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Soil microclimate changes affect soil fungal communities in a Mediterranean pine forest

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Summary

- Soil microclimate is a potentially important regulator of the composition of plant-associated fungal communities in climates with significant drought periods. Here, we investigated spatio-temporal dynamics of soil fungal communities in a Mediterranean *Pinus pinaster* forest in relation to soil moisture and temperature.
- Fungal communities in 336 soil samples collected monthly during a year from 28 long-term experimental plots were assessed by PacBio sequencing of ITS2 amplicons. Total fungal biomass was estimated by analysing ergosterol. Community changes were analysed in the context of functional traits.
- Soil fungal biomass was lowest during summer and late winter and highest during autumn, concurrent with a greater relative abundance of mycorrhizal species. Intra-annual spatio-temporal changes in community composition correlated significantly with soil moisture and temperature. Mycorrhizal fungi were less affected by summer drought than free-living fungi. In particular, mycorrhizal species of the short-distance exploration type increased in relative abundance under dry conditions, whereas species of the long-distance exploration type were more abundant under wetter conditions.
- Our observations demonstrate a potential for compositional and functional shifts in fungal communities in response to changing climatic conditions. Free-living fungi and mycorrhizal species with extensive mycelia may be negatively affected by increasing drought periods in Mediterranean forest ecosystems.

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

Introduction

Soil fungi are essential drivers of organic matter dynamics and nutrient release and uptake in coniferous forest ecosystems. However, climate changes, such as warming or increased drought, may alter the composition of soil fungal communities (Fernandez *et al.*, 2016; Solly *et al.*, 2017; Hartmann *et al.*, 2017). In Mediterranean forest ecosystems, drought stress and low nutrient availability are important determinants of functional and structural traits of plants (e.g. sclerophylly and low growth rate; Sardans & Peñuelas, 2013). However, expected increases in temperature and the unprecedented duration and intensity of drought events could exceed the tolerance of plant

communities (Collins *et al.*, 2013). Ectomycorrhizal (ECM) fungi are key players in the alleviation of drought stress for their host trees, and more so as the frequency of drought events increases (Mohan *et al.*, 2014). Mycorrhizal fungi may contribute to plant water acquisition, both directly by increasing access to soil water (Allen, 2007), and indirectly by providing their host plants with nitrogen and phosphorus (Smith & Read, 2008) and improving soil structure and porosity through the formation and stabilisation of soil aggregates and organic matter (Querejeta, 2017). The relationship may also be reciprocal, as the plant host may improve water access of its associated mycorrhizal fungi through hydraulic lift, especially during summer (Querejeta *et al*., 2003; Unestam & Sun, 1995; Querejeta, 2017). In temperate or boreal ecosystems, ectomycorrhizal fungi have been found to increase in relative abundance during summer or autumn (Wallander *et al.*, 2001; Jumpponen *et al.*, 2010; Voříšková *et al.*, 2014; Santalahti *et al.*, 2016), probably due to a higher below-ground allocation of host sugars during the growth season (Högberg *et al.*, 2010; Žifčáková *et al.*, 2017). However, there is a lack of information relating to intra-annual patterns in fungal community composition and biomass in Mediterranean forests soils.

Several studies have shown that changes in climatic conditions may affect soil fungal communities, directly or indirectly (Hartmann *et al.*, 2017; Fernandez *et al.*, 2016; Solly *et al.*, 2017). Mycorrhizal species have shown contrasting responses to changes in climate, and responses are modulated by nutrient availability (Clemmensen *et al.*, 2006; Solly *et al.*, 2017) and host tree responses (Fernandez *et al.*, 2016; Hartmann et al., 2017). Recent studies of Mediterranean ecosystems have suggested that the biomass of some ECM species is dynamic across seasons, and that mycelial production is often halted during summer and winter (Iotti *et al.*, 2014; Castaño *et al.*, 2017; Queralt *et al.*, 2017). For example, by simulating future increases in summer drought in Mediterranean areas, we have recently predicted sharp decreases in *Lactarius vinosus* soil biomass during summer but increases during winter–spring (Castaño *et al.*, 2017). Despite these findings, similar studies considering the whole soil fungal community and biomass are still lacking in Mediterranean ecosystems.

Non-mycorrhizal fungal species, such as litter saprotrophs and opportunistic moulds, are also affected by changes in climatic conditions and exhibit seasonal changes in community composition, generally increasing in relative abundance under colder conditions (Jumpponen *et al.*, 2010; Andreetta *et al.*, 2011; Voříšková *et al.*, 2014; Santalahti *et al.*, 2016). In addition to the influence of microclimatic parameters, these

fungi may also be indirectly influenced by changes in soil properties or ground cover (Vašutová *et al.*, 2016). Climate may also select for specific groups of fungal traits (Fernandez *et al.*, 2016; Treseder & Lennon, 2015). Among these traits, ECM mycelial exploration types (i.e. long-exploration, short-exploration, contact or mat-forming) have been proposed to represent several important fungal traits that affect water and nutrient acquisition (Agerer, 2001, 2006). It has been proposed that species with short- or contact-exploration types, i.e. with mycelial biomass largely concentrated to the immediate surroundings of the roots (Agerer, 2001, 2006; Deslippe *et al.*, 2011; Fernandez *et al.*, 2016), might impose a lower carbon cost on the host plant, which could be advantageous for the host plant under stressful conditions (Fernandez *et al.*, 2016). By contrast, species with more extensive mycelium (i.e. medium fringe–long distance exploration types) may be more demanding in terms of host C (Agerer, 2001; Lehto & Zwiazek, 2011; Fernandez *et al.*, 2016). The limited water availability in Mediterranean forests often results in a reduction of tree growth during summer (Sardans & Peñuelas, 2013), and likely has a negative effect on ECM fungi (Shi *et al.*, 2002).

Here we studied monthly changes in soil fungal community composition by high-throughput sequencing of amplified fungal markers (Lindahl *et al.*, 2013) across 28 long-term experimental plots over the course of a year. Our main objective was to determine whether temporal fluctuations in fungal community composition correlated with intra-annual changes in soil moisture and temperature. We also investigated micro-climatic effects on soil fungal communities by studying spatial variation between plots. The results were interpreted in the context of fungal functional guilds and traits. Responses of the total biomass of soil fungi were also assessed by analysing the fungus-specific biochemical marker ergosterol (Wallander *et al.*, 2013). We hypothesised that (i) total fungal biomass in soils would be lower during drier conditions. However, mycorrhizal fungi may be more resistant to drought, because they may use water provided by their host tree. Thus, we expected (ii) that mycorrhizal fungi would increase in abundance relative to free-living species during drier conditions. Finally, we hypothesized (iii) that responses to climate across mycorrhizal species would be related to mycelial growth form (i.e. exploration types).

Materials and Methods

Site selection

The study site was located in the Natural Park of Poblet (Northeast Spain, 41° 21' 6.4728'' E, 1° 2' 25.7496'' N), where 28 previously established 10 × 10 m long-term monitoring plots were selected for the study. This study site has been widely used as an experimental area to study the effects of climate change in Mediterranean ecosystems (Peñuelas *et al.*, 2017). The entire experiment was initiated for a long-term project, which evaluated the mushroom production in a set of plots that were randomly distributed covering a wide range of stand characteristics. Plots consisted of even-aged (60-years-old) reforested *Pinus pinaster* (Aiton) forest, with isolated *Quercus ilex* (L.) trees, sometimes forming scrub together with other understory plant species, mostly *Erica arborea* (L.), *Arbutus unedo* (L.) and *Calluna vulgaris* (L.) Hull. Herbaceous species were rare. The plots were distributed over approximately 300 ha. area across a range of different altitudes (from 594 to 1013 m above sea level) and slopes (3–23%). The soils are characterised by siliceous minerals with franc-sandy textures, pHs ranging from 6.1 to 7.3, and organic matter contents in the upper 12 cm ranging from 3.0% to 10.5%. The mean annual temperature is 11.8°C and the mean annual rainfall is 667 mm, with summer droughts usually lasting for three months (July–August–September). During the study period, the summer drought lasted for three months, with 29 mm of precipitation recorded between July and September, whereas 210 mm of precipitation were recorded in late November alone. The average temperature during the study period was 12.3°C and the total precipitation was 655.9 mm.

Soil sampling

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

Ergosterol analyses

The total fungal biomass present in the soil was estimated by quantifying the fungal-specific biomarker ergosterol. Ergosterol was extracted as described by Nylund &

Wallander (1992) and chromatographically analysed as described by Hagenbo *et al.* (2017). Ergosterol data were converted to fungal biomass using a conversion factor of 3 µg ergosterol mg^{-1} dry matter (Salmanowicz & Nylund, 1988), and a correction factor (1.62) was applied to compensate for unextracted ergosterol (Montgomery *et al.*, 2000).

Fungal community analysis

Genomic fungal DNA was extracted from 500 mg aliquots using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol, but with 900 µl of lysis buffer. The fungal internal transcribed spacer 2 (ITS2) region was PCR amplified in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) using the primers gITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990), both of which were fitted with unique 8-bp tags, differing in at least three positions. The number of PCR cycles was optimised for individual samples, with most of the samples amplifying well at 21–24 cycles. The final concentrations in the 50-µl PCR reaction mixtures were: 25 ng template, 200 µM of each nucleotide, 2.75 mM $MgCl_2$, primers at 200 nM and 0.025 U μl^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA) in 1X buffer. PCR cycling conditions were as follows: 5 min at 95°C, followed by 24–30 cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C and a final extension step at 72°C for 7 min before storage at 4°C. Samples were amplified in triplicates with negative extraction and PCR controls. PCR products were purified using the AMPure kit (Beckman Coulter Inc. Brea, CA, USA) and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled, and the mix was further purified using the EZNA Cycle Pure kit (Omega Bio-Tek). Quality control of purified amplicons was carried out using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and a 7500 DNA chip. Samples were sequenced at SciLifeLab NGI, Uppsala, Sweden on a PacBio RS II system (Pacific Biosciences, Menlo park, CA, USA) using 28 SMRT cells.

Bioinformatic analysis

Sequences were quality filtered and clustered using the SCATA pipeline (https://scata.mykopat.slu.se/). Sequences with length of <200 bp were removed, after which remaining sequences were screened for primers (requiring 90% primer match) and sample tags. After the collapse of homopolymers to 3 bp, sequences were pair-wise compared using 'usearch' (Edgar, 2011). Pairwise alignments were scored using a mismatch penalty of 1, gap open penalty of 0 and a gap extension penalty of 1. Sequences were clustered into species hypotheses (Kõljalg *et al.*, 2013) using single

linkage clustering, with a maximum distance of 1.5% to the closest neighbour required to enter clusters. Global singletons were excluded from further analyses. Sequence data are archived at NCBI's Sequence Read Archive under accession number PRJNA309233 (www.ncbi.nlm.nih.gov/sra).

Taxonomic and functional identification

We assigned putative names to the 550 most abundant Species Hypotheses (SHs), which represented 93% of the total sequences (Table S1). We selected the most abundant sequence from each SH for taxonomic identification, using the massBLASTer in PlutoF against the UNITE (Abarenkov *et al.*, 2010) and INSD databases. Taxonomic identities were assigned based on > 98.5% similarity with database references, or on supported monophyletic neighbour-joining clades that included reference sequences (Fig. S2). SHs were assigned to the following functional guilds: a) ectomycorrhizal, b) moulds, c) yeasts, d) black yeasts, e) litter saprotrophs, f) soil saprotrophs (saprotrophic taxa commonly found in N-rich mineral soils), g) pathogens, h) moss-associated fungi, i) root-associated ascomycetes, and j) unknown function, based on the UNITE database and DEEMY (www.deemy.de) or other published literature. Ectomycorrhizal species were assigned to exploration types according to Agerer (2001, 2006), Suz *et al.* (2014) and the dEEMY database (Agerer & Rambold, 2017), among others (Table S1).

Climate data

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

Data analysis

Fungal community data were subjected to analyses using CANOCO version 5.0 (Biometris Plant Research International, Wageningen, Netherlands) for ordinations and the 'nlme' R package for linear mixed effect models (LME; R version 3.0.2, R Development Core Team 2015).

Differences in soil fungal biomass were analysed using LME models after square-root transformation. Two different independent analyses were carried out to test temporal and spatial relationships between fungal biomass and soil conditions. To analyse

temporal variation in fungal biomass in relation to soil moisture and temperature, as well as their interaction, 'plot identity' was considered a random factor, whereas 'month' was defined as a fixed factor together with the climatic variables. To analyse spatial variation in fungal biomass in relation to soil temperature and moisture, as well as their interactions, 'month' was considered a random factor, whereas 'plot identity' was defined as a fixed factor together with the climatic variables. In addition, models were tested with and without accounting for 1-week temporal autocorrelation among observations (AR1), and the most parsimonious model was selected based on the Akaike Information Criterion (AIC).

SH abundance data were Hellinger transformed before all multivariate analyses to account for taxa with many ceros and low count numbers. First, the relative importance of soil climatic factors, soil biochemistry and geographical distance as predictors of soil fungal community composition was evaluated by variation partitioning analyses. The first axis (PCA1) of a Principal Coordinates Analysis (PCA) of soil biochemistry parameters (Fig. S1a) was used as a biochemical index. Similarly, the first axis of a PCA of soil temperature and soil moisture was used as a soil climate index (Fig. S1b.). As a geographical distance index, we first calculated the principal coordinates of neighbor matrices (PCNM) spatial eigenvectors, based on UTM coordinates of the sampled plots, using Euclidean distances. We used forward selection of explanatory variables to select for significant eigenvectors. The scores of the significant spatial eigenvectors (named PCOs) for each plot were used as explanatory variables in the variation partitioning analyses, together with the soil biochemical and climate indexes. Finally, in a separated variation partitioning analysis, we identified the variance explained by spatial (plot) and temporal (month) changes on the community composition, by using the "varpart" function in CANOCO."

Detrended correspondence analysis (DCA) was used to obtain graphical representations of fungal community similarity between plots and months. Temporal variation in fungal community composition was related to soil moisture and temperature, as well as their interaction, by canonical correspondence analysis (CCA). Here, 'plot identity' was defined as a covariate, and months were randomly permuted (999 permutations), without permutation between spatial replicates within single months (i.e. effective N = 12), treating 'month' as a repeated measure. We tested the effect of soil moisture, soil temperature and their interaction by forward selection of explanatory variables. Three independent tests were performed considering: (i) community composition at the SH

level, (ii) relative abundances of functional guilds and (iii) relative abundances of exploration types within the ECM community. CCA was also used to relate spatial variation in fungal community composition to soil moisture and temperature. The significance of the explanatory variables was established using Monte Carlo permutation tests (9999 permutations under the full model) without permutation of repeated observations from single plots (i.e. effective N = 28), and forward selection of explanatory variables. The same analysis was carried out using the relative proportion of each functional guild and ECM exploration type. Changes in the relative abundance of individual functional guilds and exploration types in response to variation in soil temperature and soil moisture and their interaction were assessed *post-hoc*, using LME models of square-rot transformed relative proportions. In these LME models, fungal groups were defined as response variables, whereas soil moisture, soil temperature and their interaction were defined as explanatory variables (fixed terms). Both plot identity and month were included as random factors in a single analysis.

Results

The average soil fungal biomass (dry matter), as based on ergosterol measurements, was 4.9 ± 0.1 mg g soil^{-1} (0.145 kg m^2 or 1,450 kg ha^{-1}) and varied significantly between plots (F=11.06, P<0.001) and months (F = 5.04, P < 0.001; Fig. 1a). Fungal biomass was lowest during the summer months, especially in August (3.8 mg g soil^{-1}), but increased during late summer–autumn to a maximum in October (5.9 mg g soil^{-1}). Biomass decreased progressively during the winter months to another minimum in February (4.0 mg g soil^{-1}) and increased again during the spring (Fig. 1a). At spatial scale, fungal biomass was negatively correlated with temperature (F=3.87, P=0.050). At temporal scale, fungal biomass was significantly correlated to the interaction between soil temperature and moisture (F = 5.28, P = 0.022) (Fig. 1b), with soil mycelial biomass having a negative correlation with moisture under cold conditions, a positive correlation with temperature under wet conditions, and a negative correlation with temperature under dry conditions (Fig. 1b).

A total of 408,788 out of 791,099 sequences (52%) passed quality filtering. Single-linkage clustering resulted in 3,063 SHs, of which 550 (93% of the high-quality sequences) were assessed for identification to species level, functional guild and exploration type (for ectomycorrhizal fungi). We obtained an average of 27,268±3,406 reads per month and 9,703±1,084 reads per plot. This corresponded to 977±353 (SD)

reads per sample (min= 287 reads, max= 2,368). Overall, basidiomycota dominated the fungal community (56 ± 1% of the identified sequences), followed by ascomycota (30 ± 0.6%). Regarding functional guilds, mycorrhizal species were by far the most abundant (53 ± 5%), followed by moulds (7 ± 0.6%). Other functional groups, such as yeasts or saprotrophs, together represented 12% of the total abundance, and taxa with unknown or non-determined function accounted for 28 ± 0.6% of the reads. The three most abundant mycorrhizal genera were *Inocybe* spp. (30 ± 0.6% of the mycorrhizal sequences), *Russula* spp. (23 ± 0.8%) and *Tricholoma* spp. (8 ± 0.4%). Among ECM exploration strategies, the short exploration type was most abundant (36 ± 0.3%), followed by the contact type (22 ± 0.5%), the medium-distance fringe type (20 ± 0.5%) and the long-distance type (4%± 0.5%).

Temporal changes accounted for 3.1% of the total variance in community composition, whereas plot identity accounted for 31.8% of the total variance. At the spatial scale (plot differences), the soil climate index contributed to 4.9% of the total variance, whereas the soil biochemistry index and geographical proximity contributed to 6.4% and 5.6% of the total variance, respectively (Fig. 2a, 2b).

Fungal species composition varied systematically across months according to soil moisture and temperature (total monthly inertia = 0.29, adjusted explained variation = 4.4%, Fig. 3a). The largest temporal differences in fungal community composition were observed between the cold and moist months (positive first-axis values, corresponding to winter months; Fig 3a) and the warm and dry months (negative first-axis values, corresponding to summer months; Fig. 3a). Forward selection indicated that soil moisture was more important in explaining temporal variation in community composition (59.7% of fitted variation, $P = 0.002$) than soil temperature (40.3%, $P = 0.001$), and the interaction was not significant ($P = 0.48$). Relative abundances of certain species, especially moulds (e.g. *Mortierella* and *Umbelopsis*) and yeasts (e.g. *Cryptococcus*), were higher during wetter and colder months (positive first-axis values in Fig. 4a), whereas other species, especially mycorrhizal taxa (e.g. *Russula* and *Inocybe*), were relatively more abundant during drier and warmer months (negative first-axis values in Fig. 4a).

Accordingly, many functional guilds correlated with soil temperature and moisture across months (pseudo-$F = 9.7$, $P < 0.001$), accounting for 6.7 % of the total inertia (Fig. 5a). Forward selection of explanatory variables identified that soil moisture was more important (85% of fitted variation) than soil temperature (15% of fitted variation)

in explaining changes in guild composition across months. Exploration types of mycorrhizal species also correlated significantly with soil temperature and moisture across months (pseudo-F = 4.7, P = 0.004), accounting for 3.3% of the total inertia (Table 1, Fig. 6a). Forward selection of explanatory variables indicated that soil temperature (87% of fitted variation) was more important than soil moisture (13% of fitted variation) in explaining the temporal variation in exploration types.

Spatially, fungal species composition also varied systematically across plots according to soil moisture and temperature (Fig. 3b), with significant correlations with both soil moisture (F = 3.1, P = 0.035) and soil temperature (F = 2.7, P = 0.008), accounting for 2.7% of the total inertia (Fig. 4b). Changes in the distribution of functional guilds between plots were also correlated with soil moisture (F = 14.2, P = 0.006, Fig. 5b), accounting for 5.0% of the total inertia. Spatial variation in soil moisture, but not temperature, correlated significantly with the relative distribution of exploration types (CCA: Pseudo-F = 7.4, P = 0.044, Fig. 6b), accounting for 2.7% of the total inertia.

Overall, mycorrhizal species correlated negatively with soil moisture (Table 1; Fig. 7a), whereas many free-living fungi correlated positively (Table 1; Fig 7b). The relative abundance of ECM taxa decreased from 75% of the sequences in the driest month (August) to 58% in the wettest month (February) (Fig. 7a). By contrast, the proportion of amplicons attributed to moulds (Fig. S3b), moss-associated fungi (Fig. S3c), yeasts and black yeasts correlated positively with soil moisture. The overall proportion of amplicons attributed to black yeasts (Fig. S3d) and litter saprotrophs (Fig. S3e) correlated negatively with soil temperature.

Climate effects on exploration types of fungi were particularly driven by species belonging to the long-distance exploration type, which were more abundant under wetter and colder conditions (Table 1, Fig. S3f, negative first-axis in Fig. 6b), and the short-distance exploration types, which were more abundant under drier conditions (Table 1, Fig. S3g, Positive X-axis in Fig. 6b). Temperature only affected long-distance exploration types, which were more abundant under colder conditions (Fig. S3h).

Discussion

Spatial variation in fungal community composition was much larger than temporal variation. Fungal communities were affected both by soil biochemistry and soil climate, and also structured according to geographical proximity. We found that intra-annual variation in fungal biomass and fungal community composition correlated with changes

372 in soil moisture and temperature. Intra-annual changes were also found among guilds
373 and among specific mycorrhizal exploration types. Correspondingly, spatial fungal
374 community patterns across local plots also reflected different microclimatic features.
375 Shifting balances between fungal functional guilds together with changes in fungal
376 biomass suggest potential alteration in ecosystem functioning with respect to plant
377 nutrition, soil organic matter decomposition and carbon storage (Averill *et al.*, 2014;
378 Clemmensen *et al.*, 2015; Kyaschenko *et al.*, 2017).
379 A marked seasonality of soil fungal communities has been reported previously
380 (Jumpponen *et al.*, 2010; Andreetta *et al.*, 2011; Voříšková *et al.*, 2014), with seasonal
381 changes being more pronounced in the topsoil layer than in deeper horizons (Andreetta
382 *et al.*, 2011; Voříšková *et al.*, 2014). Our results indicate that the soil fungal
383 communities of Mediterranean forest ecosystems also show temporal changes within a
384 year, although spatial variation at local scale (<3 km) is larger. Overall, soil moisture
385 seemed to be more important than temperature in explaining variation in fungal
386 community composition in this ecosystem. In contrast, studies of subarctic and alpine
387 tree line ecosystems have reported that warming may cause important fungal
388 community shifts and changes in soil fungal biomass, because higher temperatures may
389 stimulate nutrient cycling and plant production (Clemmensen *et al.*, 2006; Solly *et al.*,
390 2017). Here, soil fungal biomass was lower during summer than during autumn and
391 spring, supporting previous reports from other Mediterranean forest ecosystems (Iotti *et*
392 *al.*, 2014; Castaño *et al.*, 2017; Queralt *et al.*, 2017) and potentially reflecting
393 differences between Mediterranean and boreal forest ecosystems.
394 We observed that functional changes in the fungal community correlated with changes
395 in soil moisture and temperature. Drought conditions during summer were negatively
396 correlated with soil fungal biomass in our plots, supporting our first hypothesis that
397 summer drought would reduce soil fungal biomass. Surprisingly, however, we observed
398 high levels of fungal biomass also under conditions of low soil moisture, such as those
399 recorded in October, provided that soil temperatures were not too high. The increase in
400 fungal biomass during autumn correlated with an increase in the relative abundance of
401 ECM species. In Mediterranean forests, trees may shift their primary water source from
402 the surface soil to groundwater during the summer (Voltas *et al.*, 2015). The capacity of
403 deeper tree roots to access groundwater under dry conditions could help to maintain
404 fungal symbionts via hydraulic lift (Unestam & Sun, 1995; Allen, 2007; Querejeta *et*
405 *al.*, 2003; Querejeta, 2017). This adaptation to drought may represent an advantage for

root-associated symbionts versus free-living fungi (i.e. saprotrophs, moulds, yeasts and moss-associated fungi). Our results support this hypothesis, with a greater relative abundance of mycorrhizal fungi relative to free-living fungi during the summer period, as well as a higher relative proportion of mycorrhizal fungi in drier plots.

In other ecosystems, such as boreal or temperate forests, increasing relative abundance of ECM species has also been observed during the late growing season (Jumpponen *et al.*, 2010; Voříšková *et al.*, 2014; Santalahti *et al.*, 2016), concurrent with increases in total fungal biomass in soils (Wallander *et al.*, 2001; Nilsson *et al.*, 2007; Högberg *et al.*, 2010). Thus, the observed increase in fungal biomass during autumn (September–October) along with a higher relative representation of ECM species also supports the theory that the increasing allocation of carbon from the tree host to the roots during the autumn enhances the growth of mycorrhizal fungi (Wallander *et al.*, 2001; Högberg & Högberg, 2002; Högberg *et al.*, 2010). Potentially, there may be a link between host supply of C from photosynthesis, and water transport from roots to soil mycelium (Unestam & Sun, 1995).

We also observed a decreasing trend in fungal biomass during the winter months (November–February), together with a decreasing relative representation of mycorrhizal species but an increased relative abundance of saprotrophs, including yeasts (*Cryptococcus*, *Rhodotorula*), litter saprotrophs (*Mycena*) and moulds (*Mortierella*). Mycorrhizal fungi may decline during winter, when carbon allocation from the host is reduced (Jumpponen *et al.*, 2010; Voříšková *et al.*, 2014). Wetter and colder conditions during late autumn and winter may favour free-living fungi, such as moulds (Hartmann *et al.*, 2017; Castaño *et al.*, 2016; Santalahti *et al.*, 2016). Mould species can use a variety of carbohydrates, such as cellulose, pectin and starch (Thormann *et al.*, 2001) and they likely contribute to the turnover of dead mycorrhizal mycelium (Lindahl *et al.*, 2010; Jumpponen *et al.*, 2010).

The mycelial architecture of ectomycorrhizal fungal species has been used to differentiate species traits (Agerer, 2001, 2006) with implications for nutrient and water mobilisation, as well as for the demand for host carbon. In this study, mycorrhizal species with long-distance exploration types of mycorrhiza were less abundant under drier conditions, whereas short-distance and contact species increased. This pattern was observed temporally but also spatially across plots with varying micro-climatic conditions. From a fungal perspective, drought conditions imply a moisture gradient away from the roots, with the non-suberized root tips of deeply rooted trees constituting

"moisture hot spots" in the otherwise dry surface soil. This soil moisture gradient away from the roots could be caused by hydraulic lift, promoting more translocated water near the roots than in the bulk soil (Querejeta *et al.*, 2003). This situation may favour fungi that focus their biomass to the interior and surface of tree roots, as well as the closer rhizosphere, over both non-symbiotic fungi and ECM fungi with extensive extramatrical mycelial proliferation. Thus, when soil becomes extremely dry, it is likely that fungi that mainly reside close to roots, such as short-distance exploration types or other low biomass ascomycetes typically found in xeric environments (Smith *et al.*, 2007), have a comparative advantage over fungi with a large proportion of their mycelium in the drier bulk soil (e.g. long-distance exploration types). In addition, association with fungi with more extensive production of extraradical mycelium may imply a higher C demand on the host (Agerer, 2001, 2006) and reduce tree fitness. In contrast, 'low biomass' species of the contact or short-distance exploration types (Agerer, 2001, 2006; Deslippe *et al.*, 2011; Fernandez *et al*., 2016), as well as specific stress-resistant mycorrhizal ascomycetes (Gordon & Gehring, 2011), could be less demanding in terms of host C and may, thus, be favoured under drought or other stresses.

It should be mentioned that long-distance exploration types were represented by only 8 OTUs, all from the order Boletales, and most within the *Suillus*/*Rhizopogon* clade. It seems plausible that their typically extensive proliferation away from roots was disadvantageous in particularly dry soils. However, it is also possible that other traits of these species made them particularly sensitive to drought, and caution should be taken when extrapolating their negative response to other taxa within the long-distance exploration type.

Here, we found that below-ground fungal biomass and community composition changed in relation to intra-annual temporal and spatial climatic variation, with potential functional implications. Variable climate effects on different mycorrhizal species suggest that species may be selected or disfavoured under a climate change scenario depending on their ecological traits. Free-living fungi and mycorrhizal species with extensive mycelia could be negatively affected under increasing periods of drought. Under drought stress conditions, mycorrhizal species with mycelia concentrated more tightly around the roots may be favoured due to better water availability and lower C demand. Warmer and drier summers will most likely affect soil fungal biomass negatively, with potential to decrease saprotrophic activity. Further

research is needed to predict how shifts in dominance between fungal guilds and ECM exploration types in drier and warmer scenarios may affect host tree performance, drought resistance and nutrient cycling in Mediterranean forests ecosystems.

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Author Contributions

C. C. conducted soil sampling, community analysis and the statistical analysis of the data. B. D. L. supervised the community analysis and contributed to statistical analyses and data interpretation. J. G. A. contributed to statistical analyses and guided the main structure of the manuscript. A. H. contributed with ergosterol analyses and data interpretation. Javier Parladé and Joan Pera contributed with the design of the experiment and participated in the community analysis. PNIN plots have been regularly maintained by J. M. A. and J.A.B., who initiated the study and contributed with the design of the experiment, soil samplings, provided the environmental data and guided the main structure of the manuscript. All the authors contributed writing the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Neighbour-joining trees of representative sequences of the clusters (OTU, operational taxonomic unit) obtained and reference sequences from UNITE and INSD.

Fig. S2 Principal component analysis of the (a) soil characteristics parameters and (b) climate-related parameters.

Fig. S3 Mean relative abundances of relevant and significantly affected functional guilds of soil fungi and exploration types of ectomycorrhizal fungi in relation to soil moisture and soil temperature in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons. Data points represent monthly averages across 28 plots.

Table S1 OTU representative identities and guild assignments

Figures

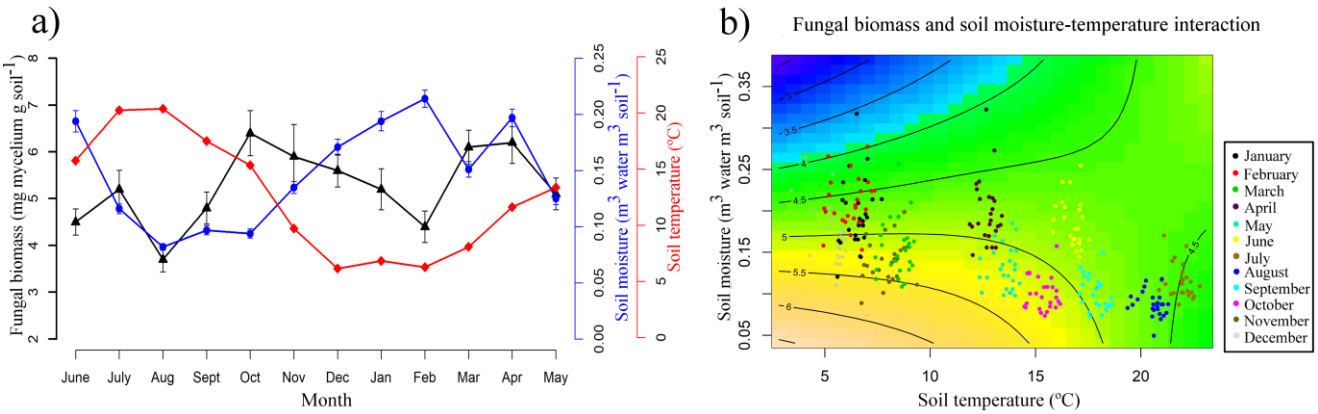

Fig. 1 (a) Monthly averaged soil fungal biomass (mg mycelia g soil^{-1}) over a year, based on ergosterol analysis, in a Mediterranean *Pinus pinaster* forest, and (b) correlations between soil fungal biomass and volumetric soil water content (m^3 water m^3 soil^{-1}). Mean values ± SE are shown for the soil fungal biomass, soil moisture and soil temperature values in (a). Background colours in (b) indicate soil fungal biomass (mg mycelia g soil^{-1}): yellow background colours represent high levels of fungal biomass (5 to 5.9 mg mycelia g soil^{-1}), green background colours represent intermediate levels of fungal biomass (4 to 4.9 mg mycelia g soil^{-1}), whereas blue background colours represent lower levels of fungal biomass (3 to 3.9 mg mycelia g soil^{-1}). Coloured dots represent data points for each month.

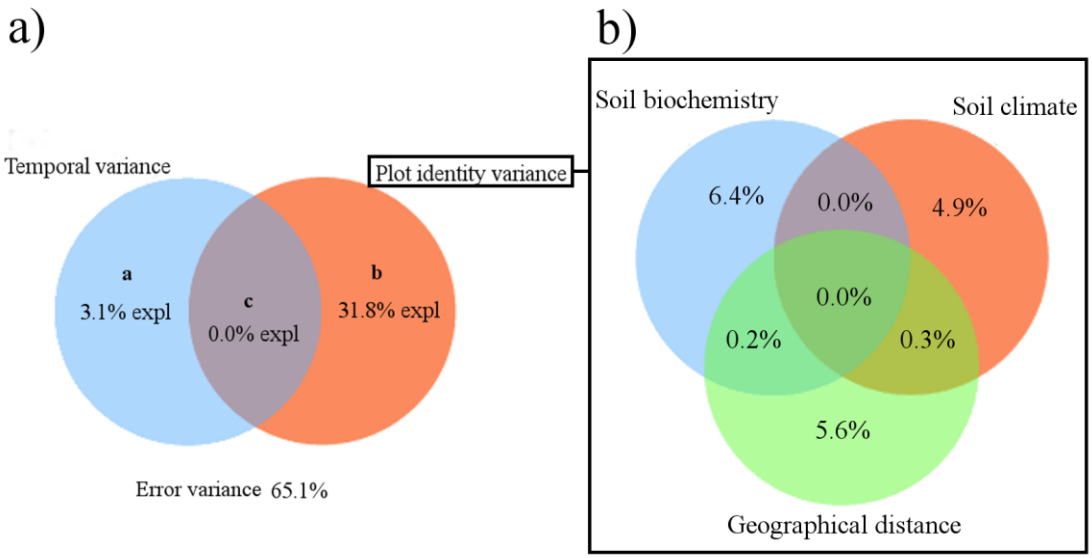

Fig. 2 Variance partitioning analyses (a) including the plot identity effect and the temporal effects, and (b) including soil characteristics, geographical distance and soil climate on the soil fungal community. Values show the fraction of variation explained by each parameter, as well as the shared contribution of each of the parameters combination.

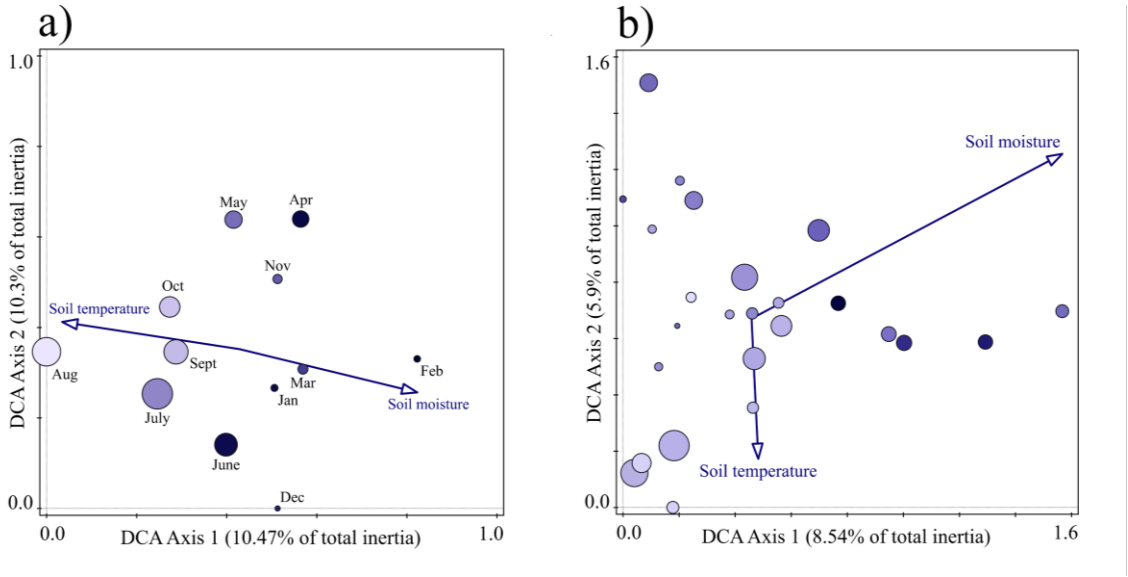

Fig. 3 Detrended correspondence analyses (DCA) of the species level community composition of soil fungi in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons. The figures illustrate the variation in community composition (a) across months and (b) across plots. The shift from light blue to dark blue colours represents the shift in gradient from low to high soil moisture, respectively. The increasing size of the circles represents the increasing soil temperature. Soil moisture and temperature are shown as supplementary variables.

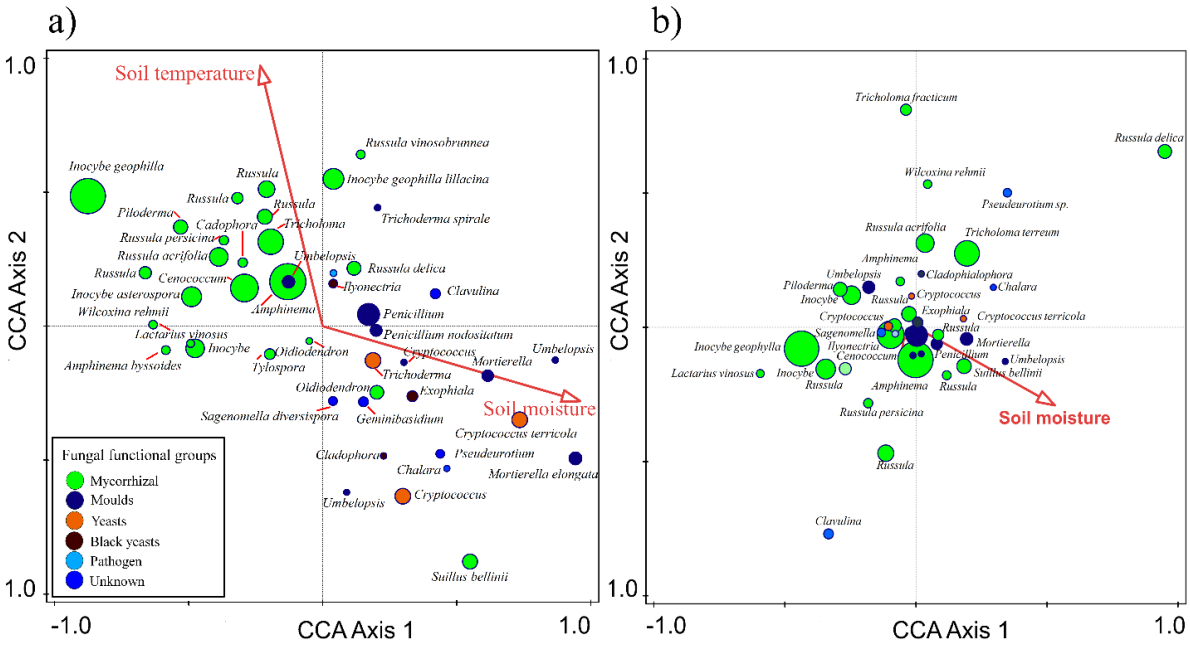

Fig. 4 Canonical correspondence analysis (CCA) species plot showing the variation in the community composition of soil fungi in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons related to temporal changes in soil temperature and moisture. Species symbols are coloured according to functional guilds, and symbol sizes are proportional to the average relative abundance. The figure only shows the 45 most abundant species hypotheses. The analyses were based on species composition permuting (a) across months and (b) across plots.

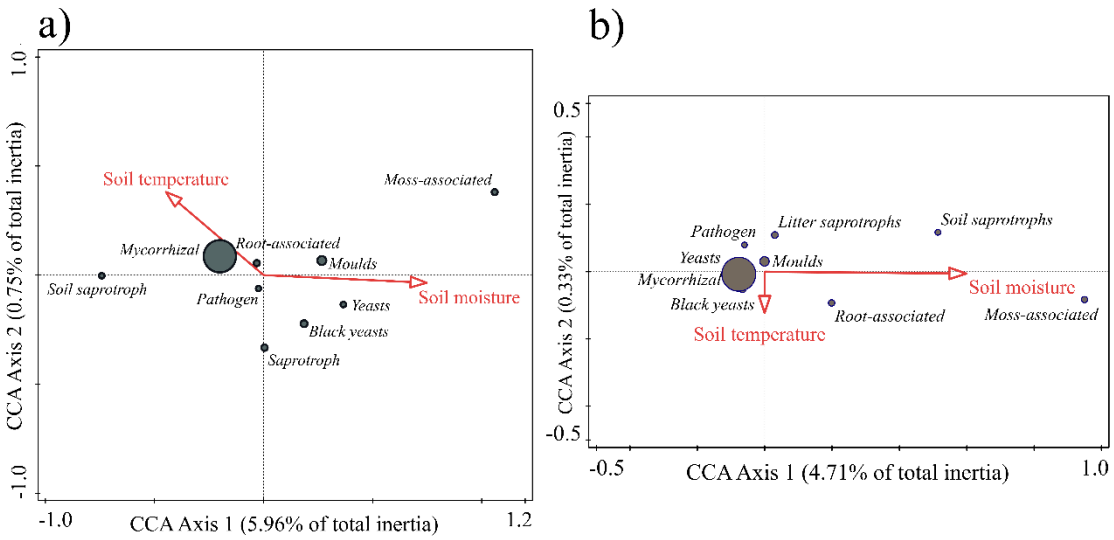

Fig. 5 Canonical correspondence analysis (CCA) plots considering relative proportions of functional guilds in relation to variation in soil temperature and moisture in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons. The analyses were based permuting (a) across months and (b) across plots. Symbol sizes are proportional to average relative abundances of each guild.

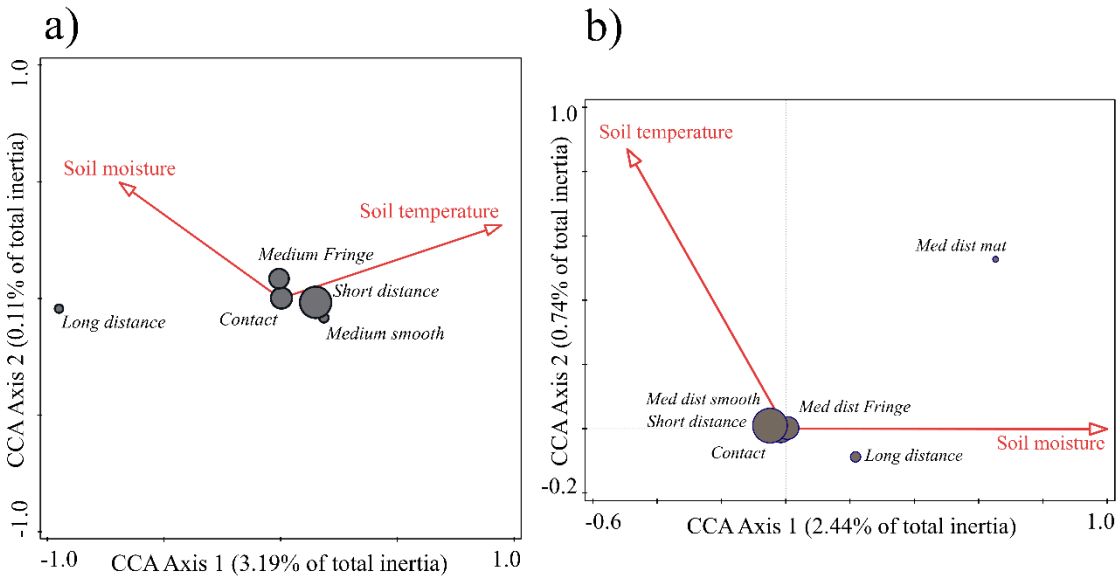

Fig. 6 Canonical correspondence analyses (CCAs) plots considering relative proportions of exploration types of ectomycorrhizal species in relation to variation in soil temperature and moisture in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons. The analyses were based permuting (a) across months and (b) across plots. Symbol sizes are proportional to average relative abundances of each guild. In (b), '*med dist'* refers to medium distance.

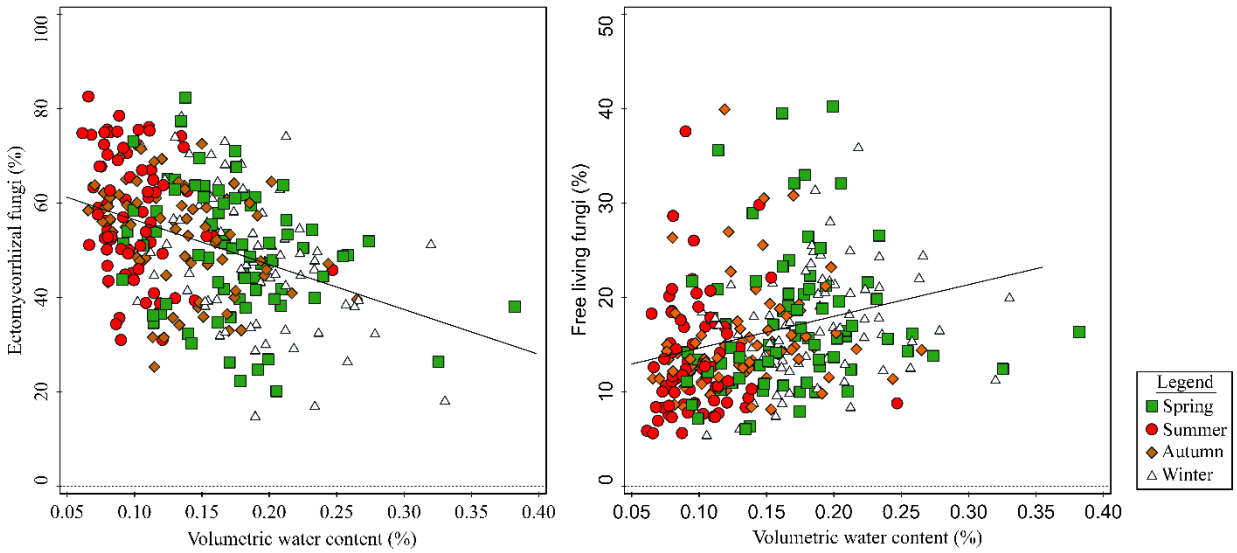

Fig. 7 Relationship between the relative abundance of (a) ectomycorrhizal fungi, (b) free-living fungi and the volumetric water content. Symbols are coloured according to season, either winter (white colour triangle), spring (light green colour square), summer (red colour circle) or autumn (brown colour diamond).

Tables

Table 1 Significance of linear mixed effect models of temporal and spatial correlations between relative proportions of specific functional guilds of soil fungi and exploration types of mycorrhizal fungi and climatic variables. Here, we show the soil moisture and soil temperature effects and their interaction, which were defined as fixed variables, whereas plot and month were defined as random factors. Numbers in bold indicate significant effects (P<0.05).

		Soil moisture		**Soil temperature**		**Moisture × Temp**	
Groups	**Response**	**F**	**p**	**F**	**p**	**F**	**p**
Functional guilds	**Black yeast**	**34.48**	**<0.001**	**7.73**	**0.006**	0.85	0.356
	Mycorrhizal	**44.88**	**<0.001**	1.165	0.281	0.28	0.597
	Moss associated	**18.20**	**<0.001**	1.39	0.240	0.00	0.982
	Moulds	**100.64**	**<0.001**	2.24	0.136	3.29	0.071
	Pathogen	0.11	0.742	0.37	0.544	0.16	0.689
	Root associated	0.26	0.610	0.29	0.589	0.08	0.781
	Saprotroph	1.04	0.307	**5.68**	**0.018**	1.12	0.291
	Soil saprotroph	10.53	0.469	0.68	0.411	0.33	0.564
	Yeast	**43.74**	**<0.001**	0.62	0.430	0.12	0.724
Exploration types	**Contact**	0.29	0.593	0.04	0.837	**4.52**	**0.034**
	Short distance	**4.09**	**0.044**	1.09	0.297	**4.12**	**0.043**
	Long distance	**13.96**	**<0.001**	**4.45**	**0.036**	**4.74**	**0.030**
	Medium Fringe	1.94	0.165	0.40	0.530	0.80	0.636
	Medium smooth	1.88	0.171	0.16	0.692	0.00	0.939
	Medium Mat	**4.53**	**0.034**	3.14	0.077	0.86	0.353