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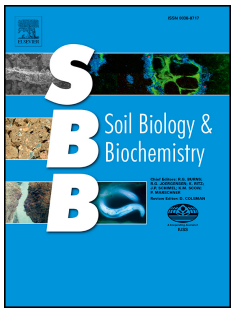

Soil physico-chemical properties have a greater effect on soil fungi than host species in Mediterranean pure and mixed pine forests

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PII: S0038-0717(21)00193-0

DOI: https://doi.org/10.1016/j.soilbio.2021.108320

Reference: SBB 108320

To appear in: *Soil Biology and Biochemistry*

Received Date: 2 July 2020

Revised Date: 23 April 2021

Accepted Date: 29 May 2021

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

Mediterranean pure and mixed pine forests

PacBio sequencing of ITS2 amplicons

P. sylvestris *P. nigra* *P. halepensis* *P. nigra-halepensis* *P. sylvestris-nigra*

Lack of host effect on soil fungal community composition and diversity

Mycorrhizal and saprotrophic proportion of community variation

Soil physico-chemistry

Geographic distance

Stand structure

Mycorrhizal fungal composition

pH

Saprotrophic fungal composition

P

Compositional drivers

CN

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

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Abstract

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

Mediterranean pine forests, soil parameters are the most important driving forces shaping soil fungal communities at the regional scale given that ectomycorrhizal and saprotrophic fungi were more influenced by soil physico-chemical parameters or geographical distance than by *Pinus* species or forest structural variables. Finally, P content in soils also emerged as a significant factor driving differences in mycorrhizal communities.

Highlights

- Soil fungal communities were profiled in 42 pure and mixed pine forests.
- Soil chemistry significantly influenced variation in soil fungal communities.
- Pine species and stand structure had no effect on the soil fungal communities.
- pH, P and the C:N ratio were the strongest predictors shaping fungal communities.

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

1. Introduction

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

Previous research from boreal and temperate ecosystems have demonstrated that soil physico-chemical properties and nutrient availability can have a strong influence in shaping soil fungal composition and diversity (Read and Perez-Moreno, 2003;

Kyaschenko et al., 2017). In this regard, pH seems to have a strong role in regulating fungal communities worldwide (Tedersoo et al., 2014; Goldmann et al., 2015; Zhang et al., 2016; Glassman et al., 2017; Tedersoo et al., 2020), and often affects nutrient cycling (Adamczyk et al., 2016), determining the availability of soil nutrients such as Nitrogen, Phosphorus and Potassium (Awad et al., 2019; Guo et al., 2020). Nitrogen is often the main limiting nutrient in soils, especially in colder terrestrial ecosystems where decomposition is limited (Read and Perez-Moreno, 2003; Kyaschenko et al., 2017). However, the primary productivity of Mediterranean terrestrial plants is generally limited by P and not by N (Du et al., 2020), suggesting that P could be a more important nutrient trader than N in these ecosystems in fungal–tree interactions. Therefore, based on the biological market theory (Konvalinková et al., 2017), P trading in these ecosystems might show similar patterns to the plant–ectomycorrhizal N trading model (Hortal et al., 2017). For example, Pérez-Izquierdo et al. (2020) found that enzymatic activity in root tips was significantly influenced by low P availability in Mediterranean *P. pinaster* and *P. halepensis* forests. In addition to the soil physico-chemistry, Mediterranean forests are highly influenced by water availability (Sardans and Peñuelas 2013; Castaño et al., 2018b), and the long-lasting dry summer periods that are typical of these ecosystems alter biologically controlled soil elements such as C and N (Jarvis et al., 2007; Delgado-Baquerizo et al., 2017). Thus, given that soil conditions and water limitation during the summer months are factors that determine plant communities (Thullier et al., 2008), soil parameters may shape fungal communities in Mediterranean soils differently than in other forest ecosystems. However, this issue remains to be explored. The tree host has also been observed to influence soil fungi, either directly via intraspecific (Pérez-Izquierdo et al., 2019) or interspecific variability that can affect tree–mycorrhizal associations (Kernaghan and Patriquin 2011; Arfi et al.,

2012; Hagenbo et al., 2020), or indirectly via changes in soil chemistry that can affect saprotrophic community structure (i.e., litter chemistry; Lladó et al., 2017). Mixed forests are of interest because they are more adaptable to climate change or disturbances than monocultures (Bravo-Oviedo et al., 2014). The coexistence of tree species may be supported by complementary niches for tree growth and nutrient uptake, increasing forest resistance to disturbances (Bello et al., 2019). Thus, due to these complementary niches, mixed forests are expected to harbour higher levels of taxonomical richness in ecosystem niches (Ishida et al., 2007, Cavard et al., 2011). In this regard, previous studies comparing soil fungal communities under distinct tree hosts in boreal and temperate ecosystems have reported higher levels of soil fungal richness in mixed stands than in pure stands (Ishida et al., 2007; Nagati et al., 2018). Although some mycorrhizal fungi are known to have relatively broad host ranges and, therefore, are rarely specific to a tree host genus (Molina et al., 1992), clear differences in soil fungal communities between pure and mixed forests have been found in studies comparing host trees with contrasting traits (i.e., deciduous vs conifers) (Ishida et al., 2007). However, it is unclear whether these differences also occur when host trees have similar traits or belong to the same genus (i.e., *Pinus*).

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

changes in tree host, geographical distance and soil physico-chemistry have not been analysed in Mediterranean ecosystems (Tedersoo et al., 2013).

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

2. Materials and Methods

2.1. Site selection

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

2.2. Soil sampling

Soils were sampled during the autumn season (October and November). Prior to this study, a 10 × 10 m plot had already been established in the centre of each of the selected forest stands for the long-term monitoring of fungal fruiting (Martínez de Aragón et al., 2007). We extracted four soil subsamples, one from the centre of each of the four sides of these plots. The upper litter layer was discarded from all soil cores to reduce the

192 sampling of needle-associated saprotrophs (Voříšková et al., 2014). We used a
193 rectangular steel drill to extract a soil core with a depth of 30 cm and a width of 6 × 4.5
194 cm. The four soil subsamples were pooled in the field and approximately 1 kg of the
195 mixed sample was stored at 4°C for < 24 h before being sieved through a 3-mm mesh
196 sieve and then stored at –20°C. A subset of the sieved sample was used to determine
197 soil physico-chemical parameters and the remainder was freeze-dried and homogenized,
198 using a pestle and mortar to form a fine powder and then stored at –20°C.

199 2.3. *Soil analyses*

200 The soil samples were analysed in the laboratory using the methodology described by
201 Alday et al. (2012). Each sample was air-dried and then sieved (≤2-mm mesh). Soil
202 texture was analysed (i.e., clay, sand and lime proportions) using the Bouyoucos-
203 method (Day, 1965). We determined the soil characteristics using the following
204 techniques: soil pH and electrical conductivity (EC) using a conductivity meter in a
205 1:2.5 soil:deionized water slurry (Allen, 1989); total N concentration using the Kjeldahl
206 method (Bremner and Mulvaney, 1982); available P concentration using the Olsen
207 method (Olsen and Sommers, 1982); total organic matter and total carbon concentration
208 using the Walkley–Black method (Walkley, 1947); and, finally, exchangeable cations as
209 sodium (Na^+), potassium (K^+) and magnesium (Mg^{2+}) using atomic absorption
210 spectroscopy after extraction with 1 N ammonium acetate (pH 7; Allen, 1989; Anderson
211 and Ingram,1993).

212 *2.3. Fungal community analyses*

213 Fungal DNA was extracted from 500 mg of homogenized soil using a NucleoSpin®
214 NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's
215 protocol. We amplified the fungal internal transcribed spacer 2 (ITS2) region in a 2720

Thermal Cycler (Life Technologies, Carlsbad, CA, USA) using the primers gITS7 (Ihrmark et al., 2012), ITS4 (White et al., 1990) and ITS4arch (Sterkenburg et al., 2018). Each primer was fitted with 8-bp tags differing in at least three positions to individually identify each sample during *a posteriori* bioinformatics analyses. We optimized the number of PCR cycles in each sample with the aim of obtaining PCR products that formed weak to medium PCR bands on agarose gels to reduce size length biases (Castaño et al., 2020), which was achieved in most of the samples by using 21–26 cycles. The final concentrations in the PCR reactions were: 25 ng template, 200 µM of each nucleotide, 2.75 mM $MgCl_2$, gITS7 primer at 500 nM, ITS4 and ITS4A primers at 300 nM and 0.025 U μL^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA) in 1X buffer in 50 µL reactions. PCR cycling conditions were as follows: 5 min at 95°C, followed by 21–30 cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C and a final extension step at 72°C for 7 min. Samples were amplified in triplicates together with negative controls obtained during the DNA extraction and PCR. Amplified products were purified using an AMPure kit (Beckman Coulter Inc. Brea, CA, USA) and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled, and the mix was purified using an EZNA Cycle Pure kit (Omega Bio-Tek) following the protocol. Amplicons were quantified and visualized using a 7500 DNA chip in a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Samples were sequenced at SciLifeLab NGI, Uppsala, Sweden on a PacBio RS II system (Pacific Biosciences, Menlo Park, CA, USA) using four SMRT cells. The PacBio RS II system was chosen because although significantly lower sequencing depths are obtained with this system compared with those obtained with other sequencing platforms, recent studies have shown that PacBio

sequencing results are less distorted than those obtained using other sequencing platforms, even at low levels of sequence output (Castaño et al., 2020).

2.4. Bioinformatics analyses

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

2.5. Taxonomic and functional identification

We taxonomically identified the 600 most abundant OTUs, which represented 93% of the total sequences. We selected the most abundant sequence from each OTU for taxonomic identification using PROTAX software (Somervuo et al., 2016) implemented in PlutoF, using a 50% probability of correct classification (considered by Somervuo et al. (2016) to be "plausible identifications"). These identifications were confirmed and some of them improved using massBLASTer in PlutoF against the UNITE database (Abarenkov et al., 2010). Taxonomic identities at species level were assigned based on >98.5% similarity to database reference sequences, or to other lower levels using the following criteria: genus based on >97% similarity, family based on >95% similarity,

265 order based on >92% similarity and phylum based on >90% similarity. OTUs were
266 assigned to the following functional guilds: (a) root-associated basidiomycetes, (b) root-
267 associated ascomycetes, (c) moulds, (d) yeasts, (e) litter-associated basidiomycetes, (f)
268 litter-associated ascomycetes, (g) pathogens, (h) moss-associated fungi, (i) soil
269 saprotrophs (saprotrophic taxa commonly found in N-rich mineral soils) or (j) unknown
270 function, based on the UNITE database, DEEMY (www.deemy.de) or FUNGuild
271 (Nguyen et al., 2016). However, for specific analyses, we used mycorrhizal community
272 (which included root-associated basidiomycetes and root-associated ascomycetes) and
273 saprotrophic fungal community (which included moss-associated fungi and soil
274 saprotrophs).

275 *2.6. Statistical analyses*

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

284 First, variation partitioning (function "*varpart"*) was used to determine the relative
285 contribution of soil parameters (i.e., sand content, pH, EC, N, P, C:N ratio, organic
286 matter, K, Mg and Na), geographical distances and stand structure (i.e., host tree
287 species, altitude, slope, number of trees per hectare and basal area) to the overall,
288 mycorrhizal and saprotrophic community composition. To avoid multicollinearity,
289 highly correlated soil variables were removed (r >0.7, i.e., EC) before variation

partitioning analysis was performed. Prior to analysis, the geographical distances were evaluated using principal coordinates of neighbours' matrices spatial eigenvectors (PCNM, *pcnm* function) based on UTM coordinates of the sampled stands with Euclidean distances. Moreover, in a second matrix we included mean annual temperature and annual precipitation to account for climatic regional and compare differences that may account for the geographic distance effect on the fungal community with and without mesoclimatic variables. Climatic data for the sampling locations were downloaded from the WorldClim database (www.worldclim.org). Thus, significant spatial eigenvectors were forward selected to be used as explanatory variables in the variation partitioning, together with soil and stand structural variables. The significance of each partition was tested using multivariate ANOVAs.

Second, differences in the overall fungal community composition between pure and mixed pine forests were assessed using permutational multivariate analyses of variance (PMAV, function "*adonis*") of a Bray–Curtis dissimilarity matrix. After that, the overall community matrix was split by main functional guilds (i.e., mycorrhizal and saprotrophs) into two matrices and analysed individually in the same way. Then, non-metric multidimensional scaling (NMDS, function "*metaMDS*") was implemented in order to visualize compositional differences in the overall, mycorrhizal and saprotrophic guilds between pine hosts. Standard deviational ellipses were used to visualize the dispersion of each forest in the ordination space. Then, the variance of the Bray–Curtis dissimilarity matrix between pine hosts for each forest type was compared using the *betadisper* function, which is an analogue of a Levene's test.

Third, we used Hill's diversity indices (Hill, 1973) to describe differences in fungal diversity between pure and mixed pine forests. The overall, ectomycorrhizal and saprotrophic communities were analysed separately using linear models. Hill's diversity

consists of three numbers: N0 is species richness; N1 is the anti-logarithm of Shannon's diversity index; and N2 is the inverse of Simpson's diversity index.

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

3. Results

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

3.1. Main drivers of fungal communities

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

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

Overall, there were no significant differences in soil fungal composition between pure and mixed pine forests (PMAV: $r^2 = 0.03$, $F_{[1,41]} = 1.01$, p-value = 0.398) given that the standard deviational ellipses of both groups were clearly superposed in the centre of the

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

3.3. Fungal diversity between forest types

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

pine forests for the overall (N2 = 11–23), mycorrhizal (N2 = 4–12) and saprotrophic fungal communities (N2 = 2–4).

Moreover, significant differences in fungal richness were detected among pine forests since the extrapolated confidence intervals did not overlap. Overall, the fungal richness of *P. sylvestris* forests (N0 = 448) was greater than that of *P. nigra–P. halepensis* (N0 = 193) or *P. halepensis* forests (N0 = 171). In the case of mycorrhizal richness, we found significant differences between *P. nigra–P. halepensis* or *P. halepensis* forests (observed richness <60) and *P. sylvestris* or *P. nigra* forests (observed richness >84; Fig. S1a). Conversely, we found significant differences in saprotrophic richness values, which were mainly due to the low richness values of the *P. nigra–P. halepensis* stands (observed richness = 16) compared with those of the *P. sylvestris* and *P. nigra* stands (observed richness >25; Fig. S1b). Finally, we found no significant effect of the environmental variables on neither fungal richness nor diversity (data not shown).

3.4. Disentangling environmental drivers of fungal communities

The general distance-based regressions showed that overall, mycorrhizal and saprotrophic community compositions were shaped primarily by soil parameters and geographical distances (Table 2). When the relative contributions of geographic, soil and stand drivers of fungal community composition were analysed, we observed that the largest proportion of overall fungal dissimilarities between forests were explained by geographical distance (28% of dissimilarities, $R^2 = 0.15$), closely followed by pH (27% of dissimilarities, $R^2 = 0.47$; Fig. 5a). In both cases, increases in pH (ranging from 4.8 to 8.5) and geographical distance between forests were associated with significantly increased compositional dissimilarity between forest stands. In addition, there were significant changes in community composition as P (ranging from 2 to 9 mg/Kg) and

414 the C:N ratio (ranging from 4 to 21.33) changed, but both variables explained smaller
415 proportions of dissimilarity (19% of dissimilarities, $R^2 = 0.09$ and 16% of
416 dissimilarities, $R^2 = 0.07$, respectively) than that explained by pH or geographical
417 distance. When considering structural variables, only altitude had a significant effect on
418 soil fungal composition (16% of dissimilarity, $R^2 = 0.10$), although this effect was also
419 lower than that explained by pH or geographical distance. The mycorrhizal community
420 showed similar general trends to that of the overall community, with geographical
421 distance being the most important driver of community dissimilarities (29%, $R^2 = 0.15$),
422 followed by pH (26%, $R^2 = 0.33$), P and the C:N ratio (17%, $R^2 = 0.07$, 17%, $R^2 = 0.06$
423 respectively), and altitude (16%, $R^2 = 0.08$, Fig. 5b). In both cases, pH was the only
424 significant factor that was positively correlated with overall richness and mycorrhizal
425 richness ($F_{[1,41]} = 23.41$, p-value <0.001, $R^2 = 0.33$). Finally, the largest proportion of
426 saprotrophic community composition variation was explained by geographical distance
427 (25%, $R^2 = 0.08$), followed by the C:N ratio, N (23% and 21% with $R^2 = 0.08$,
428 respectively) and pH (17% $R^2 = 0.07$; Fig. 5c). Thus, when the values of these variables
429 between forest stands increased saprotrophic compositional dissimilarity significantly
430 increased. In addition, N was positively associated with saprotrophic richness ($F_{[1,41]} =$
431 4.06, p-value = 0.050, $R^2 = 0.05$). Moreover, we found no significant effect of the
432 environmental variables on neither fungal richness nor diversity (data not shown).
433 Finally, the main mycorrhizal and saprotrophic species associated with these soil
434 variables are described in Figure S3.

435 **4. Discussion**

436 At regional spatial scales, niche processes, such as environmental filtering, and
437 neutral processes, such as dispersal limitation driven by spatial structure, can be
438 important determinants in structuring fungal communities (Cao et al., 2019). Our

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

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

There is a consensus that changes in soil physico-chemical properties, particularly the availability of nutrients such as N and P (Read and Perez-Moreno, 2003), shape soil fungal communities at global (Tedersoo et al., 2014), regional (Kivlin et al., 2014) and fine spatial scales (Glassman et al., 2015). Our results at regional scale agree with this given that mycorrhizal and saprophytic communities were primarily influenced by site-specific soil properties but also by geographical distance (i.e., dispersal limitation; Peay et al., 2012; Peay and Bruns, 2014). However, only a small proportion of compositional variation in this study was explained by the tree host (i.e., *Pinus* species) and stand structural variables. The lack of specificity between fungal and pine species may be related to the close phylogenetic relationships of the host trees considered here (Tedersoo et al., 2013). Moreover, the lack of stand effects on the soil fungal

communities could be because fungal networks were sufficiently preserved and all stands had enough live roots to harbour similar mycorrhizal communities (Castaño et al., 2018a; Sterkenburg et al., 2019). Our results resemble those reported by Castaño et al. (2018b) in smaller-scale Mediterranean *Pinus pinaster* stands, where fungal communities were strongly affected by soil biochemistry and geographical distance. Thus, our analyses indicate that processes such as environmental filtering produced by abiotic soil physico-chemical parameters play a dominant role in soil fungal compositional patterns in these Mediterranean pine forest ecosystems. Other processes like dispersal limitation or stochastic processes such as niche pre-emption (i.e. priority effects, Kennedy et al., 2009) might have a secondary role, potentially due to continuous forest cover promoting inoculum arrival from nearby similar forests (Redondo et al., 2020), but still mainly shaping ectomycorrhizal compositional patterns at regional spatial scales. The geographical distance effect on the soil fungal communities observed here is in accordance with distance decay similarity patterns observed in different ecosystems at local and regional scales (e.g. Bahram et al., 2013). Here, we attempted also to explore whether several other drivers could be confounded with the geographical distance effects on the soil fungal communities, such as altitude, stand structure, climate (mean annual temperature (MAT) and mean annual precipitation (MAP) and soil parameters (Fig.S4). However, only soil parameters and geographical distance emerged as significant.

4.2. Fungal community composition does not differ between pine species

As predicted, there was a lack of soil fungal compositional differences between pure and mixed forest, as well as when pure and mixed groups were split to consider the main *Pinus* host identities. Fungal community dissimilarity is tightly related to the phylogenetic distance between host tree species present in pure and mixed forests

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

The high level of heterogeneity between the selected forests in this study (high beta-diversity measured as deviational area) may explain the lack of compositional differences in the fungal communities among hosts (Fig 3). Previous studies have also reported higher community compositional dissimilarity when highly diverse and distant forests are grouped in compositional analyses (Alday et al., 2013). Therefore, recent studies have mainly focused on close homogeneous forests comprising hosts of different families or genera to detect compositional differences in soil fungal communities between pure and mixed forests (Suz et al., 2017; Nagati et al., 2018) or in common garden experiments (Pérez-Izquierdo et al., 2019). Although these considerations should not bias the conclusions from our study, care should be taken in the forest-site selection process when aiming to test the drivers of community assembly.

As observed in previous smaller-scale studies of *P. pinaster* forests (Castaño et al., 2018b), similar compositional patterns were found in the overall and ectomycorrhizal communities (Fig. 4), therefore ectomycorrhizal taxa appeared to be the main source of the overall fungal community changes in these ecosystems. In addition, saprophytic

514 community composition was similar in all pine forest types. Previous studies of
515 saprophytic fungal communities have reported that these communities are mainly
516 influenced by litter origin and chemistry (Štursová et al., 2020), which can vary with
517 forest host and stands (Li et al., 2019). Most of the soil litter in our study plots
518 originates from closely related species belonging to the *Pinus* genus, thus, the litter
519 chemistry should not differ significantly among the examined pine stands (Otsing et al.,
520 2018). Moreover, saprotrophic communities tend to be species-specific but converge
521 compositionally with forest age (Štursová et al., 2020). Therefore, the lack of a host
522 effect on the soil saprophytic community composition may be partially explained by the
523 age of the forests under study. Nevertheless, further studies of saprophytic community
524 composition should be undertaken to formally test this hypothesis in Mediterranean
525 climates.

526 *4.3. Fungal diversity across pure and mixed forest types*

527 Mixed forests were expected to harbour higher levels of diversity than pure stands
528 (Ishida et al., 2007; Cavard et al., 2011). Although the diversity of the overall and
529 mycorrhizal fungal communities significantly differed between pure and mixed stands,
530 the highest diversity values were detected in soils extracted from pure *P. sylvestris*
531 stands. Pure and mixed forests shared a great number of OTUs (i.e., more than 80; Fig.
532 S2), reducing the probability of finding compositional differences, which is likely to be
533 related to the close phylogenetic relationships of the pine species in the study stands
534 (Tedersoo et al., 2008). Nevertheless, there were significant differences in the OTU
535 richness of the overall, mycorrhizal and saprophytic fungal communities, mainly
536 because the richness values of *P. nigra–P. halepensis* stands were very low in
537 comparison to those obtained for the rest of the stands, which is the opposite of the
538 situation we had expected for a mixed forest. Unfortunately, the process behind these

extremely low richness values is unknown but could relate to differences between habitats (i.e., mixed plots were located in the poorest and driest sites). Similar results have been described by Erlandson et al. (2016), who concluded that ectomycorrhizal species richness differences were produced by soil properties and not by hosts that belonged to the same genus.

4.4. Disentangling environmental drivers of fungal communities

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

Although N has been described as an important soil element that determines fungal assemblages in boreal and temperate forests (Kyaschenko et al., 2017; Read and Perez-Moreno, 2003), in our study mycorrhizal fungal species composition was primarily driven by soil pH and P. In contrast, saprotrophic species composition was primarily associated with the C:N ratio and N. Free-living saprotrophs can have a strong influence on C:N ratios by assimilating C and N but then releasing the C into the atmosphere during respiration (Boddy et al., 2007). Conversely, some mycorrhizal fungi can affect the C:N ratio by taking N, which can then be transferred to the host trees, therefore increasing soil C:N ratios (e.g., Averill et al., 2014; Smith and Read, 2008; Clemmensen et al., 2015). P is a limiting element for primary productivity in Mediterranean ecosystems (Du et al., 2020), thus, it seems that P may be a more relevant trading element than N during the plant-fungal interactions in Mediterranean forests (Smith and Read, 2008), similar to the importance of N in other ecosystems (Hortal et al., 2017). The P gradient in our study ranged from 2 to 9 mg kg^{-1}, which are considered low values for Mediterranean ecosystems (Recena et al., 2016). Moreover, in these forests, soils are characterized by high pH values where P is mainly present in bound forms and not freely available (Antoniadis et al., 2016). Previous studies already described mycorrhizal compositional and diversity changes across P gradients in Mediterranean and temperate forests (Zavišić et al., 2016; Pérez-Izquierdo et al., 2017, Almeida et al., 2019). Therefore, in these pine forests mycorrhizal compositional dissimilarities might be explained by the intraspecific differences in the absorption of P.

4.5. Conclusions

Our analyses indicate that niche processes (soil physico-chemistry) dominate over neutral processes (geographical distance), being the main drivers of fungal community composition and are more influential than tree hosts or forest stand structure in our set

of pure and mixed *Pinus* forests along the Pre-Pyrenees. Fungal communities are not influenced by closely related congeneric host species but are primarily affected by soil properties, with pH, P and the C:N ratio the strongest predictors shaping fungal communities in these forest ecosystems. Importantly, mycorrhizal communities are significantly affected by P but not N, therefore an important nutrient trader in these ecosystems. Conversely, saprotrophic communities are significantly influenced by the C:N ratio and N. Further research should focus on a better mechanistic understanding of how variations in soil P and C:N ratios affect soil fungal communities and, consequently, Mediterranean ecosystem functioning, especially in the current climate change context.

Acknowledgements

This work was supported by the Spanish Ministry of Science, Innovation and Universities, grant RTI2018-099315-A-I00. I.A. was supported by a Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie Cofund grant agreement No. 801596, J.G.A. was supported by the Ramon y Cajal fellowship (RYC-2016-20528) and J.A.B. benefitted from a Serra-Húnter Fellowship provided by the Generalitat of Catalunya.

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959 **Tables & Figures**

960 **Table 1.** Characteristics of the study plots

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

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

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975 **Table 2** Multiple regression analyses of distance matrices on overall, mycorrhizal and

976 saprotrophic community composition

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

977 In all regressions, the Bray–Curtis community dissimilarity was used to measure fungal

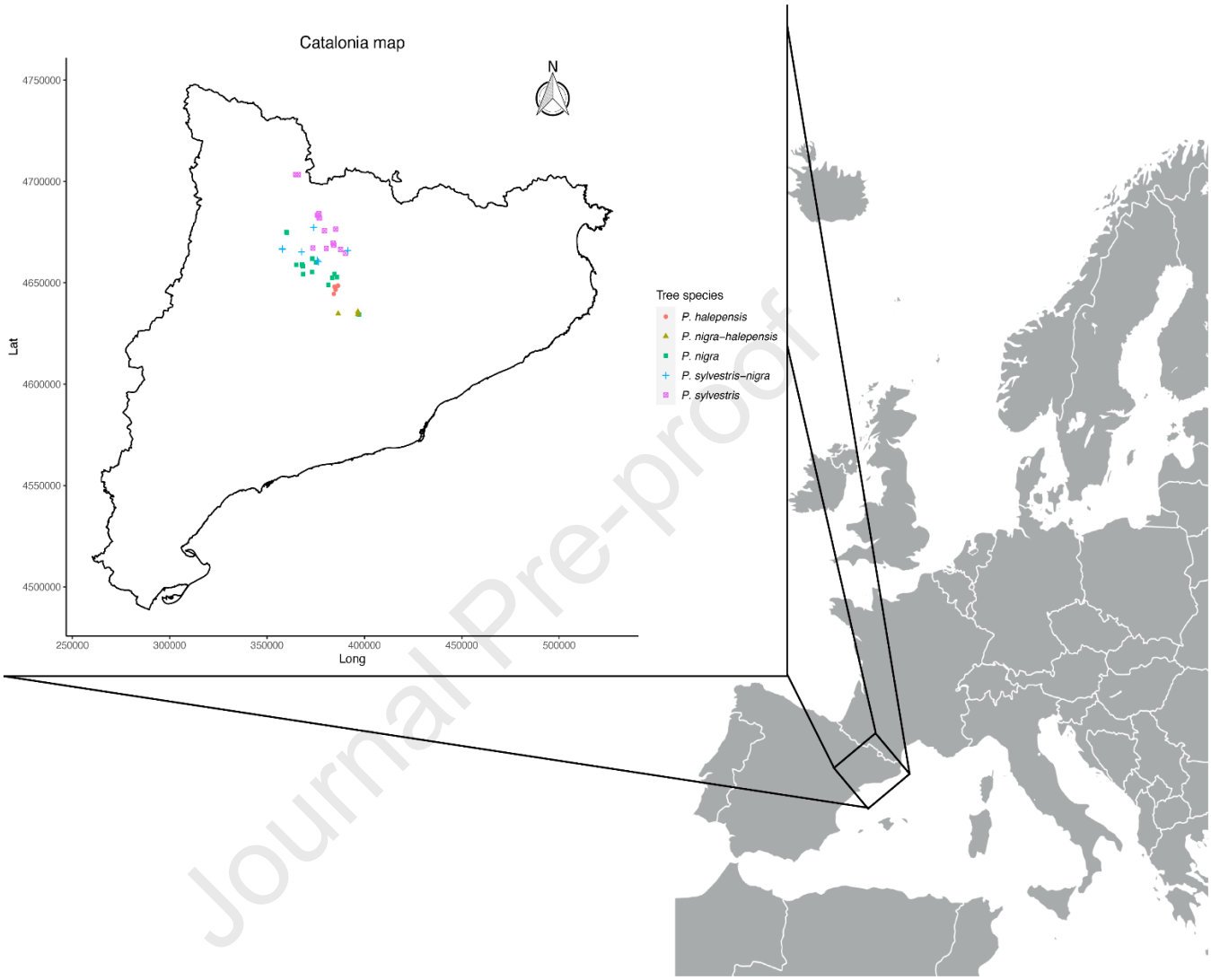
978 community distances and Euclidean distances for explanatory distance matrices, i.e.,

979 geographical distance (geo), soil, and forest variables (forest).

980

981 **Fig. 1.** Map of Catalonia showing the location of the 42 plots sampled in this study: the different

982 types of pine tree stand are indicated by different coloured symbols.



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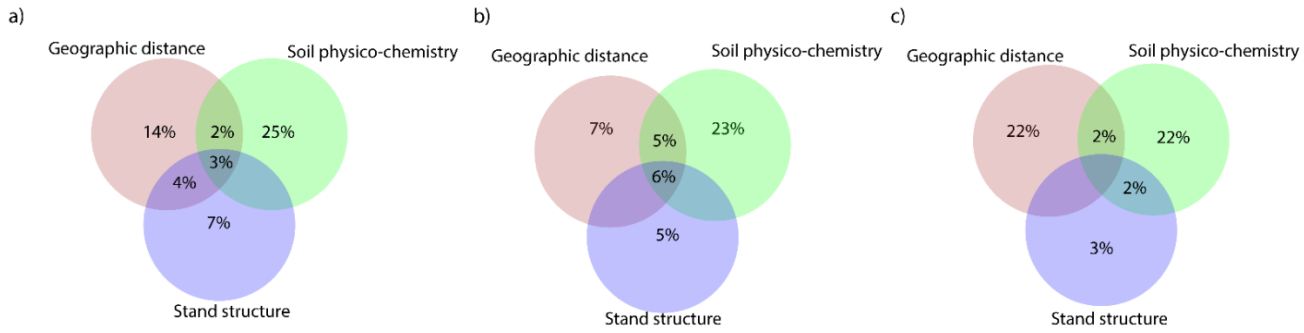

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986 **Fig. 2.** Variance partitioning analyses of (a) overall, (b) mycorrhizal and (c) saprotrophic
987 communities showing the effects of (i) geographical distance, (ii) soil physico-chemistry and
988 (iii) stand structure. Values show the fraction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

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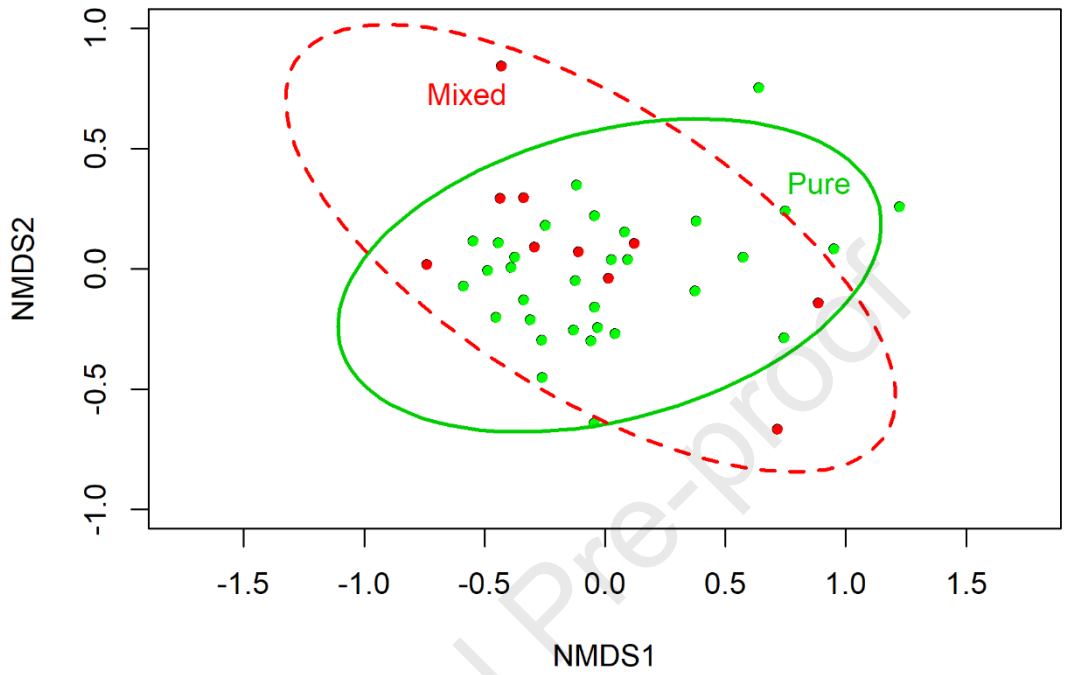

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994 **Fig. 3.** Non-metric multidimensional scaling (NMDS) showing the overall fungal

995 community similarity between pure (green) and mixed stands (red).

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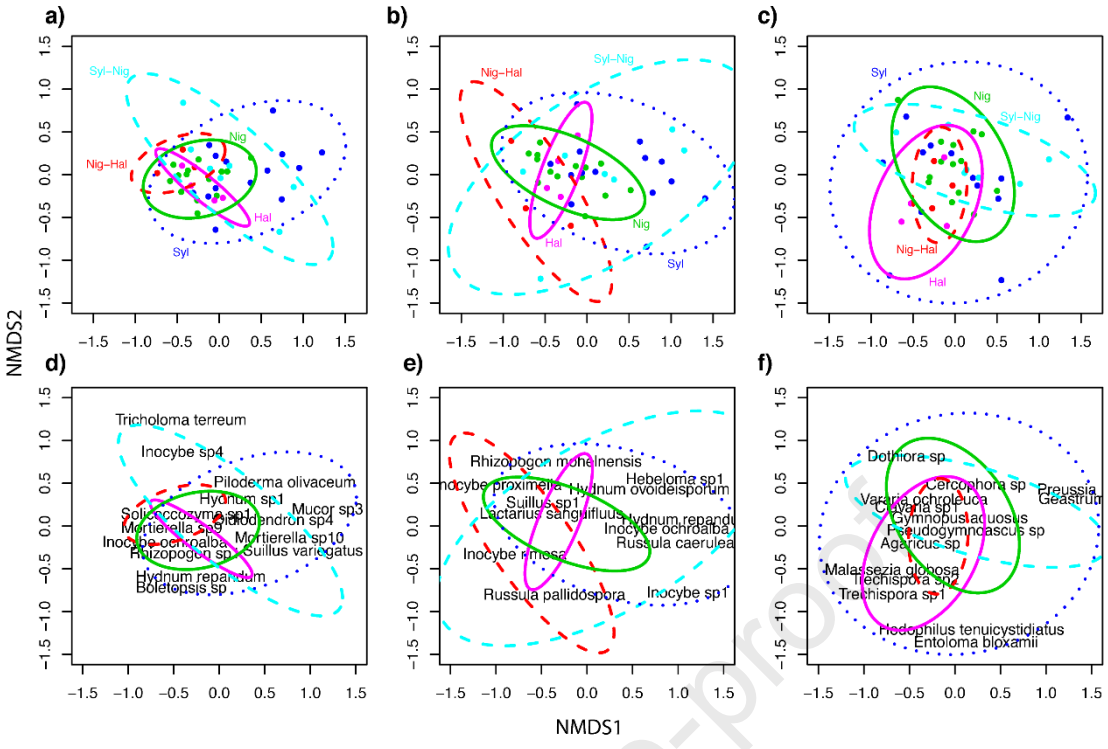

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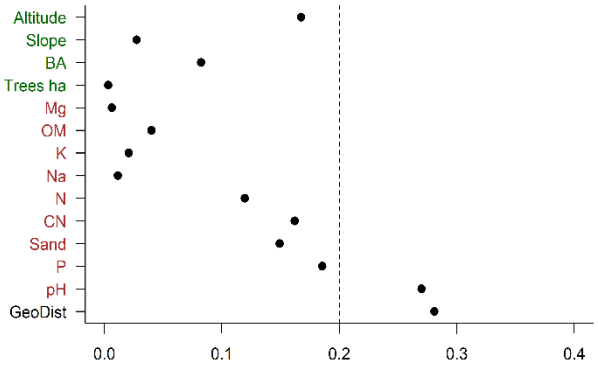
998 **Fig. 4.** Non-metric multidimensional scaling (NMDS) showing (a) overall, (b)
999 mycorrhizal and (c) saprotrophic compositional differences between forest stands: *P.*
1000 *nigra–P. halepensis* (Nig–Hal, red); *P. nigra* (Nig, green); *P. halepensis* (Hal,
1001 magenta); *P. sylvestris* (Syl, blue); *P. sylvestris–P. nigra* (Syl–Nig, cyan). (d) The most
1002 abundant species detected in the overall fungal community: *Inocybe* spp., *Piloderma*
1003 *olivaceum*, *Solicoccozyma* spp., *Hydnum* spp., *Oidiodendron* sp., *Knufia* spp.,
1004 *Mortierella* spp., *Mucor* spp., *Suillus variegatus*, *Hydnum repandum*, *Boletopsis* spp.,
1005 *Rhizopogon* spp., *Inocybe ochroalba*, *Knufia peltigerea* and *Tricholoma terreum*. (e)
1006 The most abundant species detected in the mycorrhizal community: *Rhizopogon*
1007 *mohelnensis*, *Inocybe proximella*, *Suillus* spp., *Lactarius sanguifluus*, *Inocybe rimosa*,
1008 *Russula pallidospora*, *Hebeloma* spp., *Hydnum ovoideisporum*, *Hydnum repandum*,
1009 *Inocybe ochroalba*, *Tricholoma* spp., *Russula caerulea* and *Inocybe* spp. (f) The most
1010 abundant species detected in the saprotrophic community: *Entoloma bloxamii*,
1011 *Hodophilus tenuicystidiatus*, *Trechispora* spp., *Trechispora invisitata*, *Agaricus* spp.,

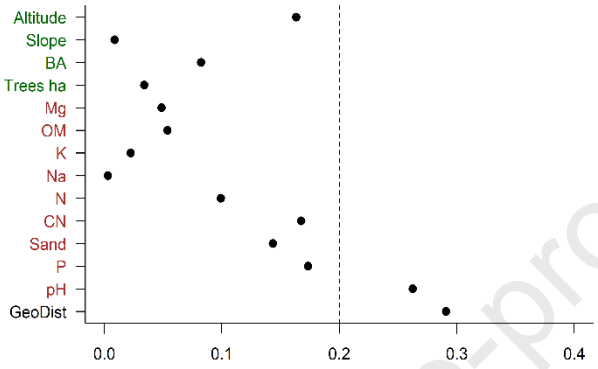
1012 *Pseudogymnoascus* spp., *Clavaria* spp., *Dothiora* spp., *Malassezia globosa*, *Gymnopus*

1013 *aquosus*, *Cercophora* spp., *Preussia* spp. and *Geastrum pectinatum*.

a)



b)



c)

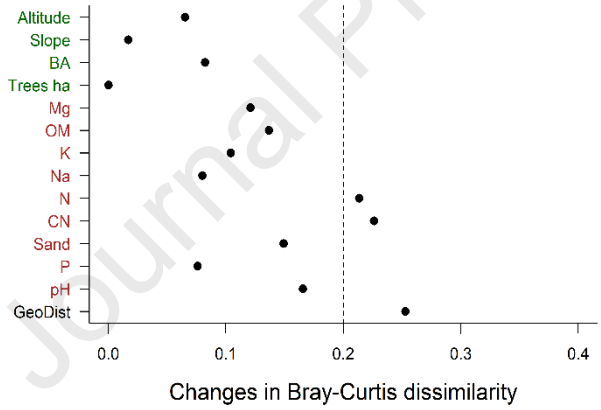
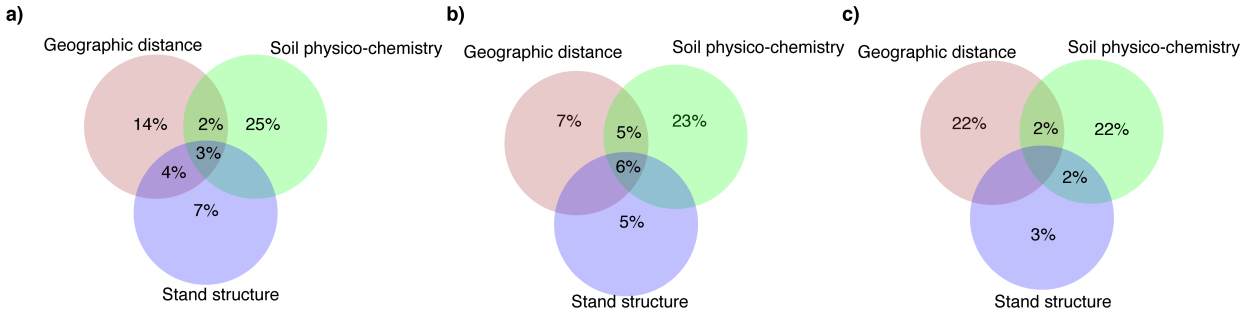
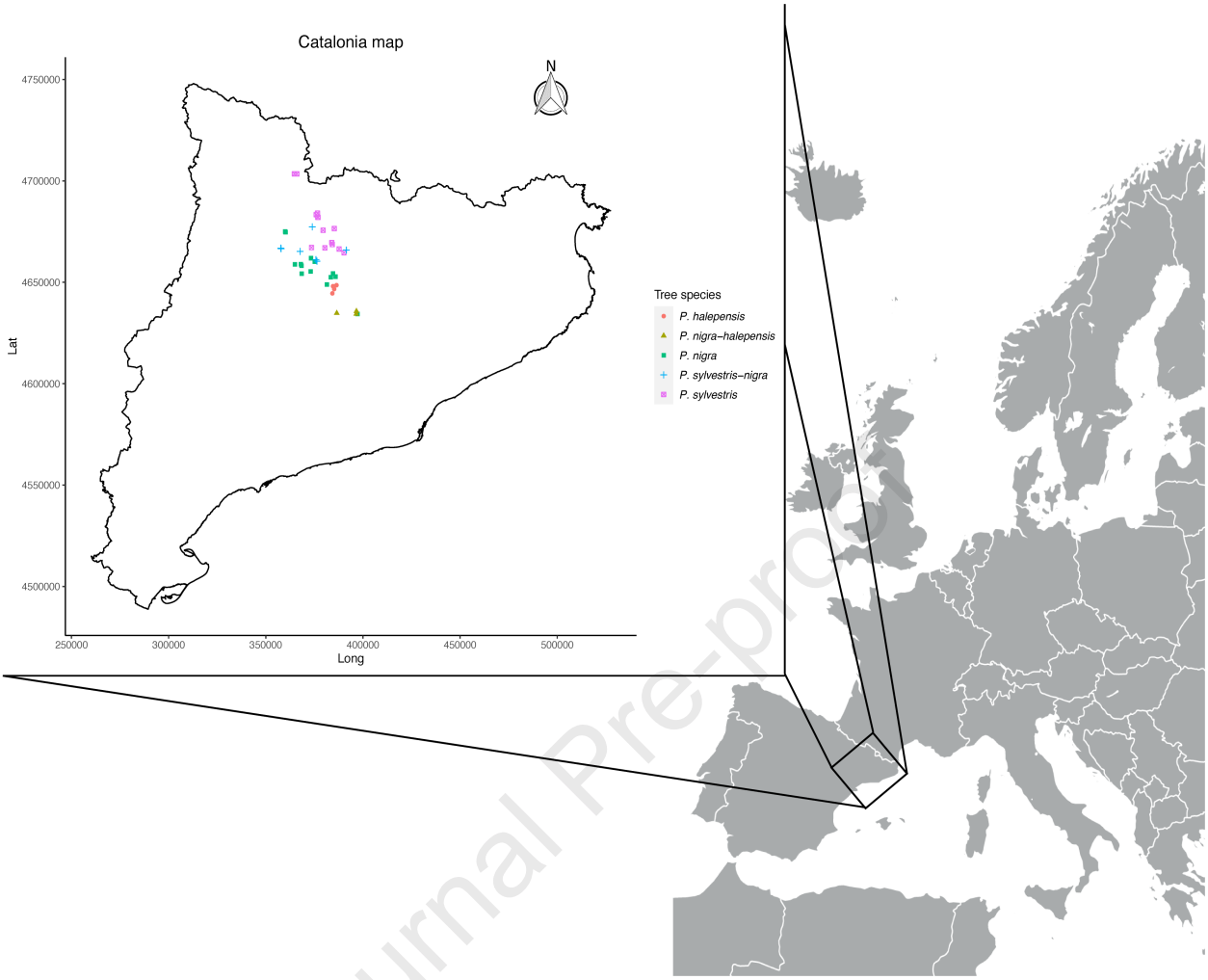
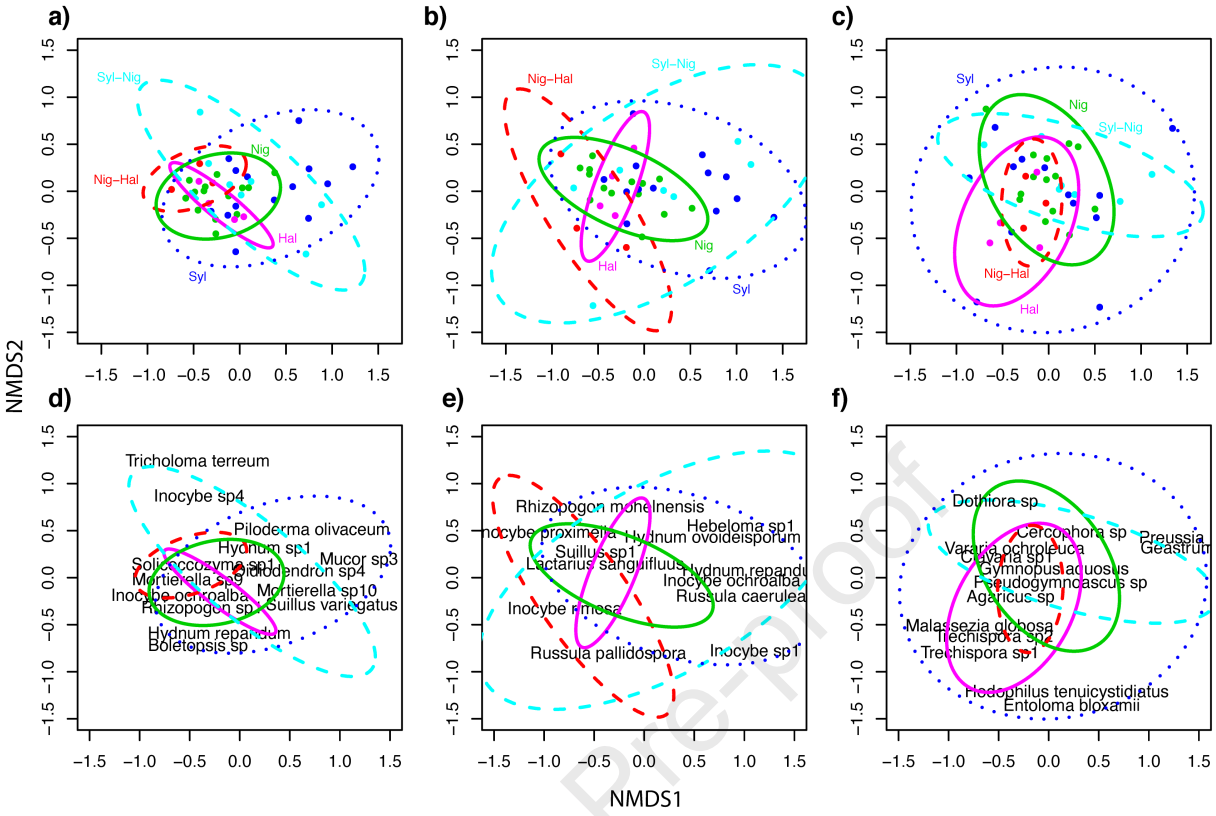


Fig. 5. Multiple regressions on distance matrices predicted changes in Bray–Curtis dissimilarity (horizontal axis) with environmental, i.e., soil physico-chemistry (brown) and stand variables (green), and geographical distance for (a) overall, (b) mycorrhizal and (c) saprotrophic community composition. The horizontal axis represents the maximum distance differences between plots of Bray–Curtis dissimilarity for each variable in isolation (y-axis). The vertical line marks the middle of the figure. Abbreviations: BA, basal area; Tree ha, number of trees per hectare; CN, C:N ratio; GeoDist, geographical distance; OM, organic matter. Considering that soil physico-

chemistry and stand structure matrices effects are decomposed in the dissimilarity explained by their vectorial parts, the overall effect for both should include all their parts. In contrast, the geographical distance effect is plotted as the overall effect including all its vectorial variables.







Highlights

- Soil fungal communities were profiled in 42 pure and mixed pine forests.
- Soil chemistry significantly influenced variation in soil fungal communities.
- Pine species and stand structure had no effect on the soil fungal communities.
- pH, P and CN ratio were the strongest predictors shaping fungal communities.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: