1 **Lack of thinning effects over inter-annual changes in soil fungal**

2 **community and diversity in a Mediterranean pine forest**

3 Carles Castaño [a, b, *], Josu G. Alday [b], Björn D. Lindahl [c], Juan Martínez de Aragón [d],

4 Sergio de-Miguel [b], Carlos Colinas [b, d], Javier Parladé [e], Joan Pera [e], José Antonio Bonet

5 [b,d]

6 [a] *Forest Bioengineering Solutions S.A. Ctra. de Sant Llorenç de Morunys, Km. 2. E-*

7 *25280 Solsona, Spain.*

8 [b] *Departament de Producció Vegetal i Ciència Forestal, Universitat de Lleida-*

9 *AGROTECNIO, Av. Rovira Roure, 191, E-25198. Lleida, Spain.*

10 [c] *Swedish University of Agricultural Sciences, Department of Soil and Environment, ,*

11 *Uppsala, Sweden.*

12 [d] *Centre Tecnològic Forestal de Catalunya. CTFC-CEMFOR. Ctra. de St. Llorenç de*

13 *Morunys km 2, E-25280 Solsona, Spain.*

14 [e] *IRTA, Centre de Cabrils, Ctra. Cabrils Km. 2, E-08348 Cabrils, Barcelona, Spain.*

15 Corresponding author at: Departament de Producció Vegetal i Ciència Forestal,

16 Universitat de Lleida-AGROTECNIO, Av. Rovira Roure, 191, E-25198. Lleida, Spain.

17 E-mail address: carles.castanyo@ctfc.cat (C. Castaño).

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Abstract

Predicted changes in global climate might negatively affect the soil microbiome and associated ecosystem processes in Mediterranean forests. Forest treatments, such as forest thinning, have been suggested to mitigate climate change impacts on vegetation by reducing competition between trees, thus increasing water availability. 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 12 pairs of thinned and un-thinned plots dominated by *Pinus pinaster* Ait. Thinning (from 30% up to 70% reduction in stand basal area) was conducted in 2009 and soil fungal community composition was studied during 4 years. Here, we used autumn precipitation and temperature to describe the impact of inter-annual weather changes. We used Pacific Biosciences sequencing of fungal ITS2 amplicons to study fungal communities in soil samples. Forest thinning did not significantly affect fungal community composition nor fungal species richness and diversity, indicating that the soil fungal community in the short-term is resistant to forest thinning regardless of its intensity. However, fungal species composition changed progressively across years, both at the species level and with regards to functional guilds. These changes in community composition were partly driven by inter-annual variation in precipitation and temperature, with free-living fungi increasing in abundance under wetter conditions, and symbiotic fungi being more prominent under drier and colder conditions. The

results indicate that mycorrhizal communities in Mediterranean forest ecosystems can resist forest thinning, if enough trees and functional roots from thinned trees are retained.

1. Introduction

Soil fungi represent an important part of the soil microbial community, and are essential drivers of many ecosystem processes, such as soil organic matter (SOM) decomposition and nutrient release as well as plant nutrient uptake and production. Mycorrhizal fungi are one of the most important functional groups of the soil microbiome, playing an important role in tree nutrition and water acquisition (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 adjacent tree hosts (Cairney, 2012). In drier ecosystems, such as Mediterranean forests, mycorrhizal fungi contribute to plant water acquisition, by providing plant roots access to less accessible water and by improving soil structure, enhancing soil water retention (Allen, 2007; Querejeta, 2017). Besides the important role of mycorrhizal fungal species, other functional guilds, such as saprotrophs, also play a paramount role in litter degradation (Baldrian et al., 2011), which may be hampered during the dry and hot summer conditions of Mediterranean forest soils. 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. In the Mediterranean basin, temperature has been forecasted to rise between 1.4°C and

71   5.1°C by 2055 (Nogués Bravo et al., 2008), and total annual precipitation projections

72   show a tendency towards less precipitation, with more extreme rainfall events (García-

73   Ruiz et al., 2011) and reduced soil moisture (Dai, 2013). Indeed, ecosystem alterations,

74   local extinctions and phenological changes in these ecosystems have already been

75   associated to current climate change (Peñuelas et al., 2002). Also, predicted drought

76   increase in Mediterranean forests will likely reduce plant growth and aboveground

77   biomass (Sardans and Peñuelas, 2013), probably with cascading effects belowground

78   (Cairney, 2012; Alday et al., 2017a). Thus, changes in climate may alter the

79   composition of soil fungal communities (Fernandez et al., 2016; Solly et al., 2017;

80   Hartmann et al., 2017; Castaño et al., 2018) and cause alterations in ecosystem

81   functioning with respect to plant nutrition, soil organic matter decomposition and

82   carbon storage (Averill et al., 2014; Clemmensen et al., 2015).

83   Climate effects on fungi may be directly driven by changes in temperature and moisture

84   (Voříšková et al., 2013; Santalahti et al., 2016) or indirectly by changes in host

85   performance (Deslippe et al., 2011; Fernandez et al., 2016; Hartmann et al., 2017), host

86   activity (Högberg et al., 2010), soil properties or litter input (Vašutová et al., 2016). In

87   drier ecosystems, some mycorrhizal ascomycetes may be more abundant (Smith et al.,

88   2007; Gordon and Gehring, 2011). Precipitation and temperature strongly influence

89   positively fruiting body emergence and production (Hernández-Rodriguez et al., 2015;

90   Alday et al., 2017b). Fire severity and thinning also affect fungal fruit body production

91   (de-Miguel et al., 2014; Salo et al., 2018) as well as community composition of fruiting

92   bodies (Mediavilla et al., 2014). Recently, we observed that intra-annual changes in soil

93   microclimate conditions strongly affected belowground fungal functional communities

94   (Castaño et al., 2018). In addition, Léon Sánchez et al., (2017) studied the climate

95   change effects on the belowground fungal community in a scrubland, and they observed

96   negative effects of drier and warmer conditions on mycorrhizal species. However,

97   studies focussed on belowground fungal responses to both thinning and climate changes

98   in Mediterranean forests are still scarce. Forest thinning has been suggested as a forest

99   management option to mitigate climate change impacts on Mediterranean forests,

100 because its potential to increase water availability and water use efficiency of trees, thus

101 changing soil microclimatic conditions. For example, Aldea et al. (2017) found that

102 thinning increased radial growth of both conifer and oak species and led to increased

103 resistance to drought and improved stand growth. Similarly, positive thinning effects

104 have been observed on the fruiting body production of economically relevant fungal

105 species (Shaw et al., 2003; Bonet et al., 2012), although the effects were species-

106 dependent. Also, sustainable forest harvesting regimes have been predicted to positively

107 influence mushroom production (de-Miguel et al., 2014). In contrast, clear-cutting and

108 associated logging disturbances in clear-cut forests have been shown to have clear

109 negative impact on soil mycorrhizal communities (Jones et al., 2003; Hartmann et al.,

110 2012; Kyaschenko et al., 2017; Parladé et al., 2017). Forest management effects on

111 mycorrhizal communities are likely to depend on whether these communities can

112 survive in symbiosis with the remaining trees (Amaranthus and Perry, 1987; Rosenvald

113 and Lõhmus, 2008). Tree removal may also affect belowground fungal communities via

114 changes in environmental conditions, such as microclimate or soil biochemistry (Jones

115 et al., 2003; Hartmann et al., 2012). Although forest thinning may have a less dramatic

116 impact than clear-cutting, its impact on belowground fungal communities has yet not

117 been assessed.

118 In this study we analysed the inter-annual dynamics of soil fungal communities during 4

119 years after forest thinning in 12 experimental plots dominated by *Pinus pinaster* Ait,

120 with 12 paired non-thinned plots as a reference. The plots represented a gradient of

121 retained stand basal area and number of trees (Bonet et al., 2012). In addition, we

122 analysed potential correlations between autumn precipitation and temperature and the

123 fungal community composition and structure. In recent studies, we analyzed the soil

124 microclimate effects on fungal communities from an intra-annual perspective, with

125 significant effects found (Castaño et al., 2018). However, here we study both the

126 climate and thinning effects from an inter-annual perspective. We specifically

127 hypothesized that i) light-medium thinning would not alter belowground fungal

128 community composition or diversity. In contrast, ii) changes in fungal species

129 composition and the relative abundance of functional guilds would be expected after

130 more intense thinning. We further hypothesized that iii) fungal community composition

131 would vary across years in relation to autumn precipitation and temperature, with mould

132 species and yeasts being stimulated under wetter conditions.

2. Material and Methods

2.1 Site selection

135 The study was carried out at a long-term experimental setup located in the natural area

136 of PNIN-Poblet (Northeast Spain, 41º 21' 6.4728'' latitude and 1º 2' 25.7496''

137 longitude), where 12 pairs of thinned and non-thinned plots were established in 2009 to

138 test the effect of forest thinning on mushroom production (Bonet et al., 2012). The plots

139 consist of even-aged *Pinus pinaster* stands (60-years-old), with isolated *Quercus ilex*

140 trees sometimes forming shrubs, while the understory is dominated by *Erica arborea*,

141 *Arbutus unedo* and *Calluna vulgaris*. Mean annual temperature at the study site is 11.8

142 ºC, and mean annual rainfall is 666.5 mm, with a pronounced summer drought that

143 usually lasts for three months (June to August). Autumn precipitation (September to

144 November) during the study years was similar across plots (136.8±3 mm), but variable

145 between years (136.8±86.4 mm), whereas temperature variation was slightly higher

146   across plots (16.06±1.13 ºC) than across years (16.06±0.84 ºC). Averaged autumn

147   rainfall was: 2009= 97.1±3.4 mm, 2012= 245.9±22.7 mm, 2013= 30.59±0.3 mm, 2014=

148   108.9±18.6 mm. Yearly averaged autumn temperature was: 2009= 17.1±0.9 ºC, 2012=

149   16.4±0.9 ºC, 2013= 15.3±1.5 ºC, 2014= 15.4±1.2 ºC. Plots are similar in soil properties,

150   but as a result of 2009 thinning, their characteristics differ considerably, with basal area

151   ranging from 16.5 to 81.7 m^2 ha^{-1} and stand density from 350 to 2,657 trees ha^{-1}. Soils

152   are siliceous with sandy loam texture, average pH 6.7±0.3, average total N 0.21±0.06%

153   and organic matter (OM) 5.5±2%.

2.2 Thinning experiment

155   Initially, 12 mushroom inventory plots of 100 m^2 (10m × 10 m) were established in

156   2008 (un-thinned plots) in an approximately 300 ha forest area (Fig. S1a). In 2009 12

157   additional inventory plots, scheduled for thinning (thinned plots), were established

158   paired (with an average distance of 50 m. from controls) with the initial plots. Each

159   thinned plot was 1600 m2 in area (40m×40m) with a central 100m^2 sampling area, to

160   reduce edge effects. In these thinned plots, three different thinning intensities were

161   employed (light: 20-30% thinned, medium: 30%-50% thinned, and heavy: 50-70%

162   thinned), resulting in basal area reductions of 30% to 70% (Fig. S1b). In un-thinned

163   plots, stand structure was more or less homogeneous, with similar tree heights and

164   diameters within plots. In thinned plots, trees were systematically removed without the

165   use of heavy machinery to avoid confounding effects caused by soil disturbance, using a

166   chainsaw and removing the cut trees from the plot. The most intense thinning resulted in

167   a remaining stand basal area of 16.5 m^2 ha^{-1} and a stand density of 350 trees ha^{-1},

168   whereas the greatest standing basal area left was 81.7 m^2 (2,552 trees ha^{-1}). Further

169   information about the thinning treatments and the stand variables before and after the

170   treatments is available in Bonet et al. (2012). A diagram of the experimental design is

171   provided as Fig S1.

172   **2.3 Soil sampling**

173   Soil sampling was conducted in all 24 plots in November 2009, 2012, 2013 and 2014

174   (Fig. S1c). Since soil was not sampled in 2010 and 2011 due to funding limitations, our

175   focus was on the immediate thinning effect (2009) and the medium term effect (2012-

176   2014). Each year, eight soil cores (12 cm deep and 5 cm in diameter) were

177   systematically sampled with a metallic probe in each plot (two along each side of the

178   plot). In these samplings, needles and partially decomposed needles were excluded,

179   since fungal community composition in the duff layer mostly consists of saprotrophic

180   fungi (Clemmensen et al., 2013; Voříšková et al., 2013), whereas humus and mineral

181   soil were sampled together. Soil samples were sieved using 3mm mesh, stored at 4ºC

182   during <24h, freeze-dried and pooled by plots. Each of the 96 composite soil samples

183   (24 plots × 4 years) was ground to fine powder using mortar and pestle. The resulting

184   fine powder was stored at -20ºC before DNA extraction.

185   **2.4 Soil fungal community analysis**

186   Genomic fungal DNA was extracted from 500 mg of soil using the NucleoSpin® NSP

187   soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol, but

188   with 900 µl of lysis buffer (SL1). The ITS2 region was PCR amplified in a 2720

189   Thermal Cycler (Life Technologies, Carlsbad, CA, USA) using the primers gITS7

190   (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990). Both primers were fitted with

191   unique 8-bp tags, differing in at least three positions. The number of PCR cycles was

192   optimized for each individual sample, with most of the samples amplifying well at 21-

193   24 cycles. Each sample was amplified in triplicates with negative extraction and PCR

194   controls. Final concentrations in the 50 µl PCR reaction mixtures were: 25 ng template,

195   200 µM of each nucleotide, 2.75 mM MgCl2, primers at 200 nM, 0.025 U µL^{-1}

196   polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA) in 1X buffer PCR.

197   PCR cycling conditions were as follows: 5 min at 95ºC, followed by 24-30 cycles of 30

198   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

199   storage at 4 ºC. PCR products were purified using the AMPure kit (Beckman Coulter

200   Inc. Brea, CA, USA) and quantified using a Qubit fluorometer (Life Technologies,

201   Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled and

202   purified using the EZNA Cycle Pure kit (Omega Bio-Tek). Quality control of purified

203   amplicons was carried out using a BioAnalyzer 2100 (Agilent Technologies, Santa

204   Clara, CA) and a 7500 DNA chip. Samples were sequenced at SciLifeLab NGI,

205   Uppsala, Sweden on a PacBio RS II system.

206   **2.5 Quality control and bioinformatic analysis**

207   Quality control, filtering and sequence clustering were conducted with the SCATA

208   pipeline (scata.mykopat.slu.se). Sequences < 200 bp in length were removed and

209   remaining sequences were screened for primers (requiring 90% sequence match) and

210   sample tags. After collapsing homopolymers to 3 bp, sequences were pair-wise

211   compared with 'usearch' (Edgar, 2011). Pairwise alignments were scored using a

212   mismatch penalty of 1, a gap open penalty of 0 and a gap extension penalty of 1.

213   Sequences were clustered into operational taxonomic units (OTUs) based on the Species

214   Hypothesis (SHs) concept (Koljalg et al., 2013) using single linkage clustering with a

215   maximum distance of 1.5% to the closest neighbour required to enter clusters. Sequence

216   data are archived at NCBI's Sequence Read Archive under accession number

217   PRJNA309233 (www.ncbi.nlm.nih.gov/sra).

2.6 Taxonomic and functional identification

We assigned putative taxonomical identities to the 500 most abundant SHs, representing 93% of the total, high-quality DNA sequence reads. The most abundant sequence from each SH was selected for taxonomical identification, using the massBLASTer in PlutoF against the UNITE (Abarenkov et al., 2010) and INSD databases. Taxonomic identities were assigned to SHs with closed matches (> 98.5% similar) to database references, or based on well supported monophyletic neighbour-joining clades including database references. Functional roles of SHs were assigned as follows: a) ectomycorrhizal b) root-associated ascomycetes, c) moulds, d) yeasts, e) black yeasts, f) other saprotrophs or litter-decay fungi, g) soil saprotrophs, h) pathogens, and i) moss-associated fungi. Classification was confirmed using FUNGuild (Nguyen et al., 2016). Ectomycorrhizal SHs were assigned to exploration types based on DEEMY (www.deemy.de) and Agerer, (2001, 2006). Taxonomical, functional and exploration types assignments are shown in Table S1.

2.7 Climate data

We obtained weather variables (precipitation and temperature) from 2009, 2012, 2013, 2014 (September, October, November) for each of the 24 plots, following the DAYMET methodology (Thornton et al., 2000), as implemented in the R package 'meteoland' (De Cáceres et al., 2017). In short, daily precipitation and temperature were estimated for each plot by averaging the values of several Catalan and Spanish meteorological stations, applying 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 average precipitation and temperature for September-October. Although samples were collected in late November, we did not

242 consider the precipitation of November because rainfall was concentrated to the third

243 and fourth weeks of the month, when sampling was already conducted.

2.8 Data analysis

245 The fungal community data set was subjected to multivariate analyses using CANOCO

246 version 5.0 (Biometris Plant Research International, Wageningen, The Netherlands) and

247 the "nlme" package for linear mixed-effects models (LME; Pinheiro *et al.,* 2016) in R

248 (version 3.0.2; R Development Core Team 2013). Species data were square-rot

249 transformed to account for taxa with many zeros and low count numbers, and only SHs

250 with more than 5 occurrences were included.

2.8.1 The effect of forest thinning on fungal community composition

252 Principal Response Curves (PRC) were used to evaluate effects of forest thinning effect

253 on fungal community composition. This method is similar to partial redundancy

254 analysis, which enables identification of time-specific treatment effects (Thinning

255 treatments × time interaction) while controlling for the overall temporal trend, using

256 time as a co-variable (Alday and Marrs, 2014). Here, year was defined as a factor with 4

257 levels (2009, 2012, 2013, 2014), whereas thinning intensity (% reduction in basal area)

258 was defined as explanatory factor with 4 levels (control: 0% thinned, light: 20-30%

259 thinned, medium: 30%-50% thinned, and heavy: 50-70% thinned). The thinning effect

260 was tested for significance using Monte Carlo simulations (999 permutations).

261 Similarly, the short-term effects of forest thinning were tested considering only data

262 from 2009 with the fungal community composition as response variable and either the

263 basal area or the number of trees removed or left as explanatory variables. Three

264 independent tests with (i) the relative abundance of SHs, (ii) the relative abundances of

265 functional guilds, and (iii) the relative abundances of exploration types within the

266 ectomycorrhizal community as response data.

2.8.2 Inter-annual changes in fungal community composition

A graphical representation of the fungal community similarity between years was obtained by Detrended correspondence analysis (DCA). The significance of changes in fungal community composition across years was tested using Canonical correspondence analysis (CCA) with plot identity as a covariate and years randomly permuted (999 permutations) in a Monte Carlo test. Year was included both as a factor and as a quantitative variable in separate analyses. Thus, tests were carried out without permutation between spatial replicates within single years. This test was performed in three independent response datasets: (i) the relative abundances of SHs, (ii) the relative abundances of functional guilds and (iii) the relative abundances of exploration types within the ectomycorrhizal community. PRC were also used to visualize changes in community composition across years. Here, plot identity was defined as a covariate, and year was defined as a factor with 4 levels and randomly permuted (999 permutations) in a Monte Carlo test. We also tested whether yearly changes in fungal community composition 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 indicated fast responses of the fungal community to changes in soil moisture and temperature (Castaño et al., 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 autumn temperature and precipitation were defined as fixed terms.

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291   **2.8.3 Thinning and climatic effects on fungal richness and diversity**

292   Hill's series of diversity indices were used to compare differences in diversity values

293   between years and between thinning intensities (Hill, 1973), considering in separate

294   analyses the whole and the ectomycorrhizal community. Hill's diversity consists of

295   three numbers: N0 is species richness; N1 is the antilogarithm of Shannon's diversity

296   index; and N2 is the inverse of Simpson's diversity index. We did not rarefy the fungal

297   community due to the potential information loss. Instead, we included square-root

298   transformed read counts as an explaining variable (Bálint et al., 2015), to account for

299   variation in sequencing depth. LME models were used to test significant changes in

300   Hill's numbers between years and due to thinning. In these analyses, plot identity was

301   defined as a random factor, whereas year identity and thinning intensity, as well as their

302   interaction, were defined as fixed factors. The same analysis based on rarefied samples

303   (richness values calculated after subsampling to 276 sequences per sample) yielded the

304   same results (data not shown). Similarly, short-term effects of forest thinning were

305   tested using LME only considering community data from 2009 with plot as a random

306   factor and thinning intensity as a fixed factor.

307   # 3. Results

308   **3.1 Sequencing output and general community composition**

309   We obtained a total of 75,608 ITS2 sequences after quality control. Single linkage

310   clustering (1.5%) resulted in 2,134 SHs after removing singletons, of which 500 (93%

311   of the high-quality sequences) were assessed for identification at the level of species,

312   functional guilds and exploration strategy. Overall, *Basidiomycota* was the most

313   abundant phyla (54±1%), followed by *Ascomycota* (32±1%). The most abundant guild

314   was ectomycorrhizal fungi, representing 65±2% of the sequences, followed by moulds

315   (13.1±0.9%), black yeasts (4.3±0.3%), root-associated fungi (3.7±0.6%), and other

316   saprotrophs, most of them classified as litter saprotrophs (3.5±0.4%). Species with short

317   and contact exploration types represented 33.2±2% and 24.6±1%, respectively, of the

318   sequences assigned to the ectomycorrhizal guild followed by medium fringe (22.2±2%)

319   and long exploration types (9.7%±2%).

320   **3.2 Fungal community responses to forest thinning**

321   Forest thinning did not have a significant effect on fungal community composition

322   (F=1.6, P= 0.621; Fig. S2), functional guilds (F=6.0, P=0.255) or the distribution of

323   exploration type among mycorrhizal fungi (F=4.7, P=0.576). Similarly, no significant

324   relationship between community composition and basal area after thinning could be

325   demonstrated (F=1.9, P=0.584). Immediate thinning effects (i.e., in year 2009) were

326   also not significant (F=0.9, P=0.895).

327   **3.3 Inter-annual variation of the fungal community**

328   Fungal species composition varied significantly across years (Fig. 1a, 1b) in a

329   progressive manner (Fig. 1a, Fig. S3), with sampling year explaining 8.2% of the total

330   inertia (CCA pseudo-F=2.2, P=0.001).

331   Relative proportions of many mould species (*Penicillium*, *Umbelopsis, Mortierella*),

332   yeasts (*Cryptococcus*, *Guehomyces*) and potential plant pathogens (*Sydowia*, *Phoma*)

333   were more abundant early in the study, whereas relative proportions from several

334   ectomycorrhizal species (*Inocybe*, *Amphinema*) increased with time (Fig 1b). In general,

335   there was a progressive increase in ectomycorrhizal species over the years, from 2009 to

336   2014 (Fig. 1b, 2a), but exploration type proportions among mycorrhizal species did not

337   change across years (pseudo-F=1.1, P=0.665).

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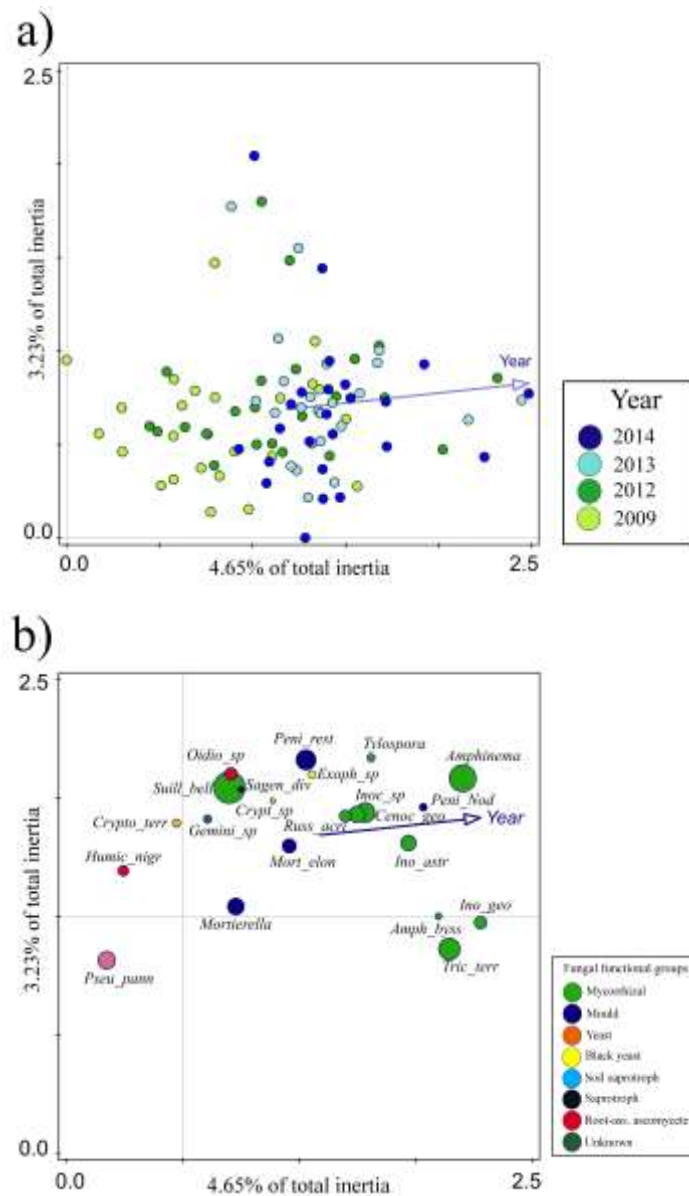
Fig. 1. Changes in soil fungal community composition across years. (a) Sample plot of a Detrended correspondence analyses (DCA) based on species level fungal community composition with different colours indicating sampling yearand (b) the corresponding species plot with colours corresponding to functional groups. Pseu_pann (*Pseudogymnoascus pannorum*), Humic_nigr (*Humicola nigra*), Crypto_terr (*Cryptococcus terricola*), Gemini_sp (*Geminibasidium_sp*), Suill_bell (*Suillus belliini*), Oidio_sp (*Oidiodendron_sp*), Crypt_sp (*Cryptococcus_sp*), Sagen_div (*Sagenomella diversispora*), Pen_restr (*Penicillium restrictum*), Exoph_sp (*Exophiala_sp*), Russ_acri

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347　(*Russula acrifolia*), Inoc_sp (*Inocybe sp*), Cenoc_gep (*Cenococcum geophilum*),

348　Pen_nod (*Penicillium nodositatum*), Ino_astr (*Inocybe asterospora*), Ino_geo (*Inocybe*

349　*geophylla*), Amph_byss (*Amphynema byssoides*), Tric_terr (*Tricholoma terreum*). Only

350　the most abundant and ecologically relevant species are included. Species names are

351　shown in italics, whereas SHs that were not identified at species level are identified to

352　the genus level.

353　Forward selection of explanatory variables identified both autumn temperature (70% of

354　fitted variation, P=0.002) and precipitation (30% of fitted variation, P=0.002) as

355　strongly related to fungal community composition across years, together accounting for

356　6.4% of the total inertia (Fig 2b). When fungal communities were evaluated according

357　to functional guilds, there was a significant correlation with autumn precipitation and

358　temperature across years (pseudo-F= 12.6, P<0.001), accounting for 26.7% of the total

359　inertia. This correlation was generally higher for temperature (82% of fitted variation)

360　than for precipitation (18% of fitted variation). The overall proportion of amplicons

361　attributed to mycorrhizal species was higher under colder and drier conditions (Table 1,

362　Fig. 2b). In contrast, the relative proportion of other functional guilds, such as black

363　yeasts, moulds and yeasts, were higher under warmer and wetter conditions (Table 1,

364　Fig 2b). The progressive change across the time span of the study, with free-living fungi

365　favoured by wet and warm conditions declining in relative abundance, was driven by

366　particularly warm autumn conditions during 2009 and particularly wet conditions

367　during 2012 (Fig. 2a). Finally, no effects of precipitation and temperature were

368　observed on litter saprotrophic taxa (Table 1). All these correlations in community

369　composition with changes in weather conditions were consistent independently of

370　whether plots were thinned or not.
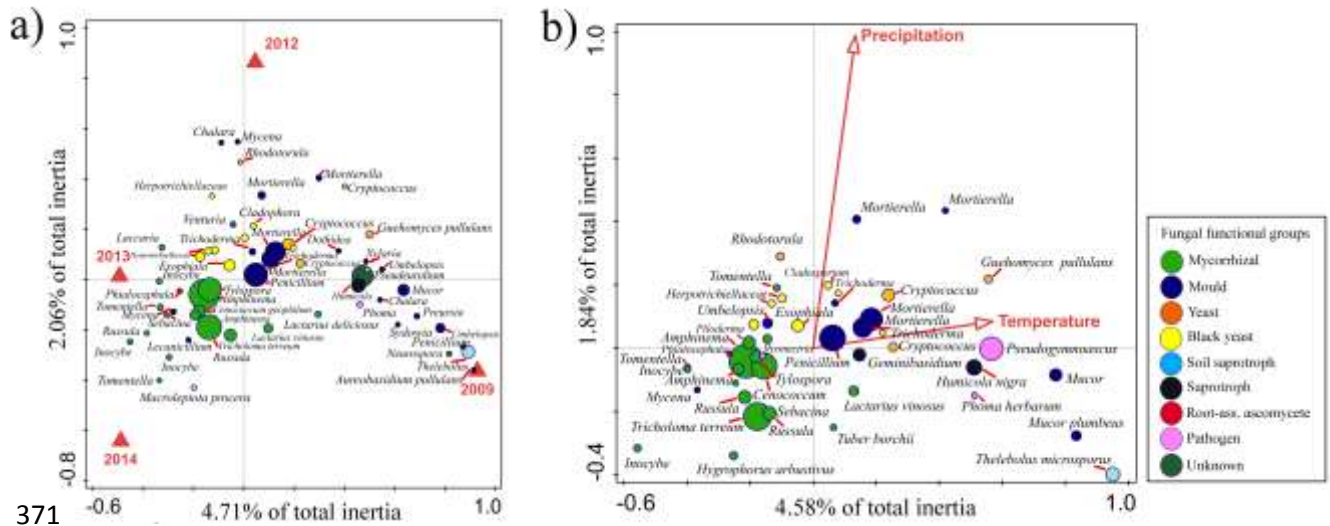
Fig. 2. CCA species plots showing correlations (a) between relative abundances of fungal SHs and the year identity, and (b) between relative abundances of fungal SHs and the variation in autumn precipitation and temperature. Species symbols in (a) and (b) are coloured according to functional guilds and symbol sizes are proportional to relative abundance.

Table 1: Fitting statistics of LME models testing correlations between relative proportions of functional group of soil fungi and inter-annual variation in autumn precipitation and temperature. Significant values are highlighted and (-) denote negative correlations.

Functional guilds	Precipitation		Temperature		Precip. × Temp.	
	F	P	F	P	F	P
Black yeast	29.37	**<0.001**	1.08 (-)	0.303	2.6	0.111
Ectomycorrhizal	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**

381 **3.4 Effects on fungal diversity**

382 There was a significant year effect on ectomycorrhizal richness and diversity (for all

383 Hill's parameters; Fig. 3a, Table 2a), with 27, 34, 35 and 37 species were detected in

384 2009, 2012, 2013 and 2014, respectively. However, there were no significant changes in

385 diversity (N1, N2) when the entire fungal community (including all functional guilds)

386 was considered, and only marginal changes in richness (N0) were found with time (Fig.

387 S4; Table 2b). Forest thinning did not have a significant effect on fungal richness and

388 diversity (Fig. 3b), nor did the interaction between thinning and year (Table 2b).
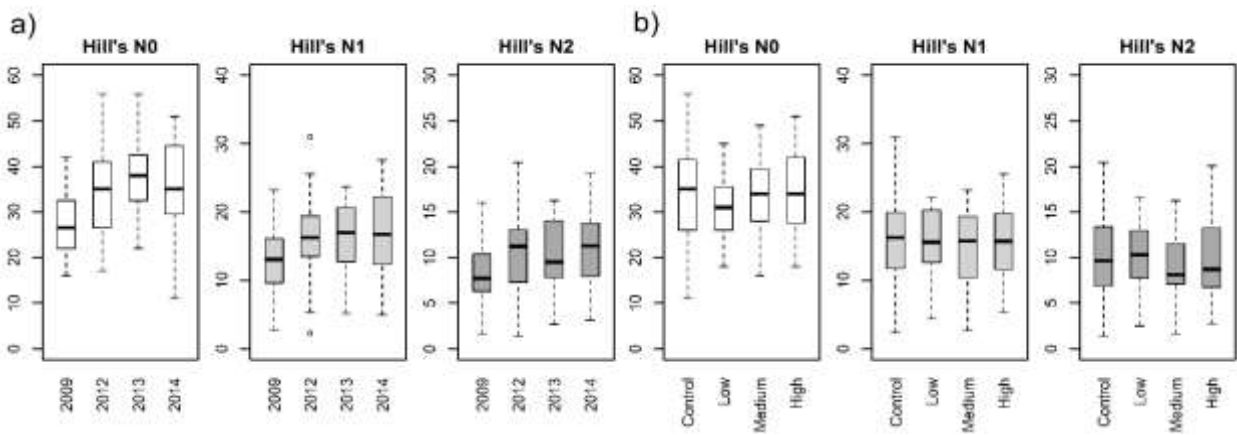
389

390 **Fig 3.** Hill's diversity values of the ectomycorrhizal community across the four years

391 considered (a) and across the thinning treatments (b).

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

Effects	dF	(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	
		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

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4. Discussion

We found a thinning-independent directional dynamics of the fungal community within the 5-year study period (Fig. 1a, 1b), related to changes in rainfall and temperature. This directional dynamic seemingly was driven by inter-annual variation in precipitation and temperature (Fig. 2a, 2b). During the warmer autumns early in the study period most non-ectomycorrhizal guilds increased their relative proportions, whereas ectomycorrhizal species were relatively more abundant during the cooler years towards the end of the study period. Moulds and yeasts also increased in abundance during wetter conditions (2012).

4.1 Lack of short-term thinning effects on the fungal community

Forest thinning did neither significantly affect fungal species composition or guild composition, nor fungal diversity. Despite that thinning may have effects on specific fungal species (Bonet et al., 2012; Liu et al., 2016), it seems that most ectomycorrhizal species can survive belowground supported by the remaining trees, seedlings or other ectomycorrhizal plants remaining in the plots (Amaranthus and Perry, 1987; Rosenvald and Lõhmus, 2008). Our results contrast with previously observed effects of more intense timber removal operations, such as clear-cutting, which usually lead to major losses of ectomycorrhizal species (Jones et al., 2003, Parladé et al., 2017), changes in ectomycorrhizal (Varenius et al., 2016, 2017) and/or overall soil fungal community composition (Hartmann et al., 2012; Kyaschenko et al., 2017) and alterations in general soil biology with potential indirect effect on fungal diversity (Colinas et al., 1994a, b). For example, tree harvesting may change soil microclimatic conditions, which in turn may affect soil fungal communities. In this regard, although soil microclimatic data measured in 2013 showed no thinning effects on soil moisture, soil temperature significantly increased in thinned plots (results not shown). Despite these positive

425   effects of thinning on soil temperature, thinning effects on belowground fungal diversity

426   and community composition were non-significant. Fungal communities may resist

427   thinning by survival on living roots of retained trees (Varenius et al., 2017) or in

428   naturally established seedlings (Cline et al., 2005). In our study, it seems that the

429   density of remaining trees in all the thinning categories sufficed to act as a post-thinning

430   'refuge' for the mycorrhizal community (Varenius et al. 2017). In addition, other

431   functional guilds, such as saprotrophic fungi, did not respond to thinning either, likely

432   because their substrates were not affected by the thinning operation. Surprisingly, even

433   heavy thinning (up to 70% reduction in stand basal area, down to 350 trees ha^{-1} left) did

434   not affect the species composition or functional composition of soil fungi, rejecting our

435   hypothesis that extensive thinning would lead to major changes in fungal species

436   composition and dominance of functional guilds. Thus, it seems as relatively sparsely

437   distributed trees may be efficient in preserving the mycorrhizal diversity as long as tree

438   roots and their associated extramatrical mycelium form a continuum across the forest

439   area. Lack of compositional effects of thinning together with climate dependent

440   temporal changes suggest that weather conditions pose a stronger environmental filter

441   on community dynamics that overshadows thinning induced reduction in C flow in this

442   system. One particularity of our study was that thinning was performed with minimal

443   disturbance to avoid soil scarification and compaction (Bonet et al., 2012; using

444   chainsaw rather than heavy machinery to fell the trees). Usually, soil compaction results

445   in a reduction of water retention capacity and it has been shown to affect the soil fungal

446   communities (Hartmann et al., 2012). Perhaps some of the effects of thinning on forest

447   fungi reported in the literature have more to do with soil disturbance than with the

448   actual reduction of basal area. This hypothesis is congruent with our results but it would

449   have to be formally tested in the future.

4.2 Inter-annual changes in community composition and correlation with changes in weather conditions

It could be possible that these directional changes in community composition were driven by changes in temperature, since we observed that temperature was also linearly decreasing across years. These changes in temperature could differently stimulate plant host activity, resulting in distinct fungal responses belowground (Högberg et al., 2010). 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 from an intra-annual perspective (Jumpponen et al., 2010; Voříšková et al., 2013) or under snow cover (Santalahti et al., 2016). In addition, we recently found similar results in Mediterranean forests, also from an intra-annual perspective (Castaño et al., 2018). In our study, yeasts belonging to Tremellomycetes (*Cryptococcus* and *Guehomyces*) decreased in relative abundance during our inter-annual study period, in parallel with decreasing temperature and precipitation, confirming their preference for moist environments (Choudhary and Johri, 2009). In agreement with our third hypothesis, and with Hartmann et al. (2017), Zygomycete fungi (mostly moulds) also responded positively to cooler and wetter climate, but litter saprotrophs did not. Litter-associated fungi accounted for less than 4% of the sequences, probably excluded by our sampling scheme that focused on the humus and mineral horizon. Despite the lack of effects on the moss-associated fungal species found in this study, they were highly represented in wetter years, and intra-annual studies from the same site found a clear positive effect of soil moisture on this group of fungi, both from a spatial and an intra-annual perspective (Castaño et al., 2018). This trend may be related to the phenology of the host trees, as carbon allocation to symbiotic fungi typically peak during the fall (Högberg et al., 2010) and may have shifted with changing weather conditions. The

475 differential response of fungi in different functional guilds might indicate a competitive

476 advantage for root-associated fungi (i.e. mycorrhizal) over free-living fungi (i.e. yeasts,

477 moulds, moss-associated) under drier conditions. Potentially, hydraulic lift may grant

478 root-associated fungi more resistance to drought, as groundwater can be supplied to

479 fungal symbionts via roots (Allen, 2007; Querejeta et al., 2003, 2017). The decrease in

480 the proportion of free-living fungi under drier conditions may be especially important

481 for Mediterranean forests soils, where water is the most limiting factor for tree growth,

482 and provide insights into future potential climate-change effects on the belowground

483 fungal community and associated processes such as decomposition and nutrient cycling.

484 5. Conclusions

485 In our study, thinning did not have a significant effect on fungal community

486 composition or diversity, indicating that these communities are resistant to forest

487 thinning if enough trees are left on site. However, our observations regarding inter-

488 annual changes in fungal composition and their relationship to changes in

489 meteorological conditions have important implications for our understanding and

490 prediction of future effects of climate change in Mediterranean forests. Further research

491 should address how the climate-related effects on fungal community will affect

492 ecosystem processes such as nutrient cycling, and thinning effects should be evaluated

493 in different tree species and forest ecosystems under different climate regimes.

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509   **Conflict of interests**

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

511   **References**

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Supplementary material
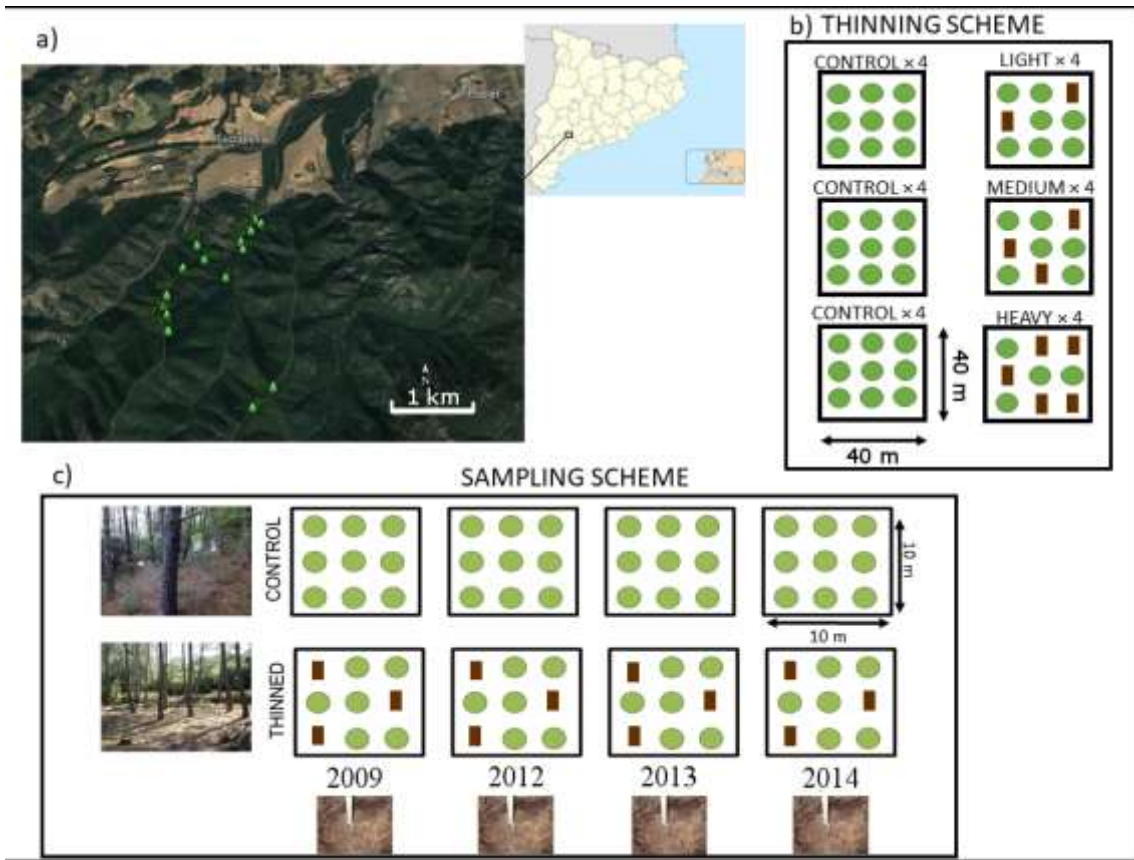
Fig. S1 (a) Geographical localization of the plots. Each of these plots consisted in two

paired plots, one thinned and another one un-thinned, except for the plots 311 and

314 (not included in this study), amounting a total of 24 plots. Map obtained from

Google earth V 6.2.2.6613 (November 2017). (c) Thinning design, consisting in

three treatment levels (light thinning, medium thinning and heavy thinning) in a 40

× 40 m. plots. (c) Sampling pattern, in which soil samples were obtained in 2009,

2012, 2013 and 2014 in both un-thinned and thinned plots. In total, 8 soil samples

were obtained in each of these plots and pooled to a composite sample.
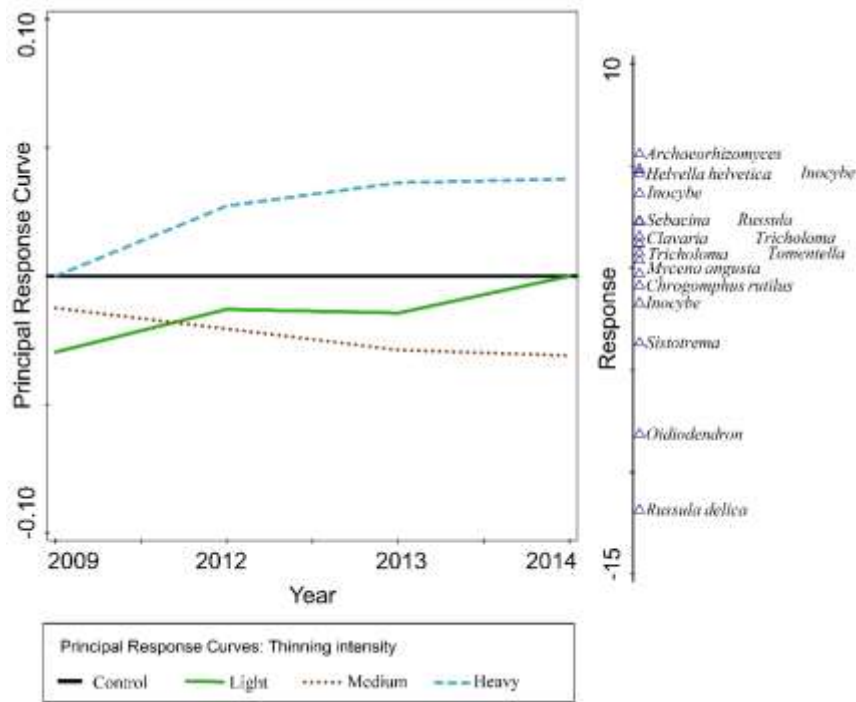
Fig. S2: Principal Response Curves obtained from the whole set of 24 plots.

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. Relativ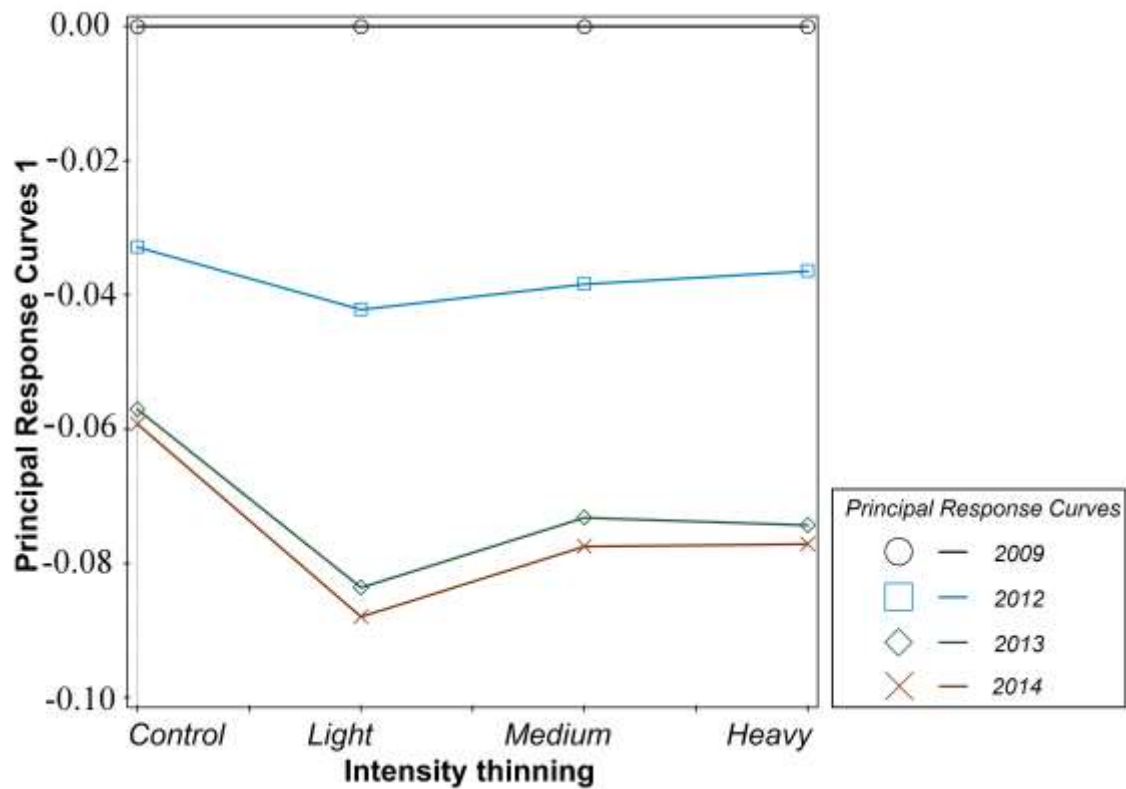e proportion increases of specific fungal taxa under certain thinning intensity treatment are shown on the right axis. Although some species became more abundant under specific forest thinning treatments (genera and species list in the right, Y-axis), the overall effects of the thinning treatments were not significant (F=1.6, P= 0.621).

Fig. S3: Principal Response Curves obtained from the whole set of 24 plots. Each

curve represents a different year (2009, 2012, 2013, 2014). Here, the factor year is

tested, and the direction of changes in community composition across treatments is

shown for all years.

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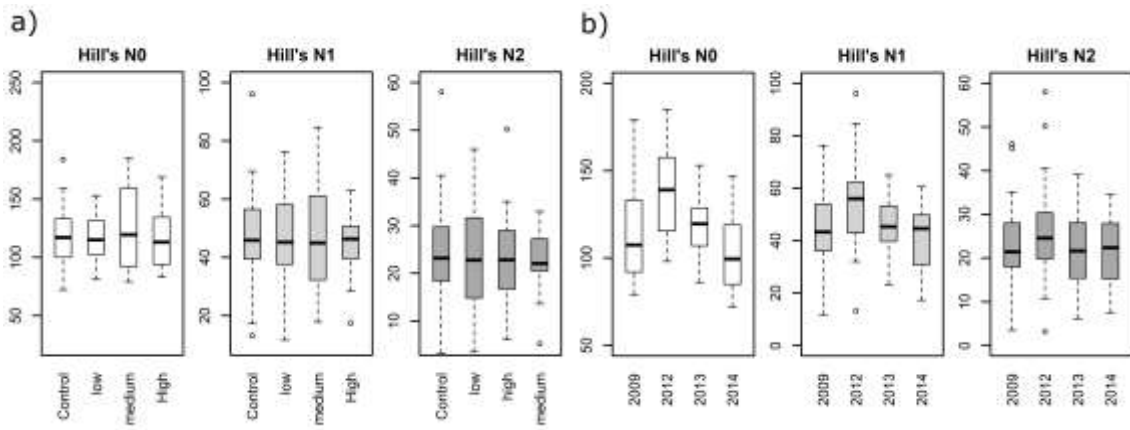Fig. **S4** Hill's diversity values of the whole fungal community across the four thinning treatments (a) and across the four years (b).

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