotiales	Leotiomycetes	ascomycota	Unknown
506	**OTU_488**	Geoglossum vleugenianum/lineare	geoglassaceae	geoglossales	Geoglomycetes	Ascomycota	Unknown
507	**OTU_493**	Chroogomphus rutilus	gomphidiaceae	Boletales	Agaricomycetes	basidiomycota	Ectomycorrhizal
508	**OTU_508**	Sebacina sp.	Sebacinaceae	Sebacinales	Agaricomycetes	Basidiomycota	Unknown
509	**OTU_546**	Unknown	Unknown	Unknown	Orbiliomycetes	Ascomycota	Unknown
510	**OTU_557**	Peziza phyllogena/polaripapulata	Pezizaceae	Pezizales	Pezizomycetes	Ascomycota	Ectomycorrhizal
511	**OTU_574**	Unknown	Unknown	Helotiales	Leotiomycetes	ascomycota	Unknown
512	**OTU_585**	Unknown	Unknown	Helotiales	Leotiomycetes	ascomycota	Unknown
513	**OTU_608**	Unknown	Unknown	Helotiales	Leotiomycetes	ascomycota	Unknown
514	**OTU_617**	Geoglossum bruneipes	geoglassaceae	geoglossales	Geoglomycetes	Ascomycota	saprotroph
515	**OTU_450**	Inocybe sp.	Inocybaceae	Agaricales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
516	**OTU_509**	Hydnum sp.	Hydnaceae	Cantharellales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
517	**OTU_532**	Cladophialophora sp.	Herpotrichiellaceae	Chaetothyriales	Eurotiomycetes	Ascomycota	Black yeast
518	**OTU_478**	Mortierella hyalina	Mortierellaceae	Mortierellales	Incertae sedis	Zygomycota	Moulds
519	**OTU_503**	Unknown	Unknown	Orbiliales	Orbiliomycetes	ascomycota	Unknown
520	**OTU_506**	Tomentella sp.	Thelephoraceae	Thelephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
521	**OTU_507**	Sclerotinia subarctica/Pycnopeziza sympodialis	Sclerotiniaceae	Helotiales	Leotiomycetes	ascomycota	Pathogen
522	**OTU_516**	Pyrenochaeta	Unknown	Pleosporales	Dothideomycetes	Ascomycota	Unknown
523	**OTU_597**	Gorgomyces honrubiae	Unknown	Unknown	Unknown	Unknown	Unknown
524	**OTU_423**	Mycena thymicola	Mycenaceae	Agaricales	Agaricomycetes	Basidiomycota	saprotroph
525	**OTU_526**	Tetracladium sp.	Unknown	Helotiales	Leotiomycetes	ascomycota	Unknown
526	**OTU_602**	Unknown	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota	Unknown
527	**OTU_672**	Cryptosporiopsis ericae/radicicola	Dermateaceae	Helotiales	Leotiomycetes	ascomycota	saprotroph

528	**OTU_405**	Unknown	Tricholomataceae	Agaricales	Agaricomycetes	basidiomycota	Unknown
529	**OTU_436**	Unknown	Unknown	Unknown	Orbiliomycetes	Ascomycota	Unknown
530	**OTU_454**	Pseudotomentella sp	Thelephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
531	**OTU_469**	Ramariopsis kunzei	Clavariaceae	agaricales	agaricomycetes	basidiomycota	saprotroph
532	**OTU_486**	Tomentella sp.	Telephoraceae	Telephorales	agaricomycetes	basidiomycota	Ectomycorrhizal
533	**OTU_494**	Russula maculata	Russulaceae	Agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
534	**OTU_505**	Unknown	Unknown	Agaricales	Agaricomycetes	basidiomycota	Unknown
535	**OTU_533**	Unknown	Unknown	Unknown	Agaricomycetes	basidiomycota	Unknown
536	**OTU_567**	Tomentella cinerascens	Thelephoraceae	Thelephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
537	**OTU_595**	Unknown	Unknown	hypocreales	Sordariomycetes	Ascomycota	Unknown
538	**OTU_659**	Pochonia sp.	Clavicipitaceae	Hypocreales	Sordariomycetes	Ascomycota	saprotroph
539	**OTU_413**	Rhodotorula sp.		Sporidiobolales	Microbotryomycetes	basidiomycetes	yeast
540	**OTU_462**	Hemimycena sp.	Mycenaceae	Agaricales	Agaricomycetes	basidiomycota	saprotroph
541	**OTU_480**	Inocybe rimosa/mimica	Inocybaceae	agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
542	**OTU_534**	Cortinarius haasi/barbarorum/subgracilis	Cortinariaceae	Agaricales	Agaricomycetes	Basidiomycota	ectomycorrhizal
543	**OTU_543**	Trichosporon moniliiforme	Trichosporonaceae	Tremellales	agaricomycetes	basidiomycota	saprotroph
544	**OTU_616**	Unknown	Unknown	Unknown	Leotiomycetes	ascomycota	Unknown
545	**OTU_634**	Tomentella sp.	Telephoraceae	Telephorales	agaricomycetes	basidiomycota	Ectomycorrhizal
546	**OTU_400**	Unknown	Unknown	Sebacinales	Agaricomycetes	Agaricomycetes	Unknown
547	**OTU_449**	Unknown	Unknown	Unknown	Agaricomycetes	Basidiomycota	Unknown
548	**OTU_525**	Unknown	Unknown	pleosporales	Dothideomycetes	Ascomycota	Pathogen
549	**OTU_637**	Xerocomus subtomentosus	Boletaceae	Boletales	Agaricomycetes	Basidiomycota	Ectomycorrhizal
550	**OTU_664**	Russula ruberrima	Russulaceae	Agaricales	Agaricomycetes	basidiomycota	Ectomycorrhizal
551	**OTU_475**	Pseudotomentella sp	Thelephoraceae	Telephorales	Agaricomycetes	Basidiomycota	Ectomycorrhizal

CHAPTER IV

Weather variation but not thinning drives inter-annual changes in fungal composition and diversity in a Mediterranean pine forest

Under review in Agricultural and Forest Meteorology

Weather variation but not thinning drives inter-annual changes in fungal composition and diversity in a Mediterranean pine forest

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Abstract

Predicted changes in global climate might negatively affect soil microbiome and associated ecosystem processes in Mediterranean forests. Simultaneously, forest treatments such as thinning have been suggested to mitigate climate change impacts on forests by reducing competition between trees, thus increasing water availability. However, studies addressing the combined effects of climate and forest thinning on belowground fungal communities are still scarce, being fundamental to elaborate adaptive strategies to global warming.

The aim of this study was to evaluate the short-term tree density reduction effects on soil fungal communities and their response to inter-annual changes in weather conditions. The temporal dynamics of soil fungal communities in relation to these two drivers (i.e., forest management and weather conditions) were studied from 2009 until 2014 in a set of 24 thinned and un-thinned paired plots dominated by *Pinus pinaster* Ait. Thinning was conducted in 2009 (from 30% up to 70% reduction in stand basal area). Here, we only used the autumn precipitation and temperature to describe the impact of inter-annual weather changes. We used Pacific Biosciences sequencing of fungal ITS2 amplicons to study the fungal communities from soil samples. Fungal species composition changed across years, both at species and functional level. These changes were partly driven by inter-annual changes in precipitation and temperature, with free-living fungi increasing their presence in wetter conditions, and symbiotic fungi under

drier and colder conditions. Thinning did not affect fungal community composition nor fungal species richness and diversity, indicating that the soil fungal community is resistant at short-term to forest thinning regardless of its intensity.
We show important shifts in community composition and function in response to inter-annual changes in weather conditions. Free-living fungi such as moulds or yeasts may be negatively affected by increasing drought autumns in Mediterranean forest ecosystems.
Keywords: forest management, mycorrhizal, climate, drought, saprotrophs, fungal diversity

1. Introduction

Fungi represent an important part of the soil microbial community, and are represented by several functional guilds that affect important ecosystem processes, such as soil organic matter (SOM) decomposition, nutrient cycling, plant production or carbon storage (Clemmensen et al., 2013; Fernandez et al., 2016a). One of the most important functional groups of the soil microbiome is represented by mycorrhizal species, because they are important components for tree nutrition and nutrient mobilization (Smith and Read, 2008). The extramatrical mycelia (EMM) of these fungi explore the soil surrounding the host tree, foraging for nutrients and forming mycorrhizae with root tips of different tree hosts (Cairney, 2012). At the same time, the high fungal turnover rates in forests contribute to SOM formation in forest soils (Fernandez et al., 2016a). In drier ecosystems such as Mediterranean forests, mycorrhizal fungi are important for plant water acquisition both, by providing plant roots with access to water otherwise less accessible to host vegetation, and by improving soil structure which enhances soil water retention capacity (Allen, 2007; Querejeta, 2017). Besides the important role of mycorrhizal fungal species, other functional guilds such as saprotrophs also have a paramount role in litter degradation (Baldrian et al., 2011), including dead mycelia (Lindahl et al., 2010). Thus, fungal community changes in these ecosystems will have important consequences for nutrient cycling and water acquisition by plants and therefore impact plant communities (Sardans and Peñuelas, 2013).

Global change is one of the most important threat for many Mediterranean ecosystems (Thuiller et al., 2008). In the Mediterranean basin, temperature has been forecasted to rise between 1.4°C and 5.1°C by 2055 (Nogués Bravo et al., 2008), and total annual precipitation projections show a tendency towards less precipitation, with more extreme rainfall events (García-Ruiz et al., 2011) and a soil moisture reduction (Dai, 2013). Indeed, ecosystem alterations, local extinctions and phenological changes in these ecosystems have already been associated to current climate change (Peñuelas et al., 2002). Also, predicted drought increase in Mediterranean forests will likely reduce plant growth and aboveground biomass (Sardans and

Peñuelas, 2013), probably causing a cascading effect belowground (Alday et al., 2017a; Cairney, 2012). Shifts in belowground fungal communities are related to changes in carbon storage, nutrient cycling and ecosystem productivity in boreal and temperate ecosystems (Clemmensen et al., 2013; Solly et al., 2017).

Several studies have shown phylogenetic and trait-specific contrasting climate responses of belowground fungal communities (Cairney, 2012; Deslippe et al., 2011; Fernandez et al., 2016b). Climate effects on fungi may be directly driven by changes in temperature and moisture (Santalahti et al., 2016; Voříšková et al., 2013) or indirectly by changes in host performance (Deslippe et al., 2011; Fernandez et al., 2016b; Hartmann et al., 2017), host activity (Högberg et al., 2010), soil properties or litter cover changes (Vašutová *et al.*, 2016). In drier ecosystems, some mycorrhizal ascomycetes may be more abundant (Gordon and Gehring, 2011; Smith et al., 2007), whereas wetter conditions may favour opportunistic species such as moulds or yeasts (Castaño et al., 2016; 2017). Climate effects on fruiting body emergence and production in Mediterranean forests have been well studied, being precipitation and temperature strongly influencing emergence and production (Hernández-Rodriguez et al., 2015; Alday et al., 2017b). However, studies on belowground fungal responses to climate changes in drier ecosystems such as Mediterranean forests are still scarce and how belowground fungal community is affected by weather is still unknown.

Forest thinning has been suggested as a management option to mitigate climate change impacts on Mediterranean forests, because it increases water availability and water use efficiency of trees, thus changing soil microclimatic conditions. For example, Aldea et al. (2017) found that thinning increased radial growth of both conifer and oak species together with an increased resistance to drought and improved stand growth. Similarly, positive thinning effects have been observed on the aboveground production of important fungal species (Bonet et al., 2012; Shaw et al., 2003), although such effects are species-dependent. Similarly, sustainable forest harvesting regimes has been predicted to positively influence mushroom production (de-Miguel et al., 2014). In contrast, clear-cutting and associated logging disturbances have shown clear negative impact on mycorrhizal communities living in soils (Hartmann et al., 2012; Jones et al., 2003; Kyaschenko et al., 2017; Parladé et al., 2017) and eventually on carbon balance (Paul-Limoges et al., 2015). Logging effects on mycorrhizal communities is likely dependent on whether these communities can survive in symbiosis with the non-thinned trees (Amaranthus and Perry, 1987; Rosenvald and Lõhmus, 2008). Tree removal effects on fungal communities may also be caused by changes on environment conditions, such as microclimate or soil biochemistry (Hartmann et al., 2012; Jones et al., 2003). Although forest thinning may have lighter impact than clear-cutting, its importance in relation

to the impact on soil fungal communities has not been quantified yet.

In this study we analysed the short-term inter-annual dynamics of soil fungal communities during 4 years in response to forest thinning in 24 long-term experimental plots dominated by *Pinus pinaster* Ait. These plots represent a gradient of stand basal area and number of trees. In addition, we analysed in both thinned and un-thinned plots whether there is a correlation between yearly changes in autumn precipitation and temperature and the fungal community composition from a functional perspective. Our objective was to test whether inter-annual changes in precipitation and temperature during autumn affect the soil fungal communities. In previous works approaching intra-annual variations in fungal communities, we observed that mycorrhizal fungi were less affected by drought than free-living fungi, whereas these free-living fungi were stimulated by wetter conditions (Castaño et al., *unpublished*). However, whether these functional changes follow an inter-annual pattern is still unknown. We specifically hypothesize that i) light-medium thinning will not produce soil fungal compositional changes nor diversity changes belowground, because enough trees remain in the plots to sustain existing fungal community. In contrast, ii) a fungal species compositional and functional changes will be expected in heavy thinning treatments since the lack of enough remaining trees to sustain populations of mycorrhizal fungi may cause a decrease in the relative proportions of mycorrhizal species. Regarding the inter-annual changes in the fungal community, we hypothesize that iii) expected changes in the fungal community across years should be partly driven by changes in autumn precipitation and temperature during autumn, with mould species and yeasts being stimulated under wetter conditions.

2. Material and Methods

2.1 Site selection

The study was carried out at a long-term experimental setup located in the natural area of Poblet (PNIN) (Northeast Spain, 41º 21' 6.4728'' latitude and 1º 2' 25.7496'' longitude), where 24 paired plots were established to test the effect of forest thinning on mushroom production since 2009 (Bonet et al., 2012). The plots consisted of even-aged *Pinus pinaster* stands (60-years-old), with isolated *Quercus ilex* trees sometimes forming shrubs, while the understory was dominated by *Erica arborea*, *Arbutus unedo* and *Calluna vulgaris*. Mean annual temperature at the study site is 11.8 ºC, and mean annual rainfall is 666.5 mm, with a pronounced summer drought that usually lasts for three months (June to August). Autumn precipitation (September to November) during the study years was very similar across plots (136.8±3), but showing much variation across years (136.8±86.4), whereas temperature variation was slightly higher across plots (16.06±1.13ºC) than across years (16.06±0.84). Plots

are similar in soil properties, but as a result of the forest thinning applied in 2009, their stand characteristics differ considerably, i.e., ranging from 16.5 to 81.7 m^2 ha^{-1} in stand basal area, and from 350 to 2,657 trees ha^{-1} in stand density. Soils are siliceous with sandy loam texture, average pH 6.7±0.27, average total N 0.21±0.06% and organic matter (OM) 5.5±2%.

2.2 Thinning experiment

Of the total of 24 paired plots, twelve plots were thinned in July and August 2009 leaving 12 nearby paired plots undisturbed. Thinned plots were 1600-m^2 plots (40m×40m). In all these plots, stand structure was more or less homogeneous, with trees having similar heights and diameters within plots. In thinned plots, trees were systematically removed without the use of heavy machinery to avoid confounding effects caused by soil disturbance, using a chainsaw and removing the cut trees from the plot. As a result, basal area was reduced from 30% to 70% in the thinned plots. The most thinned plot resulted in a remaining stand basal area of 16.5 m^2 ha^{-1} and stand density of 350 trees ha^{-1}. Further information about the thinning treatments and the stand variables before and after the treatments is available in Bonet et al. (2012).

2.3 Soil sampling

Soil sampling was conducted in all 24 plots in November 2009, 2012, 2013 and 2014. Since soil was not sampled in 2010 and 2011 due to funding limitations, our focus was on the immediate thinning effect (2009) and the medium-term effect (2012-2014). Each year, 8 soil cores (12 cm deep, 5 cm in diameter) were systematically sampled with a metallic probe in each plot (two along each side of the plot), amounting to a total of 768 soil samples. In these samplings, needles and partially decomposed needles were excluded since fungal community composition in the duff layer mostly consists of saprotrophic fungi (Clemmensen et al., 2013; Voříšková et al., 2013). Thus, only humus and mineral soil was considered for sampling. Soil samples were sieved using 3mm mesh and stored at 4ºC during <24h. Samples were then freeze-dried and pooled by plots. Each of the 96 composite soil samples (24 plots and 4 years) was ground to fine powder using mortar and pestle. The resulting fine powder was stored at -20ºC before DNA extraction.

2.4 Fungus-specific amplicon sequencing

Genomic fungal DNA was extracted from 500 mg of each soil sample using NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol, but using 900 µl of lysis buffer (SL1). Each sample was subjected to PCR-amplification of the ITS2 region from 25 ng of genomic DNA, using gITS7 and ITS4 primers (Ihrmark et al., 2012) in a 2720 Thermal Cycler (Life Technologies). Unique 8 bp tags, differencing in at least 3 positions,

were added to each primer for sample labelling. The number of necessary PCR cycles was optimized, with most of the samples amplifying well at 21-24 cycles. Each sample was amplified in triplicates with negative controls (using sterile water) from DNA extraction and PCR included. Final concentrations in the 50 µl PCR reaction mixtures were: 25 ng template, 200 µM of each nucleotide, 2.75 mM MgCl2, primers at 200 nM, 0.025 U μL^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA) in 1X buffer PCR. PCR cycling conditions were as follows: 5 min at 95ºC, followed by 24-30 cycles of 30 s at 95ºC, 30 s at 56 ºC, 30 s at 72 ºC and a final extension step at 72 ºC for 7 min before storage at 4 ºC. Each sample was amplified in triplicate, including the negative control of both the DNA extraction and PCR. PCR products were purified using AMPure kit (Beckman Coulter Inc. Brea, CA, USA) and quantified using Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled and purified using EZNA Cycle Pure kit (Omega Bio-Tek). Quality control of purified amplicons was carried out using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA) and 7500 DNA chip. Samples were sequenced at SciLifeLab NGI, Uppsala, Sweden on a PacBio RS II system based on the Real-Time (SMRT®) technology.

2.5 Quality control and bioinformatic analysis

Quality control, filtering and sequence clustering was assessed with the SCATA pipeline (scata.mykopat.slu.se). Sequences with an average quality score <20, individual bases with a quality score <10 or a sequence length < 200 bp were removed. Remaining sequences were screened both for primers (requiring 90% primer match) and sample tags. After collapsing homopolymers to 3 bp, sequences were pair-wise compared with 'usearch' (Edgar, 2010). Pairwise alignments were scored using a mismatch penalty assigned at 1, gap open penalty of 0 and a gap extension penalty with value 1. Sequences were clustered into operational taxonomic units (OTUs) based on the Species Hypothesis (SHs) concept (Koljalg et al., 2014) using single linkage clustering with a maximum distance of 1.5% to the closest neighbour required to enter clusters.

2.6 Taxonomic and functional identification

We assigned a putative taxonomical identity to the 500 most abundant OTUs, representing 93% of the total DNA sequence reads. The most abundant sequence from each OTU was selected for taxonomical identification, using massBLASTer in PlutoF against the UNITE (Abarenkov et al., 2010) and INSD database. Taxonomic identities were assigned to SHs (> 98.5% similar), or on supported monophyletic neighbour-joining clades including reference sequences from UNITE and INSD databases. Functional roles of the SHs were assigned as

follows: a) mycorrhizal b) root-associated ascomycetes, c) moulds, d) yeasts, e) black yeasts, f) saprotrophs or litter-decay fungi, g) soil saprotrophs (taxa found in mineral N-rich soils), h) pathogens, i) moss-associated fungi, and j) unknown function, based on the UNITE database and from DEEMY (www.deemy.de) or other published literature. Assignation of fungi to exploration types was done based on DEEMY (www.deemy.de) and Agerer, (2001, 2006).

2.7 Climate data

We obtained daily weather variables (precipitation and temperature) from 2009, 2012, 2013, 2014 (September, October, November) for each of the 24 plots, following DAYMET methodology (Thornton et al., 2000), as implemented in the R package 'meteoland' (De Cáceres et al., 2017). In short, daily precipitation and temperature was estimated for each plot by averaging the values of several Catalan and Spanish meteorological stations (1990-2015), weighting factors that depended on the geographic proximity to the target plot and correcting for differences in elevation between the station and the target plot. We used the total precipitation and temperature values for September-November (Average). Precipitation occurring the same sampling week (last week of November) was not considered.

2.8 Data analysis

The fungal community data set was subjected to multivariate analyses using CANOCO version 5.0 (Biometris Plant Research International, Wageningen, The Netherlands) and R software environment (version 3.0.2; R Development Core Team 2013) using "nlme" package for linear mixed-effects models (LME; Pinheiro *et al.,* 2016). Species data were square-rot transformed (Hellinger transformation) to down weight the less abundant SHs before conducting the analyses, and only OTUs with more than 5 occurrences were considered.

2.8.1 The effect of forest thinning on fungal community composition

Principal Response Curves (PRC) were used to study whether there was a forest thinning effect on fungal community composition. Here, year was defined as a factor with 4 levels (2009, 2012, 2013, 2014), whereas thinning intensity (% reduction in basal area) was defined as explanatory factor with 4 levels (control: 0% thinned, light: 20-30% thinned, medium: 30%-50% thinned, and heavy: 50-70% thinned). The thinning effect was tested using Monte Carlo simulations (999 permutations). Similarly, the short-term effects of forest thinning were tested considering only data from 2009 using the fungal community composition as response variable and either the basal area or number of trees removed or left as the explanatory variable. This test was performed in three independent tests considering, alternatively, the (i) relative abundance in species composition, (ii) relative abundances of the functional guilds, and (iii)

relative abundances of each exploration type as response data.

2.8.2 Inter-annual changes in fungal community composition

To obtain graphical representation of the fungal community similarity between years a Detrended correspondence analysis (DCA) was used. The significance of these changes on fungal community composition across years was tested using canonical correspondence analysis (CCA). Here, plot identity was defined as a covariate factor, and years were randomly permuted using Monte Carlo (999 permutations). Thus, tests were carried out without permutation between spatial replicates within single years. This test was performed in three independent response datasets: (i) the total relative abundance in species composition, (ii) the relative abundances of the functional guilds and (iii) the relative abundances of each exploration type. Similarly, we tested whether these yearly changes in fungal community were correlated with autumn precipitation and temperature using a CCA with the same permutation scheme, but using forward selection of explanatory variables to disentangle the proportion of explained variation from each variable (Precipitation and temperature). We used only autumn precipitation because previous studies using soil samples from the same site showed fast responses of the fungal community to changes in soil moisture and temperature (Castaño et al., 2016; 2017). Changes in relative abundance of each functional guild and exploration type in response to changes in precipitation and temperature were studied using linear mixed-effects (LME) models from square-root transformed relative proportions. Here, plot was defined as random factor and both autumn temperature and precipitation were defined as fixed terms.

2.8.3 Thinning and climatic effects on fungal richness and diversity

Hill's series of diversity indices were used to compare differences in diversity values between years and between thinning intensities (Hill, 1973). 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. We did not rarefy the fungal community due to the potential information loss. Instead, we used square-root transformation of read counts as an explaining variable when testing for the Hill's numbers (Bálint et al., 2015) to account for the uneven read distribution attributed to the sequencing bias. LME models were used to test significant changes on Hill's numbers between years and to test the effect of forest thinning on Hill's values. In these analyses, plot identity was defined as a random factor, whereas year identity and thinning intensity, as well as their interaction, were defined as fixed factors. Similarly, short-term effects of forest thinning were tested using LME and considering only community data from 2009, where plot was defined as random and thinning intensity as fixed factor.

3. Results

3.1 Sequencing output and general community composition

We obtained a total of 75,608 ITS2 sequences after quality control. Single linkage clustering (1.5%) resulted in 2,134 OTUs after removing singletons, of which 500 (93% of the high-quality sequences) were assessed for identification at the level of species, functional guilds and exploration strategy. Overall, *Basidiomycota* phyllum was dominant (54±1.5%), followed by *Ascomycota* (32±0.9%), and others (13%). Among the known and identified functional groups, the most abundant were mycorrhizal fungi, representing 65±2% of the total number of sequences, followed by moulds (13.1±0.9%), black yeasts (4.3±0.3%), root-associated fungi (3.7±0.6%), saprotrophs (3.5±0.4%) and others (10.4%). Among the mycorrhizal fungi, species characterised by short and contact exploration types represented, respectively, 33.2±1.7% and 24.6±1.4% of known and identified exploration types, followed by medium fringe (22.2±1.5%), long exploration types (9.7%±1.5%) and others (10.3%).

3.2 Fungal community response to forest thinning

Forest thinning did not have any effect on fungal community composition (F=1.6, P= 0.621; Fig. 1), or functional guilds (F=6.0, P=0.255) or in the distribution of exploration type groups of mycorrhizal fungi (F=4.7, P=0.576), despite the inter-annual oscillations found. PRC showed that all thinning intensities were close to control line during the study period, which means that differences in community composition are very low and not significant (Fig. 1). Similar results were obtained using remaining basal area as an explanatory factor (F=1.9, P=0.584). In this case, all plots followed the same tendency regardless of whether they had been thinned or not and regardless of the thinning intensity. Forest thinning did not cause any change in functional guilds (F=6.0, P=0.255), nor in the distribution of exploration type groups of mycorrhizal fungi (F=4.7, P=0.576), despite the inter-annual oscillations found.

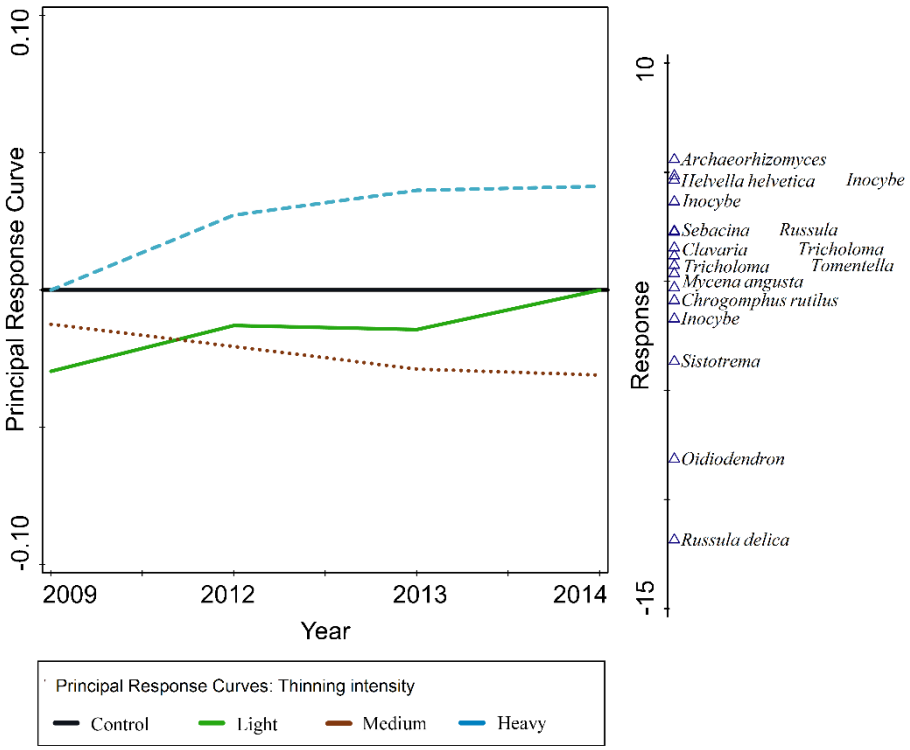

Fig. 1: Principal Response Curves obtained from the whole set of 24 plots. Thinning intensity effects (Heavy= 50-70% basal area thinned, Medium= 30-50% basal area thinned, Light= <30% basal area thinned, Control= Un-thinned) are tested, and the direction of changes in community composition across years is shown for all thinning intensity levels. Relative proportion increases of specific fungal species under certain thinning intensity treatment are shown on the right axis. Although some species were increased under specific thinning treatments (genera and species list in the right, Y-axis), the overall effects of the thinning treatments were not significant (F=1.6, P= 0.621).

3.3 Inter-annual variation of the fungal community

DCA of the 96 soil samples showed that fungal species composition varied significantly across years (Fig. 2a) with the significant year effects explaining 8.21% of total inertia (pseudo-F=2.2, P=0.001; Fig 2b). Fungal community at the first sampling year, just 3 months after tree removal, was the most different compared to 2012, 2013 and 2014, which were located at the negative end of the first CCA axis (Fig 2b).

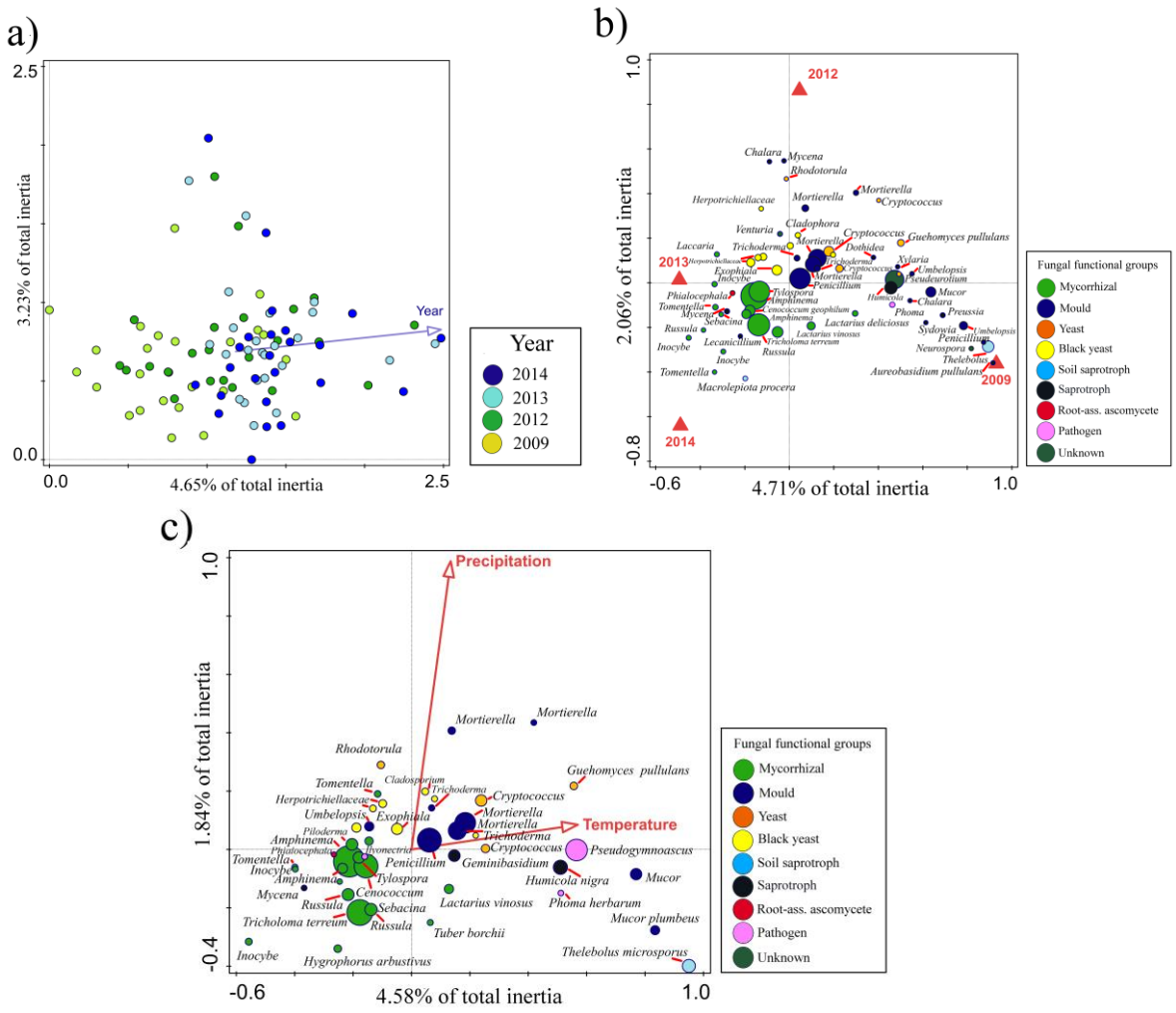

Fig. 2. Soil fungal Community compositional changes in time and in relation to precipitation and temperature. (a) Detrended correspondence analyses (DCA) of fungal community composition at species level across years. (b) Canonical Correspondence Analysis (CCA) and species plot of the yearly variation of the fungal community, in which the analysis was constrained by years, and (c) species plot showing the effects of temporal changes of temperature and precipitation. In (a), colours represent the sampling year, whereas in (b) and (c), colours represent different functional groups. In this figure, only the most abundant and ecologically relevant OTUs are shown. Species names are shown in italic in the plots. OTUs that were not identified at species level are shown at genus level.

Relative proportions of many mould species (*Mucor*, *Umbelopsis, Mortierella*), yeasts (*Cryptococcus*, *Guehomyces*) or potential plant pathogens (*Sydowia*, *Phoma*) were associated with fungal communities found in 2009 and 2012, whereas relative proportions from several mycorrhizal species (*Inocybe*, *Amphinema*) were more associated to the last sampling years (2013, 2014; Fig 2b). Forward selection of explanatory variables identified both temperature (70% of fitted variation, P=0.002) and autumn precipitation (30% of fitted variation, P=0.002) strongly related with observed changes in fungal community composition across years,

representing altogether 6.42% of the total inertia (Fig 2c).

When fungal communities were evaluated according to their functional guild identity, there was a significant correlation with autumn precipitation and temperature across years (pseudo-F= 12.6, P<0.001), accounting for 26.68% of the total inertia (Fig. 3a, 3b). This correlation was generally higher for temperature (82% of fitted variation) than for precipitation (18% of fitted variation). Again, 2009 was the most different in comparison with the other years, whereas 2013 and 2014 were more similar to each other (Fig. 3a). The overall proportion of amplicons attributed to mycorrhizal species was more abundant under colder and drier conditions (negative side of the first and second axis, respectively; Table 1, Fig. 3b). In contrast, the relative proportion of other functional guilds such as black yeasts, moulds and yeasts were more abundant under warmer and wetter conditions (positive side of the first and second axis, respectively; Table 1, Fig 3b). This was especially true during 2012, when autumn was especially wet, or 2009, when autumn was especially warm. Finally, no effects of precipitation and temperature were observed on litter saprotrophic taxa (Table 1). Despite these functional changes, exploration type proportions among mycorrhizal species did not change across years (pseudo-F=1.1, P=0.665).

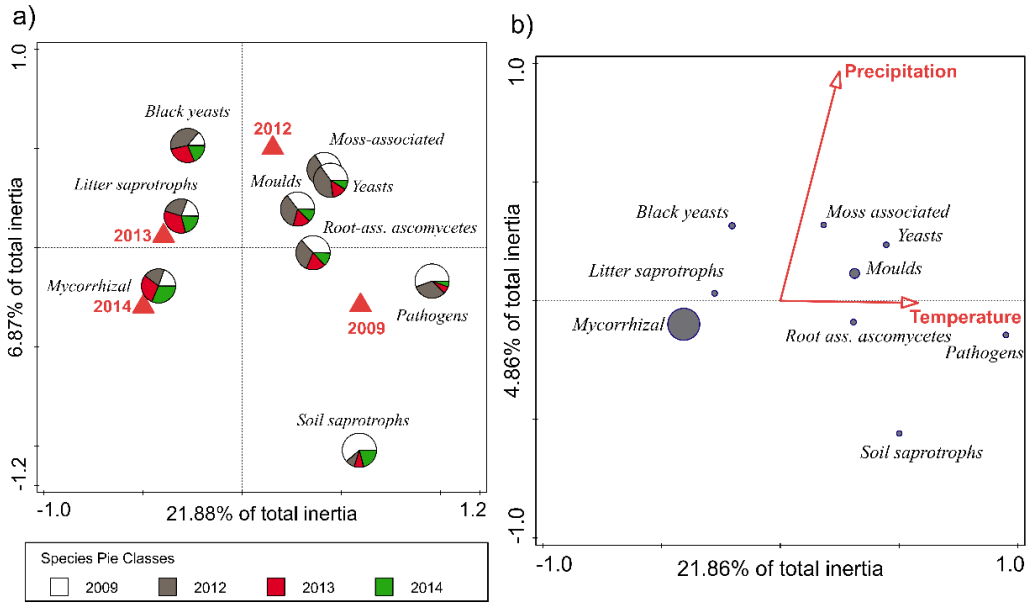

Fig. 3. CCA plots of the relative proportions from each functional guild constraining the years (a) and constraining by the averaged autumn temperature and precipitation values of each year (b). Symbols in (a) show the relative abundance of each functional guild recorded in each year. Symbol sizes in (b) are proportional to their average relative abundance. Functional identity is shown in italic.

Table 1: Fitting statistics of the LME models testing the changes in relative proportions of each functional group caused by autumn precipitation and temperature across years. In bold significant values. Negative values (-) show negative correlation.

	Precipitation		Temperature		Precip.×Temp.	
Functional guilds	F	P	F	P	F	P
Black yeast	29.37	**<0.001**	1.08 (-)	0.303	2.6	0.111
Mycorrhizal	20.16 (-)	**<0.001**	26.78 (-)	**<0.001**	0.55	0.459
Moss-associated	2.07	0.155	8.8	**0.004**	5.92	**0.017**
Moulds	23.07	**<0.001**	31.89	**<0.001**	5.08	**0.027**
Pathogen	1.99	0.163	58.96	**<0.001**	0.07	0.796
Root ass. ascomycetes	0.06	0.803	30.07	**<0.001**	4.04	**0.048**
Litter saprotroph	0.01 (-)	0.915	1.22	0.272	1.23	0.271
Soil saprotroph	2.15 (-)	0.147	2.32	0.132	0.76	0.386
Yeasts	16.94	**<0.001**	7.96	**0.006**	3.99	**0.049**

3.4 Inter-annual changes and forest thinning effects on fungal diversity

There was a significant year effect on mycorrhizal richness and diversity (all Hill's values being significant; Fig. 4a, Table 2a). Changes in mycorrhizal richness and diversity occurred mostly during the first year (2009), when the lowest richness and diversity values were observed. For instance, 27 mycorrhizal species were found in 2009, whereas 34, 35 and 37 species were found in 2012, 2013 and 2014, respectively. However, there were no significant changes in diversity (N2, N3) when considering the whole fungal community (i.e. including all functional guilds) and only richness (N0) was significantly different (Whole community section in Fig. 4: N0, Table 2). Despite the inter-annual changes in richness and diversity, forest thinning did not have any significant effect on fungal richness and diversity (Fig. 4b), nor the interaction between thinning and year (Table 2).

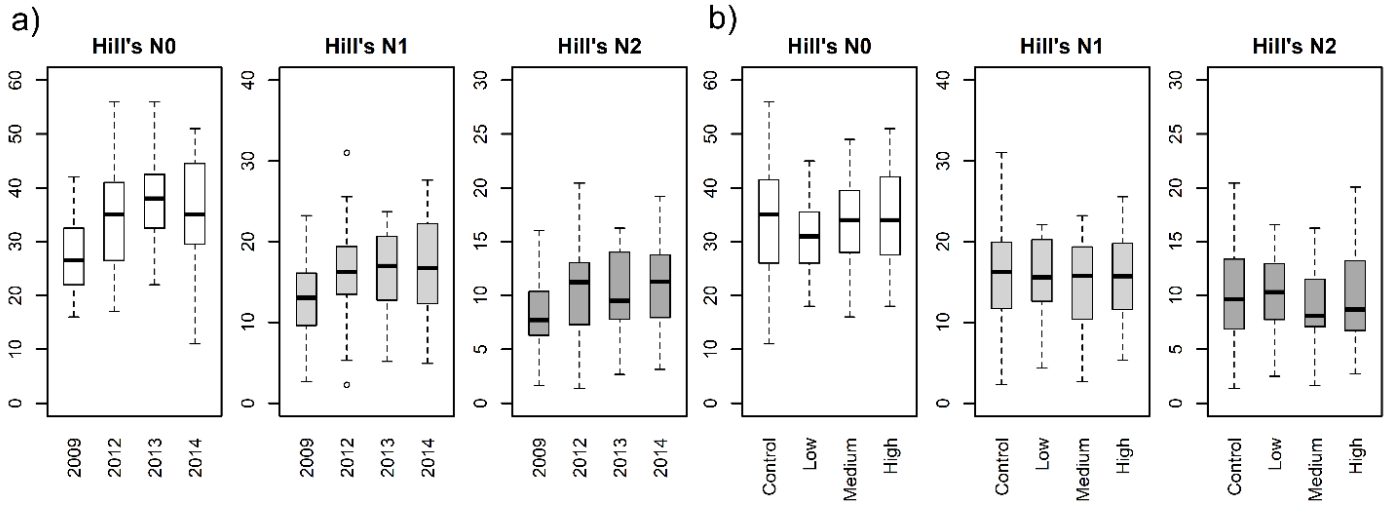

Fig 4. Hill's diversity values of the ectomycorrhizal community across the four years considered (a) and across the thinning treatments (b). In these analyses, relative proportion of each SHs was considered.

Table 2. Year and thinning effects on fungal diversity belowground observed for the (a) mycorrhizal and the (b) whole fungal community.

	(a) Mycorrhizal community							**(b) Whole fungal community**					
		Hill's N0		Hill's N1		Hill's N2		Hill's N0		Hill's N1		Hill's N2	
Effects	dF	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
Intercept	1	939.7	**<.0001**	371.5	**<.0001**	305.4	**<.0001**	3364.6	**<.0001**	647.6	**<.0001**	380.5	**<.0001**
Reads	1	66.2	**<.0001**	3.99	**0.05**	0.48	0.488	124.8	**<.0001**	6.54	**0.01**	0.24	0.62
Thinning intensity	3	0.06	0.809	0.01	0.904	0.01	0.903	0.09	0.7641	0.04	0.83	0.11	0.74
Year	3	6.42	**0.013**	6.16	**0.015**	6.17	**0.015**	3.91	**0.052**	0.77	0.38	0.49	0.48
Thinning × Year	3	0.99	0.321	0.38	0.537	0.21	0.644	0.35	0.5517	0.58	0.44	0.7	0.4

4. Discussion

Our results show a thinning-independent directional dynamics of the fungal community within the 4-year study, since fungal community composition was changing following a temporal gradient along the first CCA axis (Fig. 2a, Fig. 2b). It seems that this directional dynamic was partly driven by inter-annual changes in precipitation and temperature (Fig. 2c). Thus, during wetter autumns, and especially during the wettest autumn (2012), several non-mycorrhizal functional guilds (saprotrophs such as moulds, yeasts) increased their relative proportions, whereas mycorrhizal species were relatively more abundant during drier years. A similar response of these free-living fungi was observed under warmer autumn conditions (2009). In such case mycorrhizal species increased under colder autumns.

In line with our first hypothesis, light-medium forest thinning did not produce significant changes in fungal community composition (Fig. 1), either in fungal richness or diversity (Fig. 4). It seems that EMM from mycorrhizal species can survive into remaining trees, seedlings or with other ectomycorrhizal plants remaining in the plots (Amaranthus and Perry, 1987; Rosenvald and Lõhmus, 2008). Our results contrast with observed effects caused by more intense forest management operations such as clear-cutting, which usually causes important losses of ectomycorrhizal species (Jones et al., 2003, Parladé et al., 2017), changes in the overall soil fungal community composition (Hartmann et al., 2012; Kyaschenko et al., 2017) and general soil biology whose effect on fungal diversity may be indirect (Colinas et al., 1994a, b). It is possible that fungal community may be resistant to disturbances such as thinning by two mechanisms: i) its spore bank in the soil (Bruns et al., 2009), and ii) by survival in remaining trees or alive roots (Varenius et al., 2016) or in naturally established seedlings after thinning. We suggest that remaining trees in all the thinning categories were enough to act as a post-thinning 'refuge' for the mycorrhizal community. Surprisingly, even heavy thinning (up to 70% reduction in stand basal area) did not affect soil fungal species composition neither functional composition, rejecting our second hypothesis that fungal compositional and functional changes will be expected in heavy thinning treatments. Lack of effects on heavy thinning treatments may suggest that drought and specially changes in weather conditions occurring between years poses stronger environmental filter that overshadows reduction in C flow in this system. It is also possible that enough trees were still left in the plot to support the soil fungal community. Thus, it seems that a 70% reduction in basal area (at least 350 trees ha^{-1} left) is not enough to cross a threshold towards damaging permanently the fungal community and its composition almost in this forest area. Recently, Varenius et al. (2017) observed that forest patches were more efficient to preserve the mycorrhizal diversity than dispersed trees. Thus, it is likely that

forest patches may be still efficient in preserving the mycorrhizal diversity as long as tree roots and their associated extramatrical mycelium form a continuum along the forest soil. Additionally, medium-term effects of thinning were also not significant, likely because fungal species present in the remaining trees had a competitive advantage through priority effects compared to new colonizing fungal species (Kennedy et al., 2009). One particularity of our study was that thinning was performed with minimal disturbance and avoiding soil compaction (without using heavy machinery and only chainsaw to cut the trees). Usually, soil compaction results in a reduction of water retention capacity and it has been shown to negatively affect the soil fungal communities (Hartmann et al., 2012). Perhaps some of the effects of thinning on forest fungi reported in the literature have more to do with soil disturbance than with the actual reduction of basal area. This hypothesis is congruent with our results but it would have to be formally tested in the future.

According to our third hypothesis about the response of fungal community composition to inter-annual changes in autumn weather conditions, our results showed that fungal compositional changes across years were dependent on the functional guild considered, with many free-living fungi (yeasts, moulds) being correlated with warmer and wetter conditions. In previous studies focused on temperate or boreal forest ecosystems, free-living fungi such as yeasts, litter saprotrophs or moulds were also found to increase under wetter conditions (Jumpponen et al., 2010; Voříšková et al., 2013) or under snow cover (Santalahti et al., 2016). In our study, yeasts belonging to Tremellomycetes (*Cryptococcus*) or Leotiomycetes (*Guehomyces*) increased their proportions under higher precipitation and temperature, confirming their preference for moist environments (Choudhary and Johri, 2009). Similarly observed by Hartmann et al., (2017) and according to third hypothesis, Zygomycete fungi (mostly moulds) also responded positively to increasing precipitation, but litter saprobes did not so. Representing less than 4% of the relative fungal abundance, our sampling scheme likely excluded most of the litter-associated species, which are normally found at the needles and partially decomposed needles (Clemmensen et al., 2013; Voříšková et al., 2013). Thus, sampling should be focused on the most uppermost soil organic horizons when targeting this specific group of fungi. Despite the lack of effects on the moss-associated fungal species found in this study, they were highly represented under wetter years, and intra-annual studies from the same site show a clear positive effect of soil moisture on this group of fungi, both from a spatial and temporal perspective (Castaño et al., unpublished). Regarding the effect of temperature, we found that mycorrhizal species were more abundant during colder autumns (Fig. 2b). This trend may be caused by earlier autumn carbon reallocation to symbiotic fungi, typically occurring during the fall and coinciding with changes in tree physiology (Högberg et al., 2010), which

may be shifted under different weather conditions. Thus, we suggest that warmer autumns may have stimulated free-living fungi (yeasts, moulds) and delayed host carbon reallocation to roots and to symbiotic fungi. The differential functional response of fungi to inter-annual changes in precipitation might show a competitive advantage for root-associated fungi (i.e. mycorrhizal) *vs* free-living fungi (i.e. yeasts, moulds, moss-associated) under drier conditions. Here, hydraulic lift may grant root-associated fungi with more resistance to drought, because groundwater can be vertically transferred by symbionts under drier conditions (Allen, 2007; Querejeta et al., 2003, 2017). This adaptation could explain why all functional groups sharply decreased their proportion relative to mycorrhizal fungi in years characterised by drier autumns. Thus, we suggest that many free-living fungi may perform better under higher soil moisture levels, in comparison with root-associated fungi. Mould species, one of the most affected groups, may be potentially profiting on, and contributing to the turnover of dead mycorrhizal mycelium (Lindahl et al., 2010), and they act as soil saprotrophs, using a variety of carbohydrates such as cellulose, pectin and starch (Thormann et al., 2001). This decrease in the proportion of free-living fungi under drier conditions is especially important for Mediterranean forests soils, where water is the most limiting factor of tree growth, and provide insights into future potential climate-change effects on the belowground fungal community and associated processes such as decomposition and nutrient cycling.

5. Conclusions

In our study, thinning did not cause a significant short-term effect on fungal community composition neither on fungal species richness or diversity, suggesting that these communities are resistant to forest thinning if enough trees are left. However, our observations regarding inter-annual changes in fungal composition and their relationship with changes in meteorological conditions have important implications to understand and predict future effects of climate change in Mediterranean forests. Mycorrhizal species may be more resistant to changes in climate, but the decrease of other functional guilds under increasing drought may cause important changes in ecosystem processes such as organic matter decomposition or nutrient cycling. Further research should address how the climate-related effects on fungal community will affect ecosystem processes such as nutrient cycling, but also thinning experiments should be evaluated in different tree species and forest ecosystems under different climate regimes.

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CHAPTER V

Mushroom emergence detected by combining spore trapping with molecular techniques.

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Mushroom Emergence Detected by Combining Spore Trapping with Molecular Techniques

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Running Head: Spore detection using spore traps.

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ABSTRACT

Obtaining reliable and representative mushroom production data requires time-consuming sampling schemes. In this paper, we assessed a simple methodology to detect mushroom emergence by trapping the fungal spores of the fruiting body community in plots where mushroom production was determined weekly. We compared the performance of filter paper traps with that of funnel traps and combined these spore trapping methods with species-specific quantitative real-time PCR and Illumina MiSeq to determine the spore abundance. Significantly more MiSeq proportional reads were generated for both ectomycorrhizal and saprotrophic fungal species using filter traps than were obtained using funnel traps. The spores of 37 fungal species that produced fruiting bodies in the study plots were identified. Spore community composition changed considerably over time due to the emergence of ephemeral fruiting bodies and rapid spore deposition (lasting from one to two weeks), which occurred in the absence of rainfall events. For many species, the emergence of epigeous fruiting bodies was followed by a peak in the relative abundance of their airborne spores. There were no relationships between fruiting body yields and spore abundance in time for only two out of seven fungal species. There was no relationship between fruiting body yields and their spore abundance at plot level, indicating that some of the spores captured in each plot were arriving from the surrounding areas. Differences in fungal detection capacity by spore trapping might indicate different dispersal ability between fungal species. Further research could help to identify the spore rain patterns for most common fungal species.

Keywords: *Propagules, Ectomycorrhizal, Saprotroph, DNA barcoding, molecular identification, dispersion, fungi, Lactarius*

IMPORTANCE

Mushroom monitoring represents a serious challenge in economic and logistical terms because sampling approaches demand extensive field work at both the spatial and temporal scales. In addition, the identification of fungal taxa depends on the expertise of experienced fungal taxonomists. Similarly, the study of fungal dispersal has been constrained by technological limitations, especially because the morphological identification of spores is a challenging and time-consuming task. Here, we demonstrate that spores from ectomycorrhizal and saprotrophic fungal species can be identified using simple spore traps together with either MiSeq fungal-specific amplicon sequencing or species-specific quantitative real-time PCR. In addition, the proposed methodology can be used to characterize the airborne fungal community and to detect mushroom emergence in forest ecosystems.

INTRODUCTION

Wild edible fungi are highly important non-wood forest products and are increasingly in demand at food markets worldwide (1). Up to 268 fungal taxa have been authorized to be commercialized in Europe (2), of which the most important marketed mushrooms are *Boletus edulis*, *Cantharellus cibarius*, *Lactarius deliciosus*, *Morchella esculenta*, and *Agaricus campestris* (2, 3). Spain, the Netherlands, France, and Poland are the largest mushroom producers in Europe (2). Despite the increasing interest and importance of mushrooms as non-wood forest products, mushroom production has only recently been included as a target in forest management and planning alongside the traditional goal of wood production (4). The inclusion of mushroom production into forest management planning requires the ability to evaluate potential mushroom yields in quantifiable terms. However, the variability in mushroom biomass across years, the ephemeral cycle of several fruiting bodies that are only observable for a few days, and the time needed to sample representative mushroom populations (5) represent serious challenges for monitoring purposes (6). In this context, new techniques that do not require the collection of mushrooms could improve the estimation of mushroom yields. Except for a few cases (7), no correlations have been found between the belowground mycelial biomass of specific species and their mushroom production (8–10), even though positive correlations have been found between root mycorrhizal colonization and soil mycelia (11). In this regard, new

monitoring approaches using fungal spores, such as spore trapping, could represent a new way of addressing this challenge. Spore detection could be used to evaluate the fruiting bodies present without the need for regular or repeated samplings over time.

Traditionally, spore trapping has been used to detect and monitor airborne spores, particularly those of fungal pathogens (12). However, recently, spore trap methods have been combined with molecular techniques such as quantitative real-time PCR (qPCR) (13, 14). Spore traps need to be simple, robust devices for effective use in forest stands; they also need to be easy to handle and have low maintenance costs (14, 15). Active traps (e.g., volumetric or cyclonic traps) trap fungal spores more effectively than passive traps (12); however, they have some disadvantages when used under forest conditions, for example, they require electric power. By contrast, passive traps, such as filter or funnel traps, can be placed anywhere (13). Previous studies have shown that passive traps can capture ectomycorrhizal fungal propagules. These passive traps consisted of simple funnels attached to jars (16, 17). However, even simpler spore traps have been used to capture pathogenic fungi, such as filter traps (13). Although these traps collect spores passively, funnel-based traps collect spores in a jar, whereas filter traps retain spores within the filter, which may mean that the spore collection performance of these two traps is different. Despite the potential of these traps to capture fungal spores, to date, no quantitative relationship between fruiting bodies and airborne spores has been established.

Besides the type of trap, the dispersal capacity of the spores also affects spore detection. Unlike highly sporulating fungi, such as puffballs or several mold or yeast species that dominate the airborne community (18), ectomycorrhizal basidiomycetes seem to disperse spores less abundantly (19–21). Problems of detecting rare species can be circumvented by the use of molecular techniques such as qPCR (22), which can detect even small amounts of fungal DNA, although specific primers need to be designed for the target species (13). High-throughput DNA sequencing technologies enable the relative proportions of each of the identified operational taxonomic units (OTUs) to be determined in a given sample (23). Besides monitoring mushroom emergence, this approach could also be used to understand fungal dispersal. To sum up, spore traps combined with qPCR could be used to detect airborne fungal spores of specific species, while spore traps in combination with high-throughput sequencing approaches could be used as a generic tool to detect and identify airborne spores of different taxa.

We hypothesized that: (i) funnel and filter traps in combination with qPCR or MiSeq would differ in their capacity to detect spores; (ii) there is a relationship between fruiting body

yields and spore abundance over time (temporal relationship) and over plots (spatial relationship). To test these hypotheses, we identified the relative abundance of the spores by trapping spores during an 8-week period in the fall of 2014 in a *Pinus pinaster* forest in NE Spain. In parallel, we also determined the taxonomic identity and the yields of the fruiting body community.

First, we studied *Lactarius vinosus* as a model species to compare the two molecular techniques (qPCR vs. MiSeq). We chose this species because of its commercial interest. It belongs to the *Lactarius* group *deliciosus*, which has well-established markets across Europe, Asia, and North Africa (1). Furthermore, this species is highly appreciated both by mushroom pickers and consumers in Spain (24, 25). The commercialization of *Lactarius* group *deliciosus* has led to the sale of almost 500,000 kg of *L. vinosus* a year at the three most important Spanish markets; the estimated market value is 5.3 M € $year^{-1}$ (25).

Second, we studied the relationship between the spore abundance of a species and the yield of fruiting bodies from a temporal (week) and spatial (plot) perspective. For this, we compared the abundance of the fruiting bodies of the 12 most abundant fungal species (6 ectomycorrhizal and 6 saprotrophic fungal species) found in the fruiting body community.

RESULTS

Fungal species producing fruiting bodies in the studied plots

Among the sampled fruiting bodies, we found 71 fungal species belonging to the ectomycorrhizal or saprotrophic fungal guilds. Among these ectomycorrhizal and saprotrophic species, we identified 37 species that were also present in the spore community. Of those species, 18 species produced fruiting bodies in at least two plots for a two-week period, and the 12 most abundant species (6 ectomycorrhizal and 6 saprotrophic fungal species) were selected for analysis (Table 1). Thirteen species producing fruiting bodies did not have an internal transcribed spacer (ITS) reference in the UNITE (Unified system for the DNA based fungal species linked to classification) database or in the INSD (International Nucleotide Sequence Database) and, therefore, these species were not searched for within the spore community.

TABLE 1. Selected most abundant species (species found at least twice in two different plots). Identification was based on comparison with ITS2 reference sequences deposited in UNITE. Ecology refers to the functional guild of the species (ectomycorrhizal or saprotrophic). Frequency refers to the order of frequency in terms of the total number of MiSeq reads among the whole propagule community.

Potential species	Ecology	Reference	Species Hypothesis Concept	DOI	Frequency
Inocybe glabripes Ricken	Ectomycorrhizal	UDB000099	*Inocybe glabripes*	DOI: 10.15156/BIO/SH174191.07FU	1620
Lactarius deliciosus (L.) S. F. Gray	Ectomycorrhizal	UDB011514	*Lactarius deliciosus*	DOI: 10.15156/BIO/SH220107.07FU	227
Lactarius vinosus Quél	Ectomycorrhizal	UDB000876	*Lactarius semisanguifluus*	DOI: 10.15156/BIO/SH220111.07FU	104
Russula chloroides (Kromb.) Bres.	Ectomycorrhizal	FM999633	*Russula chloroides*	DOI: 10.15156/BIO/SH220523.07FU	260
Tricholoma terreum (Sch.) Kumm.	Ectomycorrhizal	UDB015079	*Tricholoma gausapatum*	DOI: 10.15156/BIO/SH219345.07FU	168
Russula torulosa Bres.	Ectomycorrhizal	UDB011110	*Russula torulosa*	DOI: 10.15156/BIO/SH186211.07FU	877
Clitocybe phaeophthalma (Pers.) Kuyper	Saprotrophic	JX514120	*Singerocybe umbilicata*	DOI: 10.15156/BIO/SH191019.07FU	1709
Hypholoma fasciculare (Huds.) Kumm.	Saprotrophic	KF373785	*Hypholoma fasciculare*	DOI: 10.15156/BIO/SH201439.07FU	32
Leucopaxillus gentianeus (Quél.) Kotl.	Saprotrophic	UDB011628	*Leucopaxillus gentianeus*	DOI: 10.15156/BIO/SH030478.07FU	50
Marasmius androsaceus (L.) Fr.	Saprotrophic	AF519893	*Gymnopus androsaceus*	DOI: 10.15156/BIO/SH183805.07FU	1680
Mycena pura (Pers.) Kumm.	Saprotrophic	JF908417	*Mycena diosma*	DOI: 10.15156/BIO/SH186350.07FU	79
Lycoperdon perlatum Pers.	Saprotrophic	UDB023596	*Lycoperdon perlatum*	DOI: 10.15156/BIO/SH175885.07FU	51

Spore abundance estimated by trapping techniques and molecular methods

MiSeq data revealed that the relative abundance of *L. vinosus* was significantly higher in filter traps than in funnel traps (F-value$_{[1,15]}$ = 4.982, P<0.001, Table 2), representing on average 5.55 and 0.48 per thousand of the total number of reads, respectively (i.e., nearly 10-times more abundant in filter traps than in funnel traps; Table 2). However, when using qPCR, the number of *L. vinosus* spores trapped by the filter and funnel traps did not seem to be significantly different (F-value$_{[1,15]}$ = 0.062, P = 0.951; Table 2). When using funnel trap data, the spore abundance of *L. vinosus* estimated by qPCR correlated with that estimated by MiSeq (F-value$_{[1,59]}$ = 27.53, P<0.001, R^2 = 0.3, Fig. 1a). However, there was not relationship between spore abundance of *L. vinosus* detected at the filter traps with the abundance detected at the funnel traps (F-value$_{[1,14]}$ = 0.92, P = 0.352, Fig. 1b)

The higher relative abundance of *L. vinosus* spores detected by MiSeq when using filter traps rather than funnel traps was also observed for most of the saprotrophic and ectomycorrhizal fungal species studied (F-value$_{[1,15]}$ = 5.712, P<0.001, Table 2). When comparing guilds, we found that selected yeast species were better represented in funnel traps than in filter traps (F-value$_{[1,15]}$ = 4.703, P<0.001, Table 2), but no differences between spore trap types were observed for mold species.

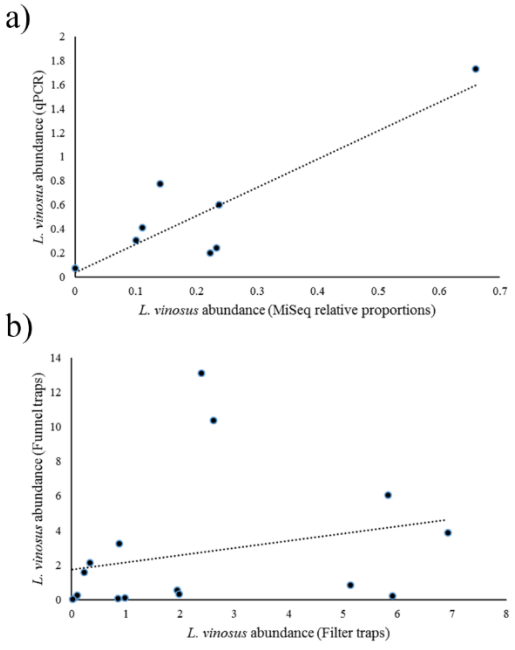

FIG 1: Relationship between the *L. vinosus* abundance measurements determined by qPCR (y-axis) and by MiSeq data (x-axis) (a) and, relationship between the *L. vinosus* abundance measurements obtained at the funnel traps (y-axis) and obtained at the filter traps (x-axis)

TABLE 2: Differences in the relative proportions of ectomycorrhizal and saprotrophic species detected by filter traps vs. funnel traps. A few of the most abundant yeast and mold species are also included in the analysis to determine whether the relative proportion of ectomycorrhizal and saprotrophic species detected by funnel traps was smaller than that detected by filter traps owing to the trapping of a larger proportion of yeast or mold species by funnel traps.

		Mean proportions		Effects				
Ecology	Species	Filter trap	Funnel trap	Value	SE	df	*t*-value	*P*-value
Ectomycorrhizal	*Russula torulosa*	0.354	0.003	–0.262	0.126	15	–2.082	0.055
Ectomycorrhizal	*Inocybe glabripes*	0.079	0.015	–0.089	0.07	15	–1.267	0.225
Ectomycorrhizal	*Lactarius deliciosus*	2.408	0.075	–1.09	0.228	15	–4.793	**<0.001**
Ectomycorrhizal	*Lactarius vinosus* (MiSeq)	5.551	0.450	–1.531	0.307	15	–4.982	**<0.001**
	Lactarius vinosus (qPCR)	2.260	2.676	–0.016	0.268	15	–0.062	0.951
Ectomycorrhizal	*Russula chloroides*	2.031	0.012	–0.898	0.254	15	–3.543	**0.003**
Ectomycorrhizal	*Tricholoma terreum*	2.207	0.033	–0.649	0.299	15	–2.17	**0.046**
Saprotrophic	*Lycoperdon perlatum*	0.715	0.695	0.068	0.212	15	0.32	0.753
Saprotrophic	*Leucopaxillus gentianeus*	13.853	2.411	–1.509	0.411	15	–3.67	**0.002**
Saprotrophic	*Clitocybe phaeophthalma*	0.022	0.000	–0.057	0.034	15	–1.689	0.112
Saprotrophic	*Hypholoma fasciculare*	10.706	2.018	–1.06	0.468	15	–2.28	**0.038**
Saprotrophic	*Gymnopus androsaceus*	0.029	0.015	–0.03	0.049	15	–0.604	0.555
Saprotrophic	*Mycena pura*	6.853	1.864	–1.107	0.23	15	–4.809	**<0.001**
Yeasts	*Cryptococcus* sp.	9.830	80.908	4.111	1.088	15	3.78	**0.002**
Yeasts	*Rhodotorula baccarum*	6.063	32.247	2.241	0.746	15	3.00	**0.009**
Yeasts	*Cryptococcus wieringae*	3.586	29.628	2.571	0.784	15	3.28	**0.005**
Yeasts	*Rhodotorula colostri*	0.389	1.348	0.502	0.146	15	3.435	**0.004**
Molds	*Mortierella elongata*	0.201	4.509	0.825	0.499	15	1.651	0.119
Molds	*Trichoderma* sp.	0.498	0.507	0.09	0.154	15	0.587	0.5658

Weekly relationship between fungal spores and the production of fruiting bodies

The spore community composition varied significantly across the weeks ($F_{[7,59]} = 3.727$, $R^2 = 0.27$, $P<0.001$) and plots ($F_{[7,59]} = 3.458$, $R^2 = 0.25$, $P<0.001$). The temporal effect (across weeks) was as high as the spatial effect (across plots), and together accounted for more than 50% of the total variation. The abundance of spores of different species found in the traps fluctuated over the sampling period, especially those of ectomycorrhizal fungal species (Fig. 2a, 2b).

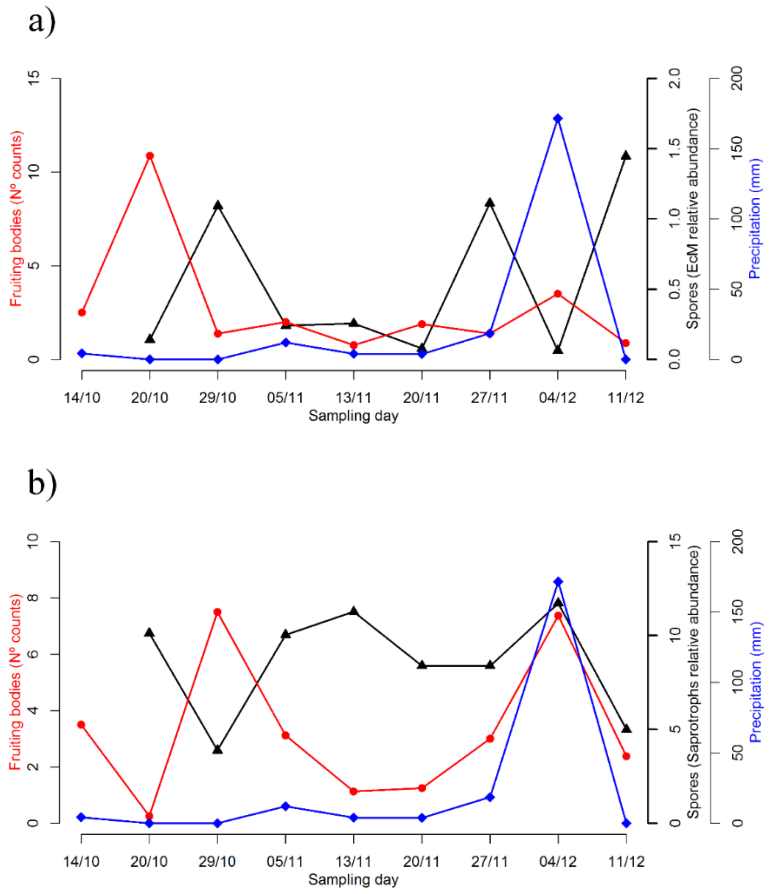

FIG 2. Temporal fluctuation of mushroom yields (red line) produced by (a) ectomycorrhizal (EcM) and (b) saprotrophic species, the relative abundance of their spores (black line), and the total precipitation recorded each week (blue line). The sampling day and month are shown along the x-axis. During the first two weeks there were no precipitation events, and only low levels of precipitation over the following weeks. However, during the first week of December, an extreme precipitation event was recorded (almost 180 mm).

Three saprotrophic (*Marasmius androsaceus*, *Clitocybe phaeophthalma*, and *Hypholoma fasciculare*) and two ectomycorrhizal fungal species (*Russula torulosa* and *Russula chloroides*)

showed signs of having highly ephemeral fruiting bodies. Spore production by most of these species peaked one or two weeks after fruiting body production but then ended abruptly (Fig. 3a, 3b). Species with at least three spore production peaks during the study period showed a significant relationship between fruiting body yields and their spore abundance; for example, *Mycena pura* (F-value$_{[1,51]}$ = 36.97, P<0.001, R^2 = 0.96), *Lactarius deliciosus* (F-value$_{[1,51]}$ = 11.56, P = 0.001, R^2 = 0.68), *Lactarius vinosus* (F-value$_{[1,51]}$ = 57.39, P<0.001, R^2 = 0.77), *Leucopaxillus gentianeus* (F-value$_{[1,51]}$ = 12.58, P<0.001, R^2 = 0.59), and *Tricholoma terreum* (F-value$_{[1,51]}$ = 20.89, P<0.001, R^2 = 0.58). This relationship was also confirmed for *L. vinosus* using qPCR data (F-value$_{[1,48]}$ = 29.24, P<0.001, R^2 = 0.79). *L. vinosus* spore abundance patterns detected using either Illumina MiSeq or qPCR data were very similar and both techniques showed that the production of *L. vinosus* spores peaked between October 20th and October 29th. This peak was estimated to represent an average of 1.5 × 10^6 *L. vinosus* spores × trap^{-1} (Fig. 4).

In contrast to the species already mentioned, *Lycoperdon perlatum* (F-value$_{[1,51]}$ = 0.01, P = 0.917) and *Inocybe glabripes* (F-value$_{[1,51]}$ = 0.89, P = 0.349) did not display a significant relationship between fruiting body yield and spore abundance. For example, the spore abundance of *L. perlatum* increased with time and did not correspond to the variable fruiting body production (Fig. 3a), and the spore abundance of *I. glabripes* peaked at the start of the season, which was not reflected at the fruiting body level (Fig. 3b).

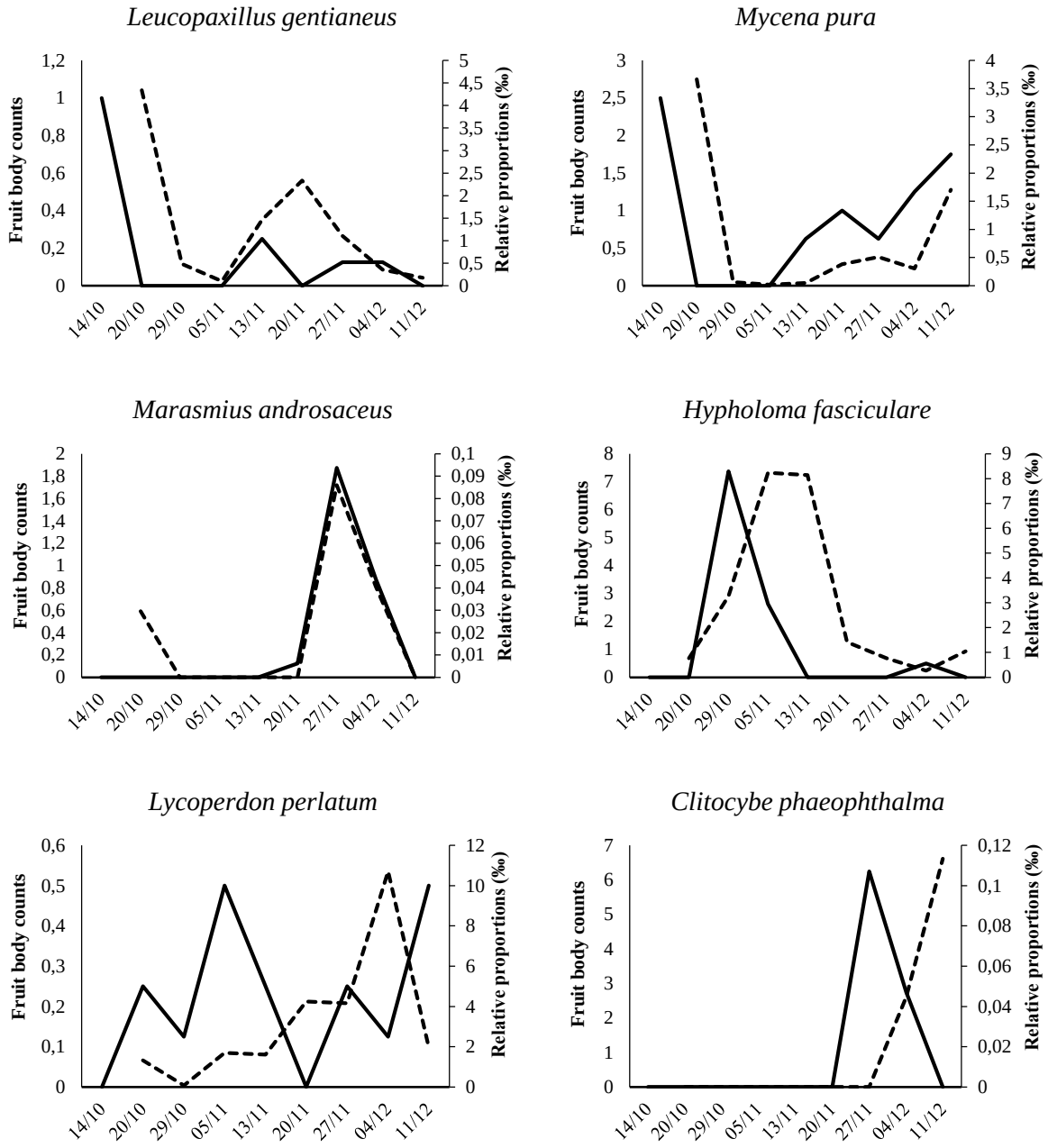

FIG 3a. Temporal changes in the number of fruiting bodies produced by saprotrophic species and their spores across the sampled weeks in all the plots considered in this study. The solid black line represents mushroom production (number of fruiting bodies × plot) and the broken black line indicates the relative spore abundance (‰).

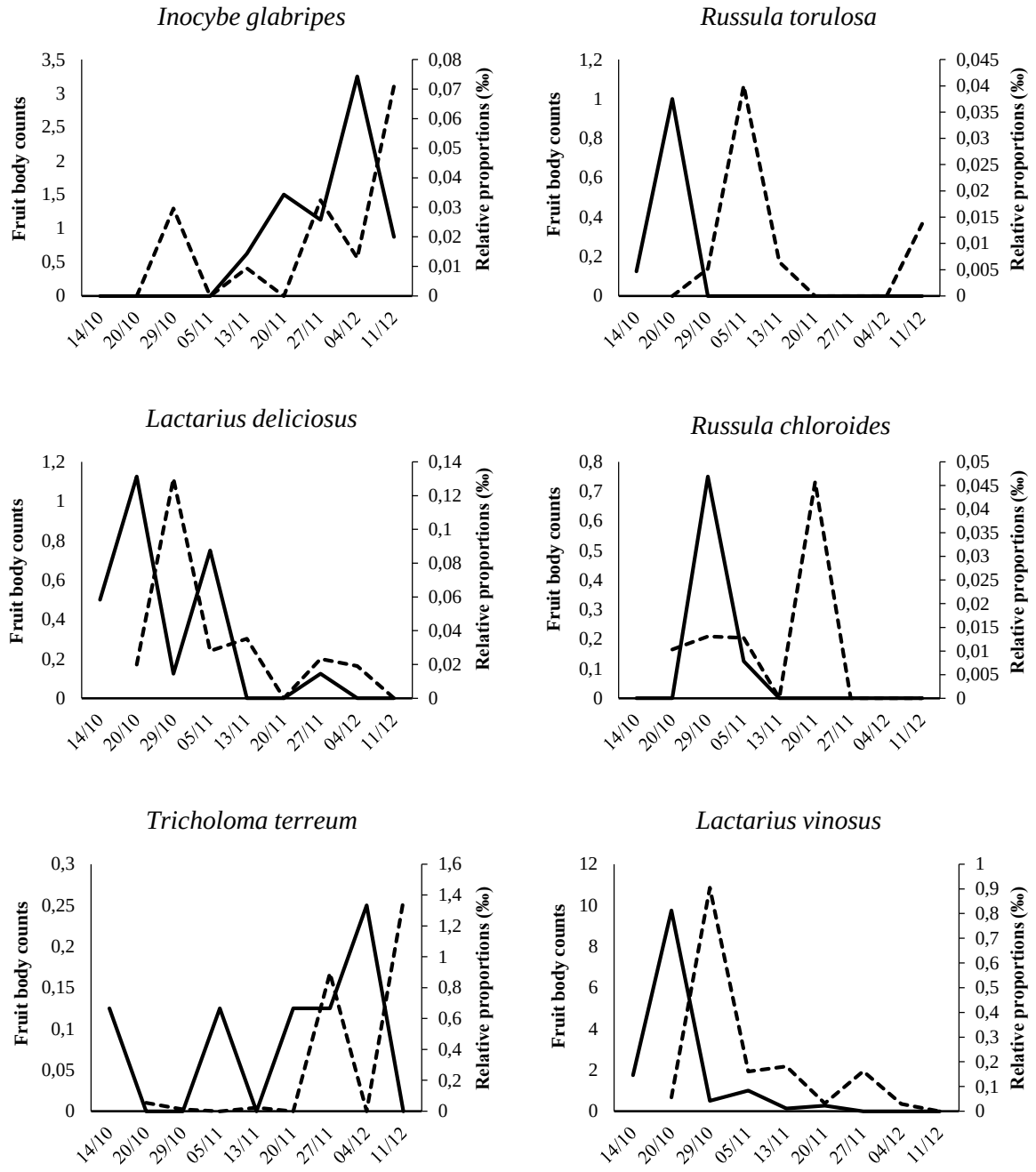

FIG 3b. Temporal changes in the number of fruiting bodies produced by ectomycorrhizal species and their spores across the sampled weeks in all the plots considered in this study. The solid black line represents mushroom production (number of fruiting bodies × plot) and the broken black line indicates the relative spore abundance (‰).

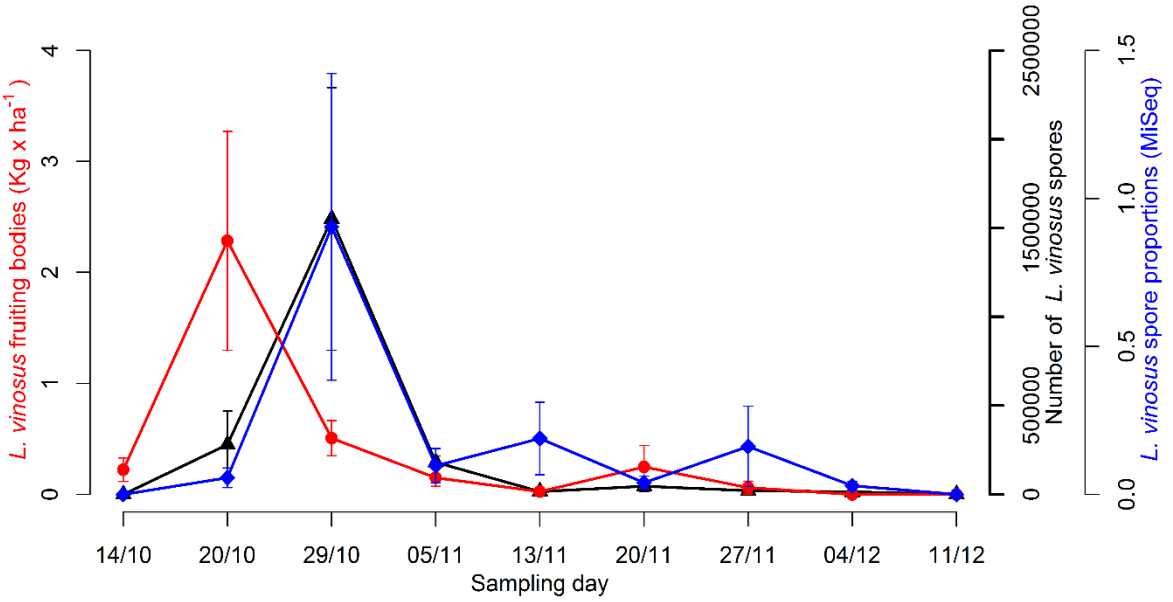

FIG 4. Relationship between mushroom biomass (red line) and the relative abundance of *L. vinosus* spores detected by the spore traps, quantified by qPCR (black line) and by determining the relative proportions of *L. vinosus* using MiSeq (blue line). Average values are shown with their standard error. The sampling day and month are shown along the x-axis. The peak in mushroom production on October 20th was detected the following week (October 29th) using both qPCR and MiSeq. The slight increase in mushroom production on November 20th was only detected by MiSeq.

Relationship between spores and fruiting body production at plot level

At plot level, only spores of saprotrophic fungal species correlated with fruiting body production ($F_{[1,48]} = 4.11$, $P = 0.048$, Fig. 5a). These significant differences were mainly attributed to the effect of one plot, in which large quantities of both spores and fruiting bodies were recorded. Nevertheless, no relationship was observed between spores of ectomycorrhizal fungi and their fruiting body yields (F-value$_{[1,48]} = 0.41$ $P = 0.527$, Fig. 5b). Only five species were found in more than two plots: *Inocybe glabripes*, *Lactarius deliciosus*, *Lactarius vinosus*, *Lycoperdon perlatum*, and *Mycena pura*. None of these species showed a significant relationship at plot level between spores and their fruiting bodies (P>0.05). Thus, in most cases, fruiting bodies were found in only a few plots but their spores were collected in a greater number of plots (e.g., *Mycena pura,* Fig. 5c). In other cases, the plots with high levels of fruiting body production also had the highest spore

abundance, but no quantitative relationships were found (e.g., *Lactarius vinosus*, Fig 5d). Amongst the saprotrophic fungal species there was a clear altitudinal pattern ($F_{[1,6]} = 2.48$, $P = 0.047$, Fig. 6), with both fruiting bodies and spores decreasing towards higher altitudes, whereas no clear altitudinal pattern was observed amongst ectomycorrhizal fungal spores ($F_{[1,6]} = -0.28$, $P = 0.785$, Fig. 6).

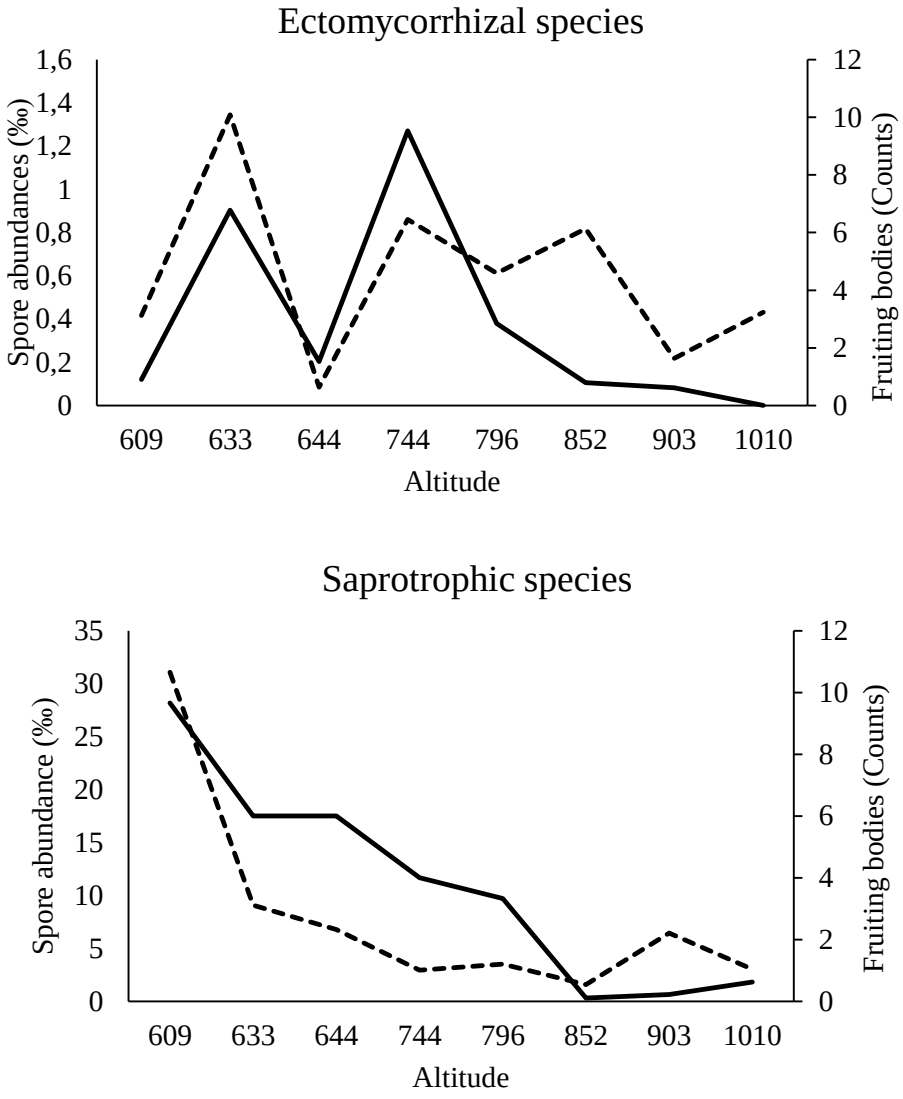

FIG 6. Relationship between fruiting body yields (solid line) and their propagules (broken line) for (a) ectomycorrhizal and (b) saprotrophic species along an elevation gradient represented by all the sampled plots considered in this study

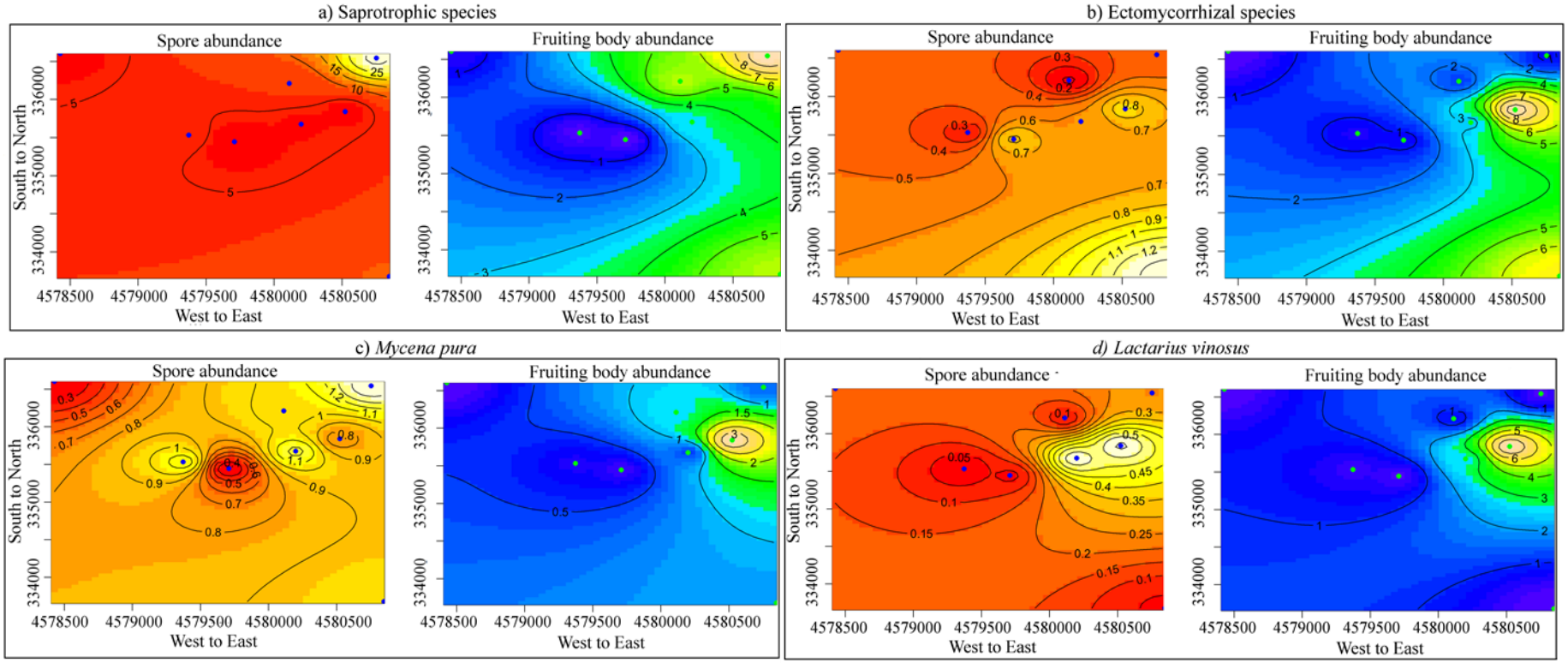

FIG 5. Spatial relationships between fruiting body yields (no. of fruiting bodies) and the relative abundance of spores evaluated by MiSeq for (a) saprotrophic species, (b) ectomycorrhizal species, (c) *Mycena pura*, and (d) *Lactarius vinosus*. Blue or green dots represent each of the 8 plots considered in this study. Reddish to yellowish colors in spore abundance represent a gradient from lower to higher spore abundance, respectively, whereas blueish to orange colors represents a gradient from lower to higher fruiting body abundance, respectively. Coordinates X (Easting) and Y (Northing) are represented in ED-50 datum.

DISCUSSION

In this study, we showed that spore trapping coupled with qPCR or MiSeq can detect the emergence of epigeous mushrooms. To our knowledge, this is the first study demonstrating that qPCR and MiSeq can be used to detect fungal emergence. In general, a one-week delay between mushroom emergence and airborne propagule detection was found using spore traps. This delay was most likely caused by a combination of i) the time needed for mushrooms to mature and produce spores, and ii) the time needed for spore ejection and deposition in the spore traps. However, an alternative explanation for the observed delay might be that our fruiting body sampling scheme likely favors the sampling of immature fruiting bodies before they initiate sporulation. It is generally accepted that spore deposition is driven by meteorological conditions. In theory, the initial phase of spore dispersal of some basidiomycete mushroom-forming species involves the discharge of basidiospores from the gill by a mechanism promoted by a droplet, also known as a Buller's drop (26), which is normally stimulated by the secretion of mannitol and other sugars (27). Then, convective airflows promoted by the pileus enhance the vertical movement of these spores, which may then reach dispersive winds (28). Finally, these spores may fall by gravity or rainfall events. However, in our study we observed that spore deposition occurred regardless of rainfall events, indicating that spore deposition is not necessarily driven by meteorological conditions such as precipitation events (29). Future studies should focus on gaining a better understanding of the potential delay in spore detection and the factors driving spore deposition, such as the effect of winds on the movement of these spores.

In this study, we observed an interspecific short temporal variation in the abundance of spores, especially those produced by ectomycorrhizal fungal species. This finding agrees with the literature because spores can remain airborne for between a day and up to a few weeks, depending on their size and aerodynamic properties (30). The temporal variation in spore abundance has been reported previously (16, 20), and this variation has been attributed to different abiotic parameters such as wind direction, rainfall, and species-specific life-history traits (29,31-32). Based on our results, spore detection was related to mushroom production peaks, thus it seems likely that seasonal differences in the composition of the airborne fungal community could also be partly driven by mushroom phenology (20, 33).

Despite the observed relationship between mushroom yields and spore abundance across the sampling weeks, there was not relationship at the spatial scale where mushroom production was assessed i.e. plots of 10 × 10 m. Spores were captured in plots without fruiting bodies of the corresponding species, indicating that these spores were produced outside the sampled plots. The spores of ectomycorrhizal fungal species are usually dispersed approximately 10 to 1000 meters (16, 19, 21) depending on the species (17). The large amount of spores produced per fruiting body, e.g., $1.1 \times 10^8 – 1 \times 10^{10}$ spores (34, 35), makes it possible for spores of these species to be dispersed long distances, particularly if carried away by turbulences. The fine mapping of spore captures in future studies could be used to indirectly estimate dispersal curves for several species and therefore understand better how these fungal species disperse.

MiSeq analysis detected significantly greater relative proportions of our 12 selected fungal species belonging to the saprotrophic or ectomycorrhizal guilds in filter traps than in funnel traps. Funnel traps trapped a greater number of species from other fungal guilds such as yeasts than the filter traps, which translated into a relative decrease in the proportion of saprotrophic and ectomycorrhizal MiSeq reads. Differences between samples could arise because of the way in which samples were handled. Spores captured at the funnel traps were first filtered *in situ* and subsequently collected inside a jar with water (17), which could favor the growth of some opportunistic fungi such as yeasts. Both fungal-specific amplicon sequencing and species-specific quantitative real-time PCR successfully detected mushroom production peaks. Both methods revealed an almost identical pattern of spore abundance across the weeks for *L. vinosus*. The use of MiSeq avoids the need to design specific primers for each target species. However, the use of high-throughput sequencing data as a semi-quantitative measure has been the subject of discussion, mostly because of the variation in ribosomal gene copy numbers among different fungal species (36), the read length biases caused by current high-throughput sequencing platforms (37), the DNA extraction efficiency (38), or biases due to interspecific primer binding differences. Despite these limitations, read abundance may still be used as a semiquantitative measure of abundance (relative proportions) (23).

We showed that a peak in ephemeral fruiting bodies was followed by a peak in the numbers of spores that were trapped. For most of the species, the spore peak mostly disappeared two weeks after mushroom emergence reached its highest level. Nevertheless, a quantitative relationship

between fruiting body yields and their fungal spores was not observed for *Inocybe glabripes* and *Lycoperdon perlatum*. Although the spore abundance and fruiting body yields observed for *I. glabripes* showed a similar pattern, the lack of relationship between these two measures was most likely caused by a peak in spore abundance that was not reflected at the fruiting body level. Interestingly, the spore abundance pattern for *L. perlatum* suggests a progressive and slow spore deposition over time; the lack of relationship between spore abundance and time could be because these spores are rarely falling unless a rainfall event occurs, such as the event occurred during the week of December 4th, which most likely washed away all the spores.

Some species produced fruiting bodies but their spores were not found among the spore community. Possible explanations for this include: i) these species had a very short range of dispersal, or ii) their sporulation was very small compared with the rest of the species. Alternatively, these species could have a very long ITS2 sequence and, thus, be underrepresented using MiSeq sequencing. We investigated whether this was a plausible explanation by studying the ITS2 region length of these species using ITS sequences from UNITE, specifically if the region theoretically amplified by the primers was longer and, if the DNA sequence where each primers binds was different for the fungal species not detected at the spore traps. However, the length of the ITS2 region of these species was similar to that of other species that were successfully captured by the spore traps. Primer sequence mismatch was also discarded as an alternative explanation because these species did not show any sequence mismatch where the primer binds. Thus, discarded these last possibilities, and given that fruiting bodies were collected <10 m from spore traps, we hypothesize that low sporulation might be the reason for the lack of detection of these species among the spore community.

Filter traps are more suitable for high-throughput sequencing approaches targeting saprotrophic or ectomycorrhizal fungal species. Our method represents a potential improvement on mushroom sampling approaches, as current sampling schemes are highly time-consuming and require expert knowledge to identify the fungal taxa (5). Here, we showed that mushroom production and fungal diversity of fungi with epigeous fruiting body structures can be monitored accurately by spore traps. Thus, there are less time-consuming alternatives for monitoring mushrooms than sampling fruiting bodies, which are fundamental for an effective management planning of non-wood forest products.

MATERIALS AND METHODS

Study area

The study was carried out at a long-term experimental set-up located in the Natural Area of Poblet (Northeast Spain, 41° 21' 6.4728" latitude N and 1° 2' 25.7496" longitude E), which is characterized by a Mediterranean climate, with an average annual temperature of 11.8°C and annual rainfall of 665.5 mm. The long-term experimental site comprises 28 fenced plots, of which we selected 8 plots (10 × 10 m), where fruiting bodies have been continuously monitored every fall since 2008 (39). Even-aged (60-years-old) *Pinus pinaster* and *Quercus ilex* are the co-dominant species growing in the plots. Additional information about the study area and the plots can be found in Table 3.

TABLE 3. Main sampling plot characteristics (altitude, location, and mean *Lactarius vinosus* mushroom biomass for the period 2008–2014). Coordinates X (represented as E-W x) and Y (represented as N-S y) are expressed as European Datum 1950 (ED50) datum.

Plot	Altitude (meters above sea level)	E-W x	N-S y	Mean *L. vinosus* mushroom yield (kg × ha^{-1})
301c	1010	4578428	336601	0.67
303c	903	4579373	335531	0.64
305c	744	4580525	335839	140.47
307c	796	4580200	335673	115.57
309c	852	4579709	335445	53.09
312c	633	4580848	333675	30.77
313c	609	4580755	336541	1.72
316c	644	4580113	336211	5.59

Fruiting body sampling

All plots were sampled for mushroom production weekly during the fall (October–December 2014) following previously described methods (5). In short, all the fruiting bodies within the plot and with a minimum cap size of 1 cm were sampled on Thursdays to minimize errors resulting from recreational weekend collectors picking mushrooms. The plots were chosen according to the

productivity gradient for *Lactarius vinosus* (from the highest to the lowest production), which was based on continuous production data, which have been recorded since 2008 (39). All the fruiting bodies from all the species found within the plots were brought to the laboratory the same day, identified, cleaned, and counted. Fruiting bodies were dried in an air-vented oven at 35–40°C, and weighed to the nearest 0.01 g. Data in this study were expressed as the number of fruiting bodies (*n*) per plot in kg × ha^{-1} to show the temporal variation of *L. vinosus* fruiting bodies over time.

Spore trapping

Two types of spore traps were installed: filter (13) and funnel traps (16). Both traps were placed one meter apart in the center of each plot and captured spores 30 cm above ground level. Funnel traps consisted of 15-cm-diameter funnels attached to 1-liter dark jars, with a 50-µm nylon mesh fixed at the bottom of the funnel. These traps were erected a week after the first mushroom of *L. vinosus* was observed (October 14th, 2014) and remained in place until December 11th, 2014, when no *L. vinosus* fruiting bodies were observed. Each week the funnels were rinsed with ultrapure water (MilliQ) to collect any spores and then the jars were immediately removed and replaced with sterile jars. Liquids from the traps were filtered using sterile filter papers (90-mm diameter: Whatman no. 1) within 48 hours of collection. Sample filtering was conducted in a flow chamber to prevent potential contamination. Filters were stored at –20ºC until further analysis.
Filter traps consisted of a filter paper (Whatman no. 1, 90 mm diameter filter paper) placed over a metal mesh, supported by a metal clamp and attached to a vertical support 30 cm above ground level. The filter papers in the filter traps were sampled simultaneously to the funnel traps during the two most productive weeks when *L. vinosus* fruiting body production was greatest (from October 14th until October 29th, 2014), and stored at –20ºC after sampling.

Spore trap sample processing and DNA extraction

Filters containing spores from the filter and the funnel traps were cut in half and placed in separate 50-ml falcon tubes. A solution of 20-ml sodium dodecyl sulfate (SDS buffer) was added to each tube before incubating at 65°C for 90 minutes. The tubes were vortexed three times before removing the filter from each tube. Twenty milliliters of 2-propanol were added to the resulting solution and then left overnight at room temperature. After centrifugation (700 × *g* for 10 min) the

supernatant was carefully removed and 700 µl of SL2 lysis buffer (NucleoSpin® NSP soil DNA extraction kit, Macherey-Nagel, Duren, Germany) was added. The resultant solution was vortexed and transferred to a 2-ml tube. After the addition of SX Enhancer (NucleoSpin® NSP soil DNA extraction kit), the spore solution was homogenized in a FastPrep®-24 system (MP Biomedicals) at 5,000 × *g* for 30 s (twice) following the instructions provided by the manufacturer. The DNA obtained was eluted in a 50-µl elution buffer.

Quantification of Lactarius vinosus using Real-Time PCR

For *Lactarius vinosus* spore quantification, we used a species-specific hydrolysis probe (40). In order to obtain comparable results between different plates, we prepared standard curves using known amounts of *L. vinosus* DNA, which were extracted with the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany). C_T values were converted to the number of *L. vinosus* spores in each reaction using serial DNA dilutions of known amounts of spores, starting with 15.10×10^6 spores and ending with 151.2 spores. Results are expressed as the number of spores × trap sample $^{-1}$.

Real-time PCR reactions were prepared using 2X Premix Ex Taq™ (Takara Bio Europe SAS, France) following the manufacturer's instructions. The reaction mix was prepared as follows: 5 µl of DNA template, 400 nM of each oligonucleotide, 200 nM of TaqMan probe, 0.8 µl of ROX, and a volume of water (HPLC) to adjust the final reaction volume to 20 µl. The cycling conditions in the StepOnePlus instrument (Applied Biosystems) were as follows: 30 s at 95°C, followed by 40 cycles at 95°C for 5 s and at 60°C for 34 s. There were three replicates of each sample and the standards were included in the analysis, as well as a negative control using HPLC water instead of the template. Only amplification efficiencies ranging from 95% to 105% were accepted and considered for analysis. qPCR reactions, processing of data, and quantification of *L. vinosus* DNA were performed following the methods described in a previous study (11).

Spore trap sample sequencing using Illumina MiSeq

Each spore trap sample was PCR-amplified using the primers fITS7 and ITS4 (41), which amplify the ITS2 region of the rDNA. Both primers were tagged using unique DNA sequences composed of eight bases. A PCR cycle test was performed prior to randomly selected samples in

order to perform the minimum number of PCR cycles possible. PCR amplifications of samples and both negative controls from DNA extraction and PCR were conducted in a 2720 Thermal Cycler (Life Technologies) in 50 µl volumes. The final concentrations in the PCR reaction mixture were: 25 ng of template, 200 µM of each nucleotide, 2.75 mM $MgCl_2$, 200 nM of each primer, 0.025 U μl^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA) in 1× buffer PCR. The cycling conditions for PCR were: 5 min at 95°C, followed by 24–30 cycles of 30 s at 95°C, 30 s at 56°C, and 30 s at 72°C, and a final extension step at 72°C for 7 min before storage at 4°C. Each sample was amplified in triplicate, purified using an AMPure kit (Beckman Coulter Inc. Brea, CA), and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA). Equal amounts of DNA from each sample were pooled before sequencing. The final equimolar mix was purified using an EZNA Cycle Pure kit (Omega Bio-Tek). Quality control of purified amplicons was carried out using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA) 7500 DNA chip. Libraries were prepared from ~10 ng of fragmented DNA using the ThruPLEX-FD Prep kit. The samples were sequenced using the Illumina MiSeq platform, with 300-bp paired-end read lengths, generating 13.4 million sequences.

Quality control and bioinformatic analysis

Quality control, filtering, and clustering were assessed using the SCATA pipeline (scata.mykopat.slu.se). Sequences were filtered to remove data with a minimum allowed base quality score of <10 at any position, an average quality score of <20, and a minimum sequence length of 200 bp, using the amplicon quality option. Sequences were also screened for primers (using 0.9 as a minimum proportional primer match for both primers) and sample tags. We used ‘usearch’ as a search engine, considering a minimum match length of 85%. Homopolymers were collapsed to 3 bp before cluster analysis. Pairwise alignments were conducted using a mismatch penalty assigned of 1, a gap open penalty of 0, and a gap extension penalty with a value of 1. Sequences were clustered in OTUs with single linkage clustering, using 1.5% as a threshold distance with the closest neighbor. After quality control and clustering, all tags were identified and tag jumps were removed from the database (42). A tag jump has been defined as the generation of artifactual sequences in which amplicons carry different tags to those originally applied. The raw sequence reads have been deposited in the NCBI Sequence Read Archive (SRA) under accession

number PRJNA352156.

Identification of the fungal clusters

From a total of 13,408,476 MiSeq DNA sequences, 1,992,529 passed the quality control. Of these, 95,104 (4.77%) sequences were discarded because they had two different tags. Finally, 1,897,425 reads were obtained, with an average of 19,361 reads per sample.

We searched among the most abundant 2,400 OTUs (representing OTUs with more than 20 read counts) for those potential fungal species representing the fruiting body community. We first identified the OTUs using the seriateBLAST option in the UNITE database (43). We included DNA sequences from both UNITE and INSD, but preference was given to the UNITE reference for identification. In case we could not find any given species, we verified that each species had a known reference ITS sequence by checking UNITE and INSD, in order to differentiate those species not found among the spore community from those without a known ITS reference sequence. Taxonomic assignment of the OTUs was given using a 98.5% sequence similarity threshold, and all the species found at or above this threshold were included in each OTU. Read count data were transformed to relative proportions and data are shown as counts per thousand of the total read numbers per sample.

Statistics

All statistical analyses were implemented in the R software environment (version 2.15.3; R Development Core Team 2013) using the “nlme” package for linear mixed models (LME (44)), the vegan package (45) for the multivariate analysis, and the “gstat” (47) and “maps” (48) packages for spatial kriging.

Comparison of spore traps and of the two molecular methods used to quantify the fungal spores

LME models were used to test differences between spore traps (filter spore traps and funnel traps) using both qPCR and MiSeq data for *L. vinosus* and only MiSeq data for the other species. In these models, the temporal dependency was considered by defining plots nested with ‘week’ as a random factor, whereas the square root spore abundance of 12 each species was defined as a fixed

factor. In these analyses, we included the 12 species considered in this study (6 ectomycorrhizal and 6 saprotrophic fungal species) in separate models and randomly selected yeast (i.e., *Cryptococcus* spp. and *Rhodotorula* spp.) and mold species (*Mortierella elongata* and *Trichoderma* sp.) to test whether the hypothetical effect of the spore trap type was due to the increase of some of these fungal guilds.

Temporal relationship between fungal spores and mushroom yields

Temporal (week) and spatial (plot) effects on community data were tested using permutational multivariate analysis of variance (PERMANOVA) based on distance matrices (using the function adonis and Bray–Curtis distances). Plot and week were included as factors, and community data were previously Hellinger transformed. For this analysis, the fruiting body community data represented species that produced fruiting bodies in at least two plots for at least two weeks.

We analyzed the temporal (week) linear relations between spore abundance and the mushroom yields using LME models, using square root transformed qPCR and MiSeq data. To test for lagged relationships, different LME models were tested, with and without accounting for one-week temporal autocorrelation among observations (corAR1(form~week)). The best model fit was selected using the Akaike information criterion (AIC) value. We confirmed this lagged relationship with LME models using data in which spores were 1-week manually lagged. Only species that produced fruiting bodies for at least two weeks were considered for analysis. As mushroom fresh weight, dry weight, and the number of mushrooms ha^{-1} were highly correlated ($P<0.001$; $R^2 = 0.9$), we only used fruiting body counts for analysis. Data were modeled with plots defined as random to deal with the intra-plot variation and either qPCR data (*L. vinosus*) or MiSeq data (for each species) were used as explanatory variables.

Relationship between fungal spores and mushroom yields at plot level

The distribution of fungal spores and mushroom counts at the spatial scale was obtained by ordinary kriging interpolating each value using the inverse distance weighting (IDW) function. Interpolated values were graphically represented using Universal Transverse Mercator (UTM) coordinates (European Datum 1950, ED50). The relationship between spores and fruiting bodies

over the plots was tested with LME models, using only species found in more than two plots. In this case, data were modeled with week defined as random to deal with the temporal variation.

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GENERAL DISCUSSION

The results obtained in this thesis provide new insights on the ecology of soil fungal communities in Mediterranean forests, with important climate effecs over fungal functional community. In addition, this thesis provides insights in forest management effects (e.g. forest thinnings), showing that soil fungal community is resistant to thinnings. Thus, fungal communities in a long term experimental set-up of 28 *P. pinaster* plots were more influenced by seasonal dynamics and soil weather changes than by thinnings. Finally, as a part of the Industrial Doctorate Programe, we also provide methodological results concerning sample handling and soil sample processing, and also new tools and methodologies to study the airborne fungal communities using simple spore trapping devices and molecular techniques.

Soil drying procedure effects on the soil fungal community and EMM biomass

Up to date, most fungal metabarcoding studies follow different methodologies from the use of primers to bioinformatics analysis (Lindahl et al., 2013). However, it is likely that future studies will be based on standardized methods, which will be supported by contrasted and scientific research. In our case, we have shown that freeze-drying of soil samples is the most reliable method for DNA preservation and the only one that prevents the growth of other opportunistic fungi. Thus, especially when soil samples are drying at room temperature, researchers should be aware of potential estimation of moulds (Lindahl et al., 2010) and potential decreases in abundance of targeted fungi (Gryndler et al., 2013; Herdina et al., 2004).

Seasonal dynamics of the soil fungal community and EMM biomass

Despite these methodological pitfails, we have validated the use of a species-specific probe to quantify soil EMM of *L. vinosus*. Here, the use of qPCR has allowed us to describe the annual cycle of *Lactarius vinosus* and estimate its soil EMM biomass. In addition, by quantifying ergosterol, we were able to quantify the total soil EMM. Our results of both *L. vinosus* and total soil EMM is in agreement with the high soil EMM turnover observed in temperate or boreal ecosystems (Ekblad et al., 2016; Hagenbo et al., 2016; Hendricks et al., 2016), highlighting that soil EMM in Mediterranean forests is also very dynamic. However, we showed that EMM fungal annual cycle was different in Mediterranean forests compared with that described in boreal or temperate forests. For example, we observed a decay in soil EMM biomass during summer months, likely caused by summer drought, which contrasts with higher soil EMM biomass observed in boreal or temperate ecosystems (Nilsson et al., 2007; Voříšková et al., 2013). This result confirm that EMM biomass may be highly vulnerable to drought (McGuire et al., 2011; Staddon et al., 2003). In contrast, we observed an increase in soil fungal EMM

during spring and autumn. Autumn soil EMM increases have been widely observed in both temperate and boreal forests (Voříšková et al., 2013; Wallander et al., 2001), probably produced by tree allocation of carbon to mycorrhizal fungi (Högberg et al., 2010).

The effect of soil moisture and temperature in soil fungal community

Our results showed that slightly changes in intra-annual soil moisture and temperature produced significant and different functional and fungal trait responses. First, ECM fungi were less affected by drought than all the other functional groups (e.g. saprotrophs, moulds). It seems that the capacity of deeper tree roots to access groundwater under dry conditions could help to maintain fungal symbionts via hydraulic lift (Unestam and Sun, 1995; Allen, 2007; Querejeta et al., 2003; Querejeta, 2017). Second, ECM species with mycelia concentrated more tightly around the roots (e.g. contact, short exploration) were less affected by drought than ECM species with extensive mycelia (e.g. long exploration). It is known that fungi with short or contact exploration types demand less C to their host trees and differ in their ability to search for soil water (Agerer, 2001; 2006). Rygiewicz and Andersen (1994) also proposed that 'high biomass' fungal species may represent a high C cost to the host tree, compared with 'low biomass' species with contact or short-distance exploration types. Thus, since trees in Mediterranean forests often reduce their growth during summer drought, we speculate that fungi with lower C demands may be favoured under drought conditions, in comparison with those with extensive mycelia (i.e. higher C demands). This finding would also explain why specific stress-resistant mycorrhizal ascomycetes are more abundant in xeric ecosystems (Gordon and Gehring, 2011). Thus, the changes in species abundances depending on their functional traits under different soil moisture and temperatures may be caused by changes in C flows of host trees to symbiotic fungi, favouring those fungi with less C demands under drought conditions. However, it may be also possible that a soil moisture gradient along the root system is favouring fungal species that life attached to roots.

Increases of moulds, yeasts and other free-living fungi during winter, autumn or under wetter conditions has already been reported (Choudhary and Johri, 2009; Jumpponen et al., 2010; Santalahti et al., 2016). Here, our results confirm that free-living fungi may be especially susceptible to drough, likely because they lack a plant symbiont. Our results also highlighted the greater importance of soil moisture changes rather than temperature in explaining fungal community compositional shifts over an intra-annual perspective. It seems that water is more limiting climatic factor for tree and fungal growth than temperature in Mediterranean climates.

Inter-annual changes in soil fungal community composition

When inter-annual changes in temperature and precipitation were considered, surprisingly we found that both were important drivers of shifts in community composition. Thus, there was a strong inter-annual effect on composition linked to precipitation and temperature variations. Again following our intra-annual findings, free-living fungi, especially moulds, were promoted under warmer and wetter conditions. In this case, the late autumn temperature was more influent in fungal community composition than precipitation. We attribute this finding to a potential earlier autumn carbon reallocation to symbiotic fungi, typically occurring during fall in Mediterranean climates. This reallocation process match with tree physiology changes in fall (Högberg et al., 2010), that might shift slightly between years caused by different weather conditions.

Forest thinning effect on soil fungal community composition

Forest thinning effect did not change fungal community composition. This result contrast with observed effects caused by more intense forest management operations such as clear-cutting, which usually causes important losses of ectomycorrhizal species (Jones et al., 2003, Parladé et al., 2017), and changes in soil fungal community composition (Hartmann et al., 2012; Kyaschenko et al., 2017). However, it is also possible that the thinning applied in our experimental plots was not strong enough to cause fungal community shifts because the trees that were left standing were likely enough to support the soil fungal community. Positive thinning effects have been observed on the aboveground production of important edible fungal species (Bonet et al., 2012; Shaw et al., 2003). Here, thinning did not cause a significant short-term effect on fungal community composition neither on fungal species richness or diversity, suggesting that these communities are resistant to forest thinning if enough trees are left.

The use of spore traps and molecular techniques to study spatio-temporal changes on spore community and fruiting body emergence

Finally, in this thesis we have shown that spore trapping coupled with qPCR or MiSeq can detect the emergence of epigeous mushrooms. In addition, we observed an interspecific short temporal variation in the abundance of spores, especially those produced by ectomycorrhizal fungal species. These results support previous assumptions, in which the composition of the airborne fungal community was assumed to be partly driven by mushroom phenology (Kivlin

et al., 2014; Levetin, 1990). Thus, the emergence of epigeous fruiting bodies is followed by a peak in the relative abundance of their airborne spores. Also, there were significant relationships in time between fruiting body yields and spore abundance for five out of seven species. However, there was no spatial relationship between fungal spores and fruiting bodies, it seems that many spores were able to arrive from the surrounding. The spores of ectomycorrhizal fungal species are usually dispersed approximately 10 to 1000 meters (Peay and Bruns, 2014; Galante et al., 2011). The large amount of spores produced per fruiting body, e.g., $1.1 \times 10^8 – 1 \times 10^{10}$ spores (Dahlberg and Stenlid, 1994; Kadowaki et al., 2010) makes it possible for a little fraction of these spores to be dispersed long distances, particularly if carried away by turbulences.

FINAL CONCLUSIONS

- **Methodological biases in soil fungal composition and biomass analyses**
  - EMM biomass quantification of specific fungal species is affected by drying procedure (Room temperature, oven and freeze-drying), being freeze-drying the most suitable procedure to ensure the recovery of the highest EMM biomass yields. In contrast, drying at room temperature is the worst option since promote specific saprotrophs, altering real biomass quantifications of other ectomycorrhizal species.
- **Seasonality and changes in climate conditions affecting the soil EMM of *Lactarius vinosus***
  - *L. vinosus* soil EMM biomass showed great monthly fluctuations. Peaks in fungal biomass during spring and autumn are linked to increases in soil moisture and temperature. In contrast, the low soil moisture and high temperatures from summer, as well as, low temperatures during winter hamper the production of EMM biomass. Using forecasted changes in climate for Mediterranean areas, *L. vinosus* soil biomass will be reduced during summer but this biomass will be increased during winter. These changes might shift or compromise the production of autumn fruiting bodies. In addition, *L. vinosus* may be replaced by other fungal species better adapted to summer conditions.
- **Seasonality and changes in climate conditions affecting the soil fungal community**
  - From an intra-annual perspective, total soil EMM biomass is negatively affected by summer drought and winter conditions, with soil EMM biomass being higher during autumn and spring months. Decreases in soil EMM biomass during summer correlates with increases in the relative abundance of ECM species, indicating that ECM species are more resistant to drought than free-living fungi. In contrast, a decrease in soil EMM biomass during winter is linked to increases in the relative abundances of free-living fungi (e.g. saprotrophs), suggesting that these species may contribute to EMM turnover under colder and moist conditions.
- **Forest thinning effects on the soil fungal community composition**
  - Fungal community composition was not affected by light, medium or heavy thinnings. It seems that if enough trees are left soil fungal EMM species are able to persist, almost in a short-term (e.g. minimum value of 350 trees ha^{-1}).
- **Spatio-temporal changes of the spore community and fruiting body detection using spore traps and molecular techniques**
  - The spore community is highly dynamic over space and time, with temporal changes in

spore composition driven by fruiting body phenology. In contrast, the spatial variation in spore composition might be caused by a high effect of the local fruiting body community changes across plots. The methodology described using spore traps and molecular techiniques was able to detect spores coming from nearby sampling plots and the emergence of fruiting bodies of several fungal species.

The important climate effects on the soil fungal community and biomass suggest potential alterations in ecosystem functioning with respect to plant nutrition, soil organic matter decomposition and carbon storage (Averill *et al.,* 2014; Clemmensen *et al.,* 2015). The observed lack of thinning effects on the soil fungal community indicate that this forest management operation is compatible with the conservation of soil fungal communities. The high spatio-temporal changes in spore community composition highlight the importance of fruiting body community phenology in spore community dynamics. Studies addressing dispersal limitation and the factors afecting fungal dispersal and spore stablishment could be achieved using spore trapping combined with with molecular techniques.

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