CONTRASTING SOIL FUNGAL COMMUNITIES IN MEDITERRANEAN PINE FORESTS SUBJECTED TO DIFFERENT WILDFIRE FREQUENCIES

Erika Buscardo & Susana Rodríguez-Echeverría & Helena Freitas & Paolo De Angelis & João Santos Pereira & Ludo A. H. Muller

E. Buscardo : S. Rodríguez-Echeverría : H. Freitas : L. A. H. Muller

Centro de Ecologia Funcional (CEF), Departamento de Ciências da Vida, Universidade de Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal

E. Buscardo : P. De Angelis

Department for Innovation in Biological, Agro-Food and Forest Systems (DIBAF), University of Tuscia,Via S. Camillo de Lellis snc, 01100 Viterbo, Italy

E. Buscardo

Escritório Central do LBA, Instituto Nacional de Pesquisa da Amazônia (INPA), Av. André Araújo, 2936, Campus II, Aleixo, 69060-001 Manaus, Amazonas, Brazil

J. S. Pereira

Departamento de Engenharia Florestal, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Tapada da Ajuda, 1349-018 Lisbon, Portugal

L. A. H. Muller (*)

Institut für Biologie—Botanik, Freie Universität Berlin, Altensteinstrasse 6, 14195 Berlin, Germany e-mail: ludo.muller@fu-berlin.de

Abstract

Mediterranean forest ecosystems are characterized by various vascular plant groups with their associated mycorrhizae and free living soil fungi with various ecological functions. Fire plays a major role in Mediterranean ecosystem dynamics and impacts both above- and below-ground community structure and functioning. However, studies on the effects induced by altered disturbance regimes (associated with recent land use and climate extremes) on fire ecology and especially on its below-ground impacts are few. The objectives of this study were to evaluate the effects of different wildfire regimes on soil fungal community structure using two different molecular methods. We investigated the long-term effects of wildfire on soil fungal communities associated with Pinus pinaster forests in central Portugal, by comparing the results of denaturing gradient gel electrophoresis (DGGE)-based profiling with those obtained with 454 pyrosequencing. Four forest stands with differing fire history and fire return interval, and vegetation cover (mature forest, early successional stage of pine regeneration, and forest converted to scrubland) were sampled 6 years after the last fire event. The

pyrosequencing-based approach indicated ca. eight-fold higher numbers of taxa than DGGE. However, fungal community fingerprinting data obtained for the different study stands with DGGE were congruent with those obtained with pyrosequencing. Both short (7.6 years) and long (24 years) fire return intervals (indicated by the presence of ericaceous shrubs in the understorey) induced a decrease in the abundance ratio between basidiomycetes and ascomycetes and appeared to reduce the frequency of ectomycorrhizal fungal species and saprophytes. Wildfire significantly reduced the frequency of late stage successional taxa (e.g. Atheliaceae and Cantharellales) and known or putative saprophytes belonging to the Clavulinaceae and the Archaeorhizomycetaceae. Conversely, early successional fungal species belonging to the Thelephoraceae were favoured by both fire return intervals, while the abundance of Cortinarius and Hebeloma, which include several Cistus-specific species, increased with short wildfire return intervals. This last finding highlights the relationship between post-fire vegetation composition and cover (vegetation successional stage), and fungal symbionts. We hypothesise that these changes could, in the long term, exhaust the resilience of Mediterranean pine forest vegetation and associated soil fungal communities by preventing pine regeneration.

Introduction

Wildfire events can have a large impact on the structure and functioning of ecosystems through a variety of effects on soil physical and chemical properties and on the above-ground vegetation. They can cause for example direct and/or indirect soil heating, remove organic matter and nutrients from the soil, alter moisture and pH, deteriorate soil structure, increase erosion and change the above-ground vegetation. Depending on the fire regime (size, intensity, duration and frequency), these effects can be more or less pronounced and affect the above- and below-ground communities of plants, animals and microorganisms accordingly (Kozlowski and Ahlgren 1974; Certini 2005). In forest ecosystems, small surface or litter fires usually affect the undergrowth but not the canopy-forming species, resulting in little impact on ecosystem integrity. Intense fires, on the other hand, can reconfigure above- and below-ground communities, resulting in major shifts in community structure and ecological functioning (DeBano et al. 1998; Keeley et al. 2012). Fungi form a dominant component of the soil microbial community in terms of biomass (Thorn 1997) and, acting as mutualists, decomposers and pathogens, fulfill important and diverse functions in soil ecosystems (Bardgett and Wardle 2010). Although soil fungal communities are known to be affected by wildfires, the effects of wildfire disturbance on the below-ground fungal community and, in turn,

the consequences for ecosystem sustainability are complex and remain largely unknown (Neary et al. 1999; Mataix-Solera et al. 2009). Most studies conducted so far have focused on the effects of single wildfire events and these have been reported either not to alter or to reduce the amount of soil fungal propagules or biomass, depending on their intensity (Vázquez et al. 1993; Bellgard et al. 1994; Bååth et al. 1995; Bárcenas-Moreno et al. 2011), to reduce fungal hyphal length and decrease the activities of hydrolytic extracellular enzymes (Holden et al. 2013), to alter microfungal communities (Bettucci and Alonso 1995) and to affect saprotrophic fungi and decomposition rates (Holden et al. 2013) through a reduction of the amount of coarse woody debris (Robinson et al. 2008). Wildfires were also found to influence the structure of arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) fungal communities (Baar et al. 1999; Grogan et al. 2000; Allen et al. 2003; Buscardo et al. 2010, 2011, 2012; Rincón and Pueyo 2010; Rincón et al. 2014), to decrease EM fungal colonization (Treseder et al. 2004; De Román and De Miguel 2005) and to induce drastic changes in the EM fungal community composition by shifting the post-fire fungal communities from more complex and stable to low-diversity communities dominated by a relatively small number of r-selected species (Visser 1995; Baar et al. 1999). The effects of recurrent wildfires on below-ground fungal communities are less known than those of single wildfires. While repeated prescribed burning appears to significantly alter the structure of the total fungal community in the upper soil layer (Bastias et al. 2006a), most of what is known so far relates to mycorrhizal fungi and indicates that repeated prescribed fire events may reduce EM fungal biomass and taxonomic richness and cause more pronounced changes to the EM fungal community than single fire events (Tuininga and Dighton 2004; Hart et al. 2005a; Bastias et al. 2006b; Anderson et al. 2007). The effects of recurrent wildfires on below-ground fungal communities have only been reported from Mediterranean Portugal for EM fungi. Frequent wildfire events were shown to affect both the EM fungal resistant propagules structure (Buscardo et al. 2010) and the potential facilitation offered by EM fungal networks for pine regeneration (Buscardo et al. 2012). Fire return interval is an important factor, as short return intervals of prescribed fires have recently been shown to have significantly larger impacts on soil fungal communities than long return intervals (Brown et al. 2013). Insights into the effects of recurrent wildfires on the diversity and structure of fungal communities are required to obtain a better understanding of the roles of these communities in above-ground/belowground interactions and their impact on the dynamics of secondary succession after fire. Since several fungal functional groups are strongly linked to their host plants, fire-induced changes in vegetation diversity and structure are expected to be mirrored in the diversity and abundance of symbiotic soil

fungi. An essential first step to establish the resilience of soil fungal communities to fire frequency, from both structural and functional perspectives, is to understand how they respond to changing disturbance regimes (Taylor et al. 2010). In our study, we assessed the impact of recurring wildfire events on the communities of soil fungi in Mediterranean pine forest ecosystems. Due to the marked seasonality of the Mediterranean climate, with precipitation and mild temperatures in the winter versus drought and high temperatures in the summer, Mediterranean forest ecosystems have always been fire-prone and are dominated by fire-adapted vegetation (Keeley et al. 2012). However, changes in wildfire regimes associated with recent land use and climate extremes may compromise the resilience of these ecosystems and may lead to large-scale shifts in vegetation types that affect biodiversity patterns and dynamics of both plant and fungal communities (Pausas 2004). Under this scenario, Mediterranean pine forests may be driven to a tipping point beyond which they are permanently replaced by communities of early/arrested successional stages that are, both above- and below-ground, of reduced complexity. In order to address the relative lack of knowledge on the diversity and the composition of the total soil fungal community in ecosystems affected by recurrent wildfires, our study aimed at characterizing the soil fungal communities in pine forests subjected to different wildfire regimes in central Portugal, using 454 pyrosequencing (Margulies et al. 2005) of nuclear ribosomal internal transcribed spacer 1 (ITS1) amplicon libraries and denaturing gradient gel electrophoresis (DGGE; Muyzer et al. 1993), and to generate hypotheses regarding shifts in soil fungal community structure in response to different fire regimes.

Material and methods

Study area and sampling sites

Our study area is situated in central Portugal between Alvito da Beira (39° 48! N, 7° 49! W, altitude: 500–600 m) and Isna de Oleiros (39° 51! N, 7° 51!W, altitude: 750–850 m), a region characterized by a Mediterranean climate (a subtropical climate with hot, dry summers and cool, wet winters), lithosol (a stony soil lacking horizon and structure development) and a plant community dominated by Pinus pinaster Aiton. Four stands characterized by three different wildfire histories were selected for sampling (Fig. 1). Stand UB had not been affected by wildfire in the past 40 years and corresponded to an open, uneven-aged maritime pine forest (trunk diameter of dominant trees at breast-height ~ 40– 45 cm), with an understorey shrub community dominated by Erica spp., Halimium spp. and Pterospartum tridentatum (L.) Willk., and a soil with an incipient organic layer.

Stand B was characterized by an average fire return interval of about 24 years, with the last wildfire event occurring in 2003, and showed extensive natural pine regeneration with the same understorey shrub species as in stand UB. Stands B1 and B2, located approximately 5 km from stands UB and B, both had an average fire return interval of 7.6 years and were affected by wildfires in 1992 and 2003. While the long fire return interval allowed the typical succession response of Mediterranean plant ecosystems in stand B, the short fire return intervals hindered pine regeneration in stands B1 and B2. The recurring wildfires in stands B1 and B2 resulted in a shrubby vegetation dominated by either Cistus ladanifer L. (B1) or Erica spp., Halimium spp. and P. tridentatum (B2). The chemical properties of the soils at these four stands were analyzed previously by Buscardo et al. (2012).

Soil sampling and DNA extraction

Within each of the stands UB, B, B1 and B2, three 10 m × 10 m plots at least 300 m apart were established. Although wildfire events could not be replicated, these plots provided independent replicates of soil fungal communities as spatial autocorrelation among these communities has been shown to occur at lower distance scales (Lilleskov et al. 2004; Smithwick et al. 2012). In June 2009, nine evenly spaced soil samples were collected in each of the plots. Soil samples were extracted, after removing litter, with a shovel to a depth of about 20 cm, and samples were pooled per plot, mixed, cleared of debris and sieved using a 2 mm mesh. For each plot, two 50 mL subsamples of soil were stored at 4 °C. The following day, three DNA extractions were carried out for each of the 24 soil subsamples using 0.4 g of soil. DNA was extracted with the UltraCleanTM Soil DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's instructions except for an extra incubation step at 70 °C for 10 min after the addition of the Inhibitor Removal Solution and a subsequent vortexing step of 5 min.

454 Pyrosequencing

Amplicon libraries for 454 pyrosequencing were prepared by PCR amplification of the variable region of the fungal internal transcribed spacer 1 (ITS1) with the fungus-specific oligonucleotide primer ITS1F (5!-CTTGGTCATTTAGAGGAAGT AA-3!; Gardes and Bruns 1993) and the universal primer ITS2 (5!-GCTGCGTTCTTCATCGATGC-3!; White et al. 1990), respectively combined through a two base pair (bp) linker with a Roche 454 A pyrosequencing adapter (5!-GCCT CCCTCGCGCCATCAG-3!) extended with an 8-bp errorcorrecting barcode sequence (Hamady et al. 2008) and with a Roche 454 B sequencing adapter (5!-GCCTTGCCAGCC CGCTCAG-3!; Table S1). PCR amplification of each of

the 72 samples was performed in 50µL reactions, containing 10 ng template DNA, 200 nM of each of the forward and the reverse primers, 200µM of each deoxynucleotide triphosphate (dNTP) and 2 U FastStart Taq DNA polymerase (Roche) in 1× FastStart PCR Master buffer (Roche), and the following PCR temperature profile was used: initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 50 °C for 45 s and 72 °C for 1 min; final extension at 72 °C for 2 min. Amplicon libraries were examined by agarose gel electrophoresis, purified with the High Pure PCR Product Purification Kit (Roche) and quantified on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). Equimolar amounts of the three amplicons for each subsample of pooled soil were combined to account for possible heterogeneity introduced by DNA extraction and/or PCR amplification. Equal amounts of the 24 combined amplicon libraries were sent to the Advanced Sequencing Services unit of Biocant (Portugal) to be processed by the Roche FLX 454 pyrosequencing system. ITS1 sequence data were deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under study accession PRJEB5987.

Denaturing gradient gel electrophoresis

Fungal DNA fragments containing ITS1 and ITS2 were PCR amplified using the oligonucleotide primers ITS1F and ITS4 (5!-TCCTCCGCTTATTGATATGC-3!; White et al. 1990). PCR amplification was performed separately for each of the 72 DNA samples in 25µL reactions, containing 1µL template DNA, 400 nM of each of the forward and the reverse primers, 200µM of each dNTP, 0.5 U DFS-Taq DNA polymerase (Bioron) and 100µg of bovine serum albumin (BSA) in 1× PCR buffer (Bioron). The following PCR temperature profile was used: initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; final extension at 72 °C for 30 min. PCR products were examined by agarose gel electrophoresis and subsequently used as templates in a nested PCR to generate fragments of appropriate length (<500 bp) for DGGE analysis. Nested PCR was performed using the primer combination ITS1F, extended with a GC-clamp (5!-CGCCCGCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGGGG-3!; Muyzer et al. 1993) at the 5!- end to prevent complete denaturation, and ITS2 with PCRs set up as described above except for a reduced final extension step of 10 min at 72 °C and the omission of BSA from the PCR mixture. Equimolar amounts of the three amplicon libraries for each subsample of pooled soil were combined and approximately 15µL of each sample was used for DGGE analysis with the DGGE-2001 system (CBS Scientific). Polyacrylamide gels (8 %) were prepared with a 30 % (2.1 M urea, 12 % (v/v) formamide) to 60 % (4.2 M urea, 24 % (v/v) formamide) vertical gradient and eight samples, corresponding to two replicates of the samples obtained from a single stand, were loaded along with a reference

ladder on each gel. The reference ladder was obtained by pooling the DNA fragments resulting from a PCR amplification of the ITS1-ITS2 region with the primer combination ITS1F and ITS2 in nine known EM species previously found in the study area (Buscardo et al. 2010). DGGE was performed in 1× TAE buffer at 20Vfor 15min, followed by 16 h at 70Vand gels were maintained at a constant temperature of 60 °C. Gels were stained for 20 min in 1× GelStar® (Lonza Bioscience) and washed for 30 min in distilled water prior to visualization. Bands of interest were excised, reamplified using the same PCR conditions as described above and purified using a QIAquick PCR Purification kit (Qiagen). Purified PCR products were sequenced by Macrogen Co. Ltd. (South Korea) and the DNA sequences were deposited in the GenBank nucleic acid sequence repository (http://www.ncbi.nlm.nih.gov/genbank) under accession numbers HQ201718 - HQ201751.

Data analysis

Pyrosequencing reads were quality trimmed and reads with unresolved nucleotides, without a valid primer sequence or without a valid DNA tag were removed. In order to prevent distorted sequence clustering and similarity searches due to the presence of highly conserved ribosomal DNA sequences flanking the ITS1 sequence (Nilsson et al. 2009), ITS1 sequences were extracted from the pyrosequencing reads that met the forementioned quality control requirements using Fungal ITS Extractor v1.1 (Nilsson et al. 2010). Extracted ITS1 sequences smaller than 100 bp were excluded from further analyses and the remaining sequences were tested for the presence of chimeras using the UCHIME method (Edgar et al. 2011). The collection of representative and reference sequences obtained by clustering ITS sequences in the UNITE sequence database with a dynamic clustering threshold (Kõljalg et al. 2013) was used as a reference set for the detection of chimeric sequences. All obtained ITS1 sequences were simultaneously grouped into operational taxonomic units (OTUs) based on sequence similarity using CD-hit v4.0 (Li and Godzik 2006) with a threshold of 97 % similarity over at least 90 % of the shortest sequence length in each pairwise comparison (Hughes et al. 2009). In order to minimize the risk of including sequencing artefacts, singleton OTUs were excluded from further analysis by randomly removing one sequence read from each OTU (Dickie 2010; Unterseher et al. 2011). Rarefaction curves were generated for each plot using the vegan v1.17-3 package (Oksanen et al. 2011) in R v3.0.2 (R Development Core Team 2011) and as the sequencing depth differed between plots, 50 rarefied subsamples, each consisting of 4,609 sequence reads (the number of sequence reads in plot B2.3 after randomly removing one sequence read per OTU), were generated for each plot from the combined sets of sequence reads of the replicate amplicon libraries with vegan v1.17-3 in R v3.0.2. Plot frequencies of non-singleton OTUs

(pi) were calculated as average frequencies across the 50 rarefied subsamples and reproducibility of the pyrosequencing approach was assessed by pairwise comparison of the OTU frequencies calculated separately for the replicate amplicon libraries. The overall OTU richness (S) was calculated for each plot as the average number of OTUs found across the 50 rarefied subsamples. In a similar way, the extrapolative richness estimator Chao1 (Chao et al. 2005), which is particularly useful for datasets skewed towards low-abundance classes (Hughes et al. 2001) and Shannon's diversity (H! = − " pi ln(pi)) were calculated using vegan v1.17-3 in R v3.0.2. Averages of the different diversity and richness indices were calculated for each sampling stand and significance of the differences in diversity or richness across the stands was assessed by one way analysis of variance (ANOVA). Correspondence analysis was performed with plot frequencies of non-singleton OTUs using the "dudi.coa" routine of the ade4 v1.4-14 package in R v3.0.2 in order to visualize similarities in fungal taxa composition among the 12 sampled plots. Because between-species comparisons based on sequence read abundance can be biased by innate sequence structure (Amend et al. 2010; Unterseher et al. 2011), correspondence analysis was also performed using binary recoded OTU presence/absence data. The OTUs were taxonomically identified using the Ribosomal Database Project (RDP) Classifier (Wang et al. 2007) with the aforementioned collection of representative and reference sequences obtained from the UNITE sequence database as a reference set (minimum confidence level for assignment = 0.80). OTU frequencies were summed hierarchically across different taxonomic levels and significances of differences in OTU frequencies across and between stands were respectively assessed by one-way ANOVA and Tukey's multiple comparison tests. Significances were corrected for multiple comparisons using the false discovery rate (FDR) method (Benjamini and Hochberg 1995). Digital images of DGGE gels were analysed with GelCompar II v4.0 (Applied Maths) and subsequent statistical analyses were performed with R v3.0.2. Species richness (S) was calculated as the total number of DGGE bands in a sample and Shannon's diversity (H!) was calculated by assigning each DGGE band in a sample to one of five possible intensity classes. Differences in taxon diversity among sampling stands were tested using one-way ANOVA and correspondence analysis using DGGE band intensity and presence/ absence data was performed using the "dudi.coa" routine to visualize differences in fungal taxa composition between the different stands. DNA sequences from excised DGGE bands were manually checked and, if necessary, edited using BioEdit v7.0.9.0 (Hall 1999). After testing for the presence of chimeric sequences using the UCHIME method, taxonomic identification of the DGGE bands was obtained using their DNA sequences and the RDP classifier with the same reference set of sequences as used

for the identification of the 454 pyrosequencing based OTUs (minimum confidence level for assignment = 0.80). Correlations between pyrosequencing- and DGGE-based diversity estimates (OTU richness S and Shannon's diversity H!) were assessed by calculating Pearson's correlation coefficient in R v3.0.2. Pairwise dissimilarities between the pyrosequencing- and DGGE-based community profiles at the different sampling plots were calculated as pairwise asymmetric binary distances or Jaccard dissimilarities (presence/ absence data) and euclidean distances (abundance data), and compared by Mantel tests as implemented in the "mantel.rtest" routine of the ade4 v1.4-14 package in R v3.0.2 (number of permutations = 9,999).

Results

454 pyrosequencing-based fungal community assessment

A total of 92,062 partial or complete ITS1 sequences, with a length between 101 and 370 bp (average = 172 bp), were retained after quality control of the pyrosequencing reads and removal of the conserved flanking regions. The number of retained sequences per plot varied between 4,743 and 9,269 (average = 7,672, Table 1). Combining the sequences into OTUs using a threshold of 97 % sequence identity resulted in a total of 2,176 OTUs

across all sampling plots, including 716 singleton and 1,460 non-singleton OTUs (see Table S2 for an overview of all nonsingleton OTUs, the name of their representative sequence, their sequence read abundances in the sampling plots and their assigned taxonomy). Excluding singleton OTUs and correcting for differences in sequencing depth, the species richness per plot varied between 215 and 349 (average = 262) while Shannon's diversity varied between 2.85 and 4.54 (average = 3.83; Table 1). High (average Pearson correlation coefficient = 0.91) and significant (P-value < 0.05) levels of correlation between the OTU frequencies in replicate amplicon libraries were found, indicating good reproducibility of the pyrosequencing approach. The rarefaction curves showed that the number of OTUs increased with the number of sequences without reaching a plateau for most plots (Fig. 2) and the Chao1 extrapolative richness estimator predicted maximum numbers of OTUs per plot ranging from 272 to 468 (average = 340; Table 1). A significant difference (P-value < 0.05) in diversity or richness among the stands was only found for Shannon's diversity, and multiple

comparison testing showed this to be due to a significantly (P-value < 0.05) lower diversity in stand B compared to stands UB and B2. The 12 sampling plots clustered together according to their stand of origin in a correspondence analysis based on OTU frequency (Fig. 3) and OTU presence/absence (Fig. S1): the most informative axes of variation in the graphical representations of the two correspondence analyses represented respectively 20 and 15 % of the total variance and clearly separated the plots of the control stand (UB) from the plots of the stands subjected to wildfires (B, B1 and B2), while the second most informative axes represented respectively 15 and 11 % of the total variance and distinguished the plots subjected to wildfires according to their respective stands of origin, with stand B2 placed between stand B (characterized by the same post-fire shrub cover) and stand B1 (characterized by the same wildfire return interval length). The first axis of the correspondence analysis based on OTU frequencies was dominated by (in descending order of importance) OTUs 10 (Archaeorhizomyces sp.), 11, 17 (Russula sp.), 1 and 22, whereas the second axis was dominated by OTUs 4 (Rasamsonia brevistipitata), 3 (Cortinarius sp.), 6, 1 and 5 (Hebeloma cistophilum; see Table S2 for OTUs and their taxonomic identity). Using the RDP classifier, taxonomic assignment to phylum or lower level was possible for 885 OTUs (61 %; Tables S2 and S3). Four hundred and twenty-seven OTUs (29 % of OTUs; 25 % of the sequence reads) were assigned to the Ascomycota and 406 OTUs to the Basidiomycota (28 % of the OTUs; 50%of the sequence reads), whereas only a few of the OTUs were assigned to the Zygomycota (41), Glomeromycota (7) or Chytridiomycota (4). The identified OTUs were distributed across 42 fungal orders, the most frequently encountered being Agaricales (140 OTUs; 21 % of the sequence reads), Thelephorales (37 OTUs; 9 % of the sequence reads) and Russulales (54 OTUs; 6 % of the sequence reads), and 34 families, the most common being Thelephoraceae (35 OTUs; 9 % of the sequence reads), Cortinariaceae (24 OTUs; 6 % of the sequence reads) and Strophariaceae (23 OTUs; 6 % of the sequence reads). Genus and species level assignments were possible for respectively 385 (26 %) and 205 (14 %) OTUs, with the most commonly encountered genera being Cortinarius (24 OTUs; 6 % of the sequence reads), Hebeloma (18 OTUs; 6 % of the sequence reads) and Russula (34 OTUs; 5%of the sequence reads), and the most common species being Hebeloma cistophilum (10 OTUs; 4 % of the sequence reads), Rasamsonia brevistipitata (11 OTUs; 3 % of the sequence reads) and Russula sardonia (6 OTUs; 2 % of the sequence reads).

Major shifts in fungal diversity across fire regimes - The most common fungal orders in the individual stands were Russulales (UB, 17 % of the sequence reads), Thelephorales (B, 21 % of the sequence reads) and Agaricales (B1 and B2, respectively 50 and 16 % of the sequence reads), whereas the

most common families in the individual stands were Russulaceae (UB, 17 % of the sequence reads), Thelephoraceae (B, 21 % of the sequence reads), Cortinariaceae (B1, 23 % of the sequence reads) and Strophariaceae (B2, 8 % of the sequence reads; Table 2). Taxonomic assignment to the genus level was possible for 387 OTUs (UB, 52 %; B, 34 %; B1, 56 %; B2, 27 %). The most commonly encountered genera and species in the individual stands were Russula and R. sardonia (UB, respectively 13 and 4 % of the sequence reads), Rasamsonia and R. brevistipitata (B, both 14 % of the sequence reads), Cortinarius and H. cistophilum (B1, respectively 23 and 9 % of the sequence reads), and Hebeloma and H. cistophilum (B2, respectively 8 and 6 % of the sequence reads; Table 2). At the genus level, EM fungi accounted for 37 % of the sequence reads in UB, 16 % in B, 54 % in B1 and 16 % in B2. Putative and known decomposers, parasites and pathogens represented 13 % of the sequence reads in UB, 17 % in B, 2 % in B1 and 7 % B2. After correcting significances for multiple comparisons using the FDR method, one-way ANOVA indicated 41 taxa to be distributed differently across the four forest stands (Table S3). The sequence read abundances of the following taxonomic units were higher in the control stand UB than in the stands subjected to wildfires: Archaeorhizomycetes, Archaeorhizomycetales, Archaeorhizomycetaceae, Archaeorhizomyces, Atheliales, Atheliaceae, Tylospora, Cantharellales, Clavulinaceae, Microbotryomycetes and Mortierella humilis. Higher abundances in at least one of the stands characterized by low fire return intervals (B1 and B2) compared to the control stand, on the other hand, were observed for: Dothideomycetes, Capnodiales, Teratosphaeriaceae, Devriesia, Devriesia shelburniensis, Leohumicola, Pezizaceae, Terfezia, Terfezia alsheikhii, Agaricales, Cortinariaceae, Cortinarius, Gymnopilus, Gymnopilus arenophilus, Clitocybe and Clitocybe metachroa. Finally, higher abundances in the stand subjected to low fire frequencies (B) compared to the other stands were observed for: Cucurbitariaceae, Curreya, Curreya grandicipis, Rhizopogonaceae and Rhizopogon.

DGGE-based fungal community assessment

DGGE analysis generated community profile patterns with 25 to 38 scorable bands per sampling plot (average = 33) and a total of 56 different bands across all plots (Figs. S2, S3 and S4). The DGGE profiles were highly reproducible, with an average of 91 % of the bands being identical between independent DGGE runs. Two of the 56 DGGE bands occurred exclusively in the profile of just a single plot, while 4 bands occurred in the profiles of all 12 sampling plots. Species richness in the four stands, averaged over the sampling plots, varied between 30 and 35 (average = 33), while Shannon's diversity varied between 3.21 and 3.61 (average = 3.39). No significant differences in diversity were found between the four stands (Table 1). The results of the correspondence analyses

based on the intensity (Fig. 4) and the presence/absence (Fig. S5) of the DGGE bands showed a clear separation of the sampling plots, with the two most informative axes of variation in the graphical representations of the correspondence analyses representing 21 and 19 % of the total variation using the band intensity data versus 25 and 19 % using the band presence/ absence data. Although sampling plots were loosely grouped according to their stand of origin, the separation between control stand and stands subjected to wildfire events was not as pronounced when compared to the results obtained with 454 pyrosequencing data. Extraction from gel, PCR reamplification and DNA sequencing were succesful for 34 different DGGE bands and taxonomic assignment at the phylum or lower level was possible for 24 (71 %) of these (Table S4). Although comigration of bands representing different DNA sequences is known to occur (Sekiguchi et al. 2001), it wasn't detected in our study as DNA sequences of bands cut out from the same position in the DGGE profiles of different sampling plots were found to be identical or highly similar and resulted in significant matches with the same entries in the reference set. However, DNA sequences of bands found at different positions in the DGGE profiles could produce significant hits with sequences obtained from the same fungal species as reported in other studies (e.g. Dowd et al. 2008). Nevertheless, the following fungal genera and species were confirmed to be present in the different stands included in this study: Amanita torrendii (UB, B1), Boletus badius (UB), Cortinarius sp. (B1), Hebeloma cistophilum (B1, B2), Lactarius sp. (UB), Penicillium sp. (B), Rasamsonia brevistipitata (B), Russula sp. (B), Russula albonigra (B1), Russula sardonia (UB) and Tricladium patulum (B2).

Comparison of 454 pyrosequencing- and DGGE-based community profiles

No significant correlation was found between pyrosequencing- and DGGE-based diversity estimates for the 12 sampling plots, with Pearson's correlation coefficients equal to 0.31 (OTU richness S; P-value = 0.32; Fig. S6) and 0.38 (Shannon's diversity H!; P-value = 0.22; Fig. S7). However, removal of the outlying plot B2.3 rendered the correlation between the OTU richness estimates significant ($r = 0.78$; P-value < 0.05). Mantel tests indicated significant correlations between pyrosequencing- and DGGE-based dissimilarity estimates among the 12 sampling plots using both abundance ($r = 0.36$; P-value < 0.05; Fig. S8) and presence/ absence ($r = 0.44$; P-value < 0.05; Fig. S9) data.

Discussion

In this study, we characterised the below-ground fungal communities in maritime pine forests in central Portugal with vegetation dynamics influenced by different wildfire frequencies. We

compared the profiling of soil fungal communities obtained with DGGE and 454 pyrosequencing using ITS1 amplicon libraries generated with DNA extracted from soil samples collected in forest stands subjected to different wildfire regimes. Estimates of fungal species richness obtained by pyrosequencing were about eight-fold higher than estimates based on DGGE community profiling. Similar ratios were observed in studies of bacterial communities in mangrove habitats (Cleary et al. 2012) and in kefir grains (Leite et al. 2012) and indicate that pyrosequencing technology allows a more complete determination of microbial diversity, whereas diversity assessments made by DGGE are restricted to the more abundant taxa (Ercolini 2004). Nevertheless, DGGEbased estimates of fungal diversity were correlated with the results of estimates based on pyrosequencing, indicating that the relative taxon diversities were comparable between the two profiling methods. The relationships between the fungal community profiles were very similar across the sampling plots. Correspondence analysis illustrated that fungal community profiles were in most cases more similar when compared within forest stands than when compared among forest stands. Pairwise differences in DGGE- and pyrosequencing-based community profiles of the sampling plots were significantly correlated. In addition, 18 of the 34 DGGE OTUs were among the fungal OTUs that provided the 100 highest sequence read abundances in the corresponding pyrosequencing-based profiles. The use of both DGGE and pyrosequencing for quantitative profiling has been shown to be questionable as biases can be introduced by differences in DNA extraction efficiency between fungal taxa and preferential PCR amplification (Amend et al. 2010; Medinger et al. 2010), and need to be interpreted with caution. Nonetheless, some of our results on read frequency data from soil samples were in line with previous quantitative studies examining resistant EM fungal propagule communities, assessed by bioassays using Pinus pinaster and Quercus suber seedlings, and EM root tip fungal communities of P. pinaster and the dominant shrub species in the same study area (Buscardo et al. 2010, 2012). The abundance trend found in the present study was found for several taxa including Inocybe jacobi, Rhizopogon luteolus and Archaeorhizomyces spp. in the bioassay study, and Cadophora finlandica, Hebeloma cistophilum and an uncultured Atheliaceae in the EM fungal community study. High levels of fungal species richness were found in all stands with values that ranged between 242 and 288 OTUs. Comparable levels of fungal richness have been observed in large-scale sequencing studies (O'Brian et al. 2005; Orgiazzi et al. 2012; McGuire et al. 2012, 2013; Neher et al. 2013) and may indicate that the long-term effects of wildfires on soil fungal taxon richness are limited and independent of wildfire regime. This is possibly owing to extensive (re)colonization by fungal species that allow recovery from initial reductions of species richness and

which are common after stand-replacing fire events (Certini 2005). Contrary to fungal species richness, composition and read frequency of soil fungal communities were variable among stands and were probably due to changes induced by fire on edaphic properties and vegetation structure which indirectly affected soil fungi. Stand-replacing wildfires have been reported to cause temporal shifts, lasting up to 6 years, in dominance from basidiomycetous to ascomycetous fungi in EM fungal communities (Torres and Honrubia 1997; Baar et al. 1999; Grogan et al. 2000; Holden et al. 2013), probably due to changes in the edaphic characteristics and the amount or type of inoculum (Visser 1995). The ratio of basidiomycetes to ascomycetes (BM:AM), calculated by using pyrosequencing read frequencies, were in range of the ratios reported for undisturbed temperate forest soils (O'Brian et al. 2005; Buée et al. 2009) in all forest stands included in this study. After ca. 6 years since the last wildfire, the abundance ratio ofBM:AMdecreased from the control stand included (2.2) to 1.6 in stand B and 1.1 in B2, owing largely to the decrease in the abundance of Russulaceae, Cantharellales, Atheliaceae and Clavulinaceae, and the increase in the abundance of Dothideomycetes in all burnt plots, the increase in the abundance of Leohumicola spp. in B2 and the increase in the abundance of Terfezia spp. in B and B2. These ascomycetes include heat resistant hyphomycetes (Hambleton et al. 2005) and fungi with hypogeous fruiting bodies, characterized by persistent thick-walled ascospores that preferentially inhabit mineral soils and are able to survive surface fires (Taylor and Bruns 1999; Tedersoo et al. 2006). The relatively high ratio of BM:AM at 4 in the stand subjected to high fire frequencies and dominated by Cistus spp. (B1) was caused by a high abundance of Cortinarius and Hebeloma species (37 % of sequence reads), several of which are Cistus-specific (Comandini et al. 2006). This result highlights the effect of post-fire vegetation cover on the soil fungal communities, especially on the mycorrhizal fungal component, which is directly related to factors such as host specificity in the recruitment of symbiotic partners (Anderson et al. 2003). Ectomycorrhizal (EM) fungi represented 37 % of the sequence reads in the control stand (UB) and decreased to 16 % in the burnt stands that were characterised by the same shrub cover, while they constituted the most abundant ecological functional group in B1 with 54 % of the sequence reads. Members of Thelephoraceae dominated all stands subjected to wildfires, irrespective of frequency. Burnt stands were also characterised by a high abundance of Cortinarius (B1), Hebeloma (B1, B2), Rhizopogon (B) and Terfezia (B, B2). Some of these taxa, and those of Thelephoraceae include several pioneer species that may have benefited from the release from competition induced by fire (Baar et al. 1999; Smith et al. 2004; Martín-Pinto et al. 2006; Buscardo et al. 2010). In contrast, species that are typical of undisturbed forests (Walker and Jones 2013) and are favoured by the

presence of litter, humus and decayed wood, such as Russula and Tylospora spp. (Tedersoo et al. 2003, 2008; Taylor and Bruns 1999) dominated the control stand. As suggested by Walker and Jones (2013), Tylospora spp. may not be efficient at colonizing from airborn spores and seem to migrate slowly from mature forests into disturbed stands. The unburnt stand was also characterised by a high abundance of members of the family Clavulinaceae that comprises species diverse in habitat and ecology, from EM symbionts (e.g. Clavulina spp. and Membranomyces spp.) to saprotrophic fungi (Uehling et al. 2012), and of the genus Archaeorhizomyces. The latter belongs to the class Archaeorhizomycetes that occurs ubiquitously in different terrestrial ecosystems and has been found to colonize typical EM habitats, i.e. soil and roots of pine and understory ericaceous plants (Rosling et al. 2003, 2011; Urban et al. 2008; Grelet et al. 2010; Rincón and Pueyo 2010; Buscardo et al. 2010, 2012). Although the precise ecological niches of these fungi remain undetermined and there is no evidence that they form mycorrhizal structures, results from culture experiments suggest that they may be involved in decomposition and do not require direct carbon transfer from the plant through symbiosis (Rosling et al. 2011). Long-termfire-induced changes on soil chemical and physical properties, such as loss of organic matter and nutrients, alteration of soil moisture and pH, and deterioration of soil structure may also influence soil fungal communities (Hart et al. 2005b; LeDuc et al. 2013; Rincón et al. 2014). Soil analyses indicated significant reductions of organic carbon, Ca and Mg in all the burnt stands compared to the unburnt control, and significantly lower N concentrations in both short return interval stands (B1, B2; Buscardo et al. 2012), which may have influenced soil fungal communities. A scarcity of saprotrophic species was apparent in both stands subjected to short fire return interval. Values of sequence read frequency at the genus level for putative and known decomposers, parasites and pathogens were lower in B1 and B2 compared to UB and B. Significantly higher sequence read abundances for parasitic or saprobic fungal species belonging to the family Mortierellaceae were found in stands UB and B2, while Rasamsonia brevistipitata, a thermophilic species belonging to the Trichocomaceae and known to be predominantly saprobic (Houbraken et al. 2012) had a significantly higher value in stand B. This could be due to the decrease of the C:N ratio in these soils (Buscardo et al. 2012), which may have reduced the competitiveness of saprotrophic fungi and favoured their replacement by mycorrhizal fungi that are known to be influenced by N availability (Lilleskov et al. 2002; Kjøller et al. 2012), as they do not depend on litterderived energy (Lindahl et al. 2007) and are less affected by environmental stress (Villeneuve et al. 1989). However, the scarcity of saprotrophic fungi may also be due (at least partly) to seasonal effects that lead to an increase of the abundance of EM fungi

during the growing season (Jumpponen et al. 2010), and/or to the under-representation of saprobic fungi in environmental studies and public DNA sequence databases (Ryberg et al. 2009). The latter is supported by the fact that the taxonomic assignment to the genus level was up to twofold higher for the stands dominated by EM fungi (UB, B1). In conclusion, this study has shown that DGGE- and pyrosequencing-based profiling of fungal communities provide, at least for the dominant fungal taxa, consistent results. Although pyrosequencing-based profiling has the advantage of generating DNA sequence data that allow direct taxonomic identification, DGGE remains a rapid and cost-effective alternative to pyrosequencing-based profiling when assessment of the variation in frequent/abundant taxa in fungal community composition is the primary objective. However, new and relatively inexpensive technologies, such as the Ion Torrent Personal Genome Machine (PGM; Life Technologies, Guilford, CT), are now available and could remove the economic advantage of DGGE and, if supported by curated databases, further improve the understanding of microbial diversity (Whiteley et al. 2012; Brown et al. 2013; Kemler et al. 2013). Our results confirm the hypothesis that the persistence of high fire frequency could, in the long term, alter soil properties and host plant distribution, as well as the associated belowground fungal communities. These changes could drive pine forests to a tipping point beyond which they are permanently replaced by shrub communities representing arrested successional stages (see Fig. 5). Previous research conducted in the study area support the results obtained here by showing that short fire return intervals affect both the structure of EM fungal resistant propagules (Buscardo et al. 2010) and the potential facilitation offered by EM fungal networks for pine regeneration by significantly reducing the frequency of occurrence and the proportion of common symbiont species (Buscardo et al. 2012). Post-fire natural regeneration of maritime pine forests depends on the existence of adequate soil conditions and a source of dispersal of forest species. Changes in the fire regime may reduce the potential for re-colonization of mycorrhizal fungi and/or mycorrhiza-dependent species (Neary et al. 1999; Buscardo et al. 2012). Such changes are likely to cause a loss of biodiversity and the breakdown of important ecosystem services, such as water retention or regulation of the local climate (Leadley et al. 2010). Whether the long-term effects of different wildfire regimes on the soil fungal communities of maritime pine forests across the full distribution area of the species are akin to that reported here remains to be confirmed by future studies. We were unable to replicate wildfire events, which are highly variable in intensity and impact. At best, replicate fire treatments can be achieved by using controlled burning or by using areas subject to recurrent prescribed fire, however in the Mediterranean controlled burning is impractical because of the hazard they pose

when escaping control and prescribed recurrent fires are inexistent. The sampling design, with its scale (grain size and extent) ensured that the sample plots represented independent samples (i.e. the fungal communities sampled were almost surely spatially independent). Therefore the independence of samples within stands legitimated an inter-stand comparison. We admit that in the absence of fire event replication our results cannot be taken as proof of differences in fire history, although soil analyses data indicate differences between UB and all the other fire treatment stands that are most likely to have been caused by fire. Nonetheless, our results on fungal community diversity and the between-method comparison are valid, with the above caveats.

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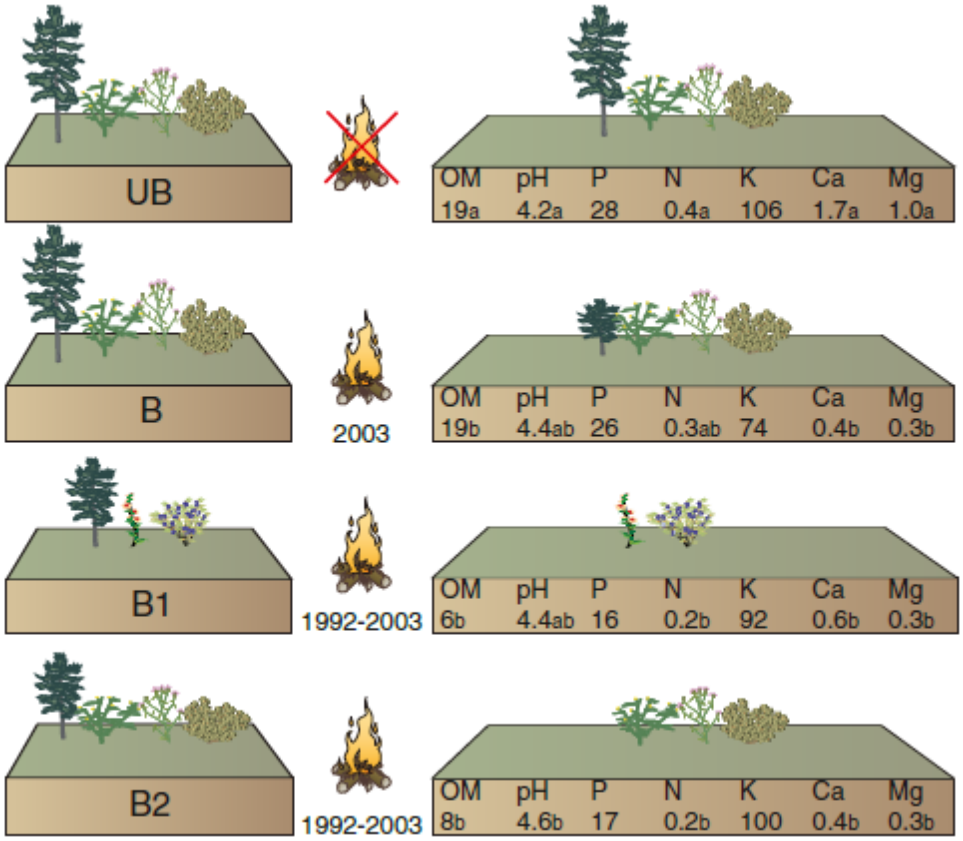

Fig. 1 Vegetation composition, fire history and soil properties of four forest stands subjected to different fire regimes: UB, unburnt stand; B, long fire return interval; B1 and B2, short fire return intervals. The symbols for plant species in stands UB, B and B2 indicate (from left to right) Pinus pinaster (where present, from largest to smallest: adult, 17- year-old and 6-year-old tree), Halimium spp., Erica spp. and Pterospartum tridentatum. In B1 the symbols indicate P. pinaster, Cistus ladanifer and Arbutus unedo. Soil analysis values after Buscardo et al. (2012). Values are in units as follow: OM, %; P, ppm; N, %; K, ppm; Ca, me/ 100 g; Mg, me/100 g

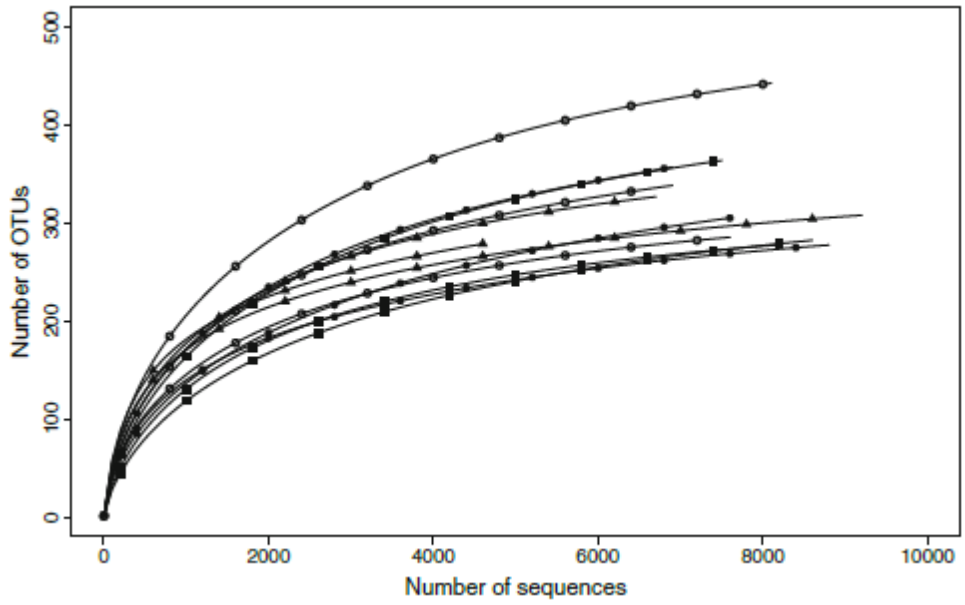

Fig. 2 Rarefaction curves for each of the 12 sampling plots included in this study, illustrating the dependence of the number of operational taxonomic units (OTUs), assigned at 97 % sequence similarity, on the number of sequence reads obtained by 454 pyrosequencing of fungal internal transcribed spacer 1 (ITS1) amplicon libraries generated from soil extracted DNA. Plots are located in four stands subjected to different fire regimes (UB, unburnt stand; B, long fire return interval stand; B1, short fire return interval stand dominated by Cistus ladanifer; B2, short fire return interval stand dominated by Erica spp., Halimium spp. and Pterospartum tridentatum). Stands: !, UB; ", B; #, B1; $, B2

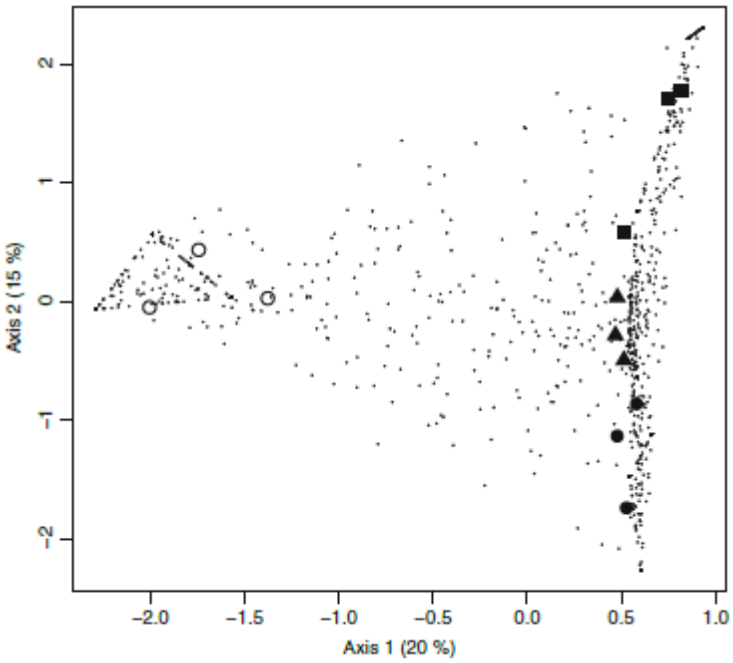

Fig. 3 Graphical representation of the results of a correspondence analysis using 454 pyrosequencing-based fungal operational taxonomic unit (OTU, assigned at 97 % sequence similarity) frequency data in 12 sampling plots with the “dudi.coa” routine of the ade4 v1.4-14 package in R v3.0.2. Sampling plots are located in four stands subjected to different wildfire return intervals (UB, unburnt stand; B, long fire return interval stand; B1, short fire return interval stand dominated by Cistus ladanifer; B2, short fire return interval stand dominated by Erica spp., Halimium spp. and Pterospartum tridentatum. Stands: !, UB; ", B; #, B1; $, B2; ·, OUT

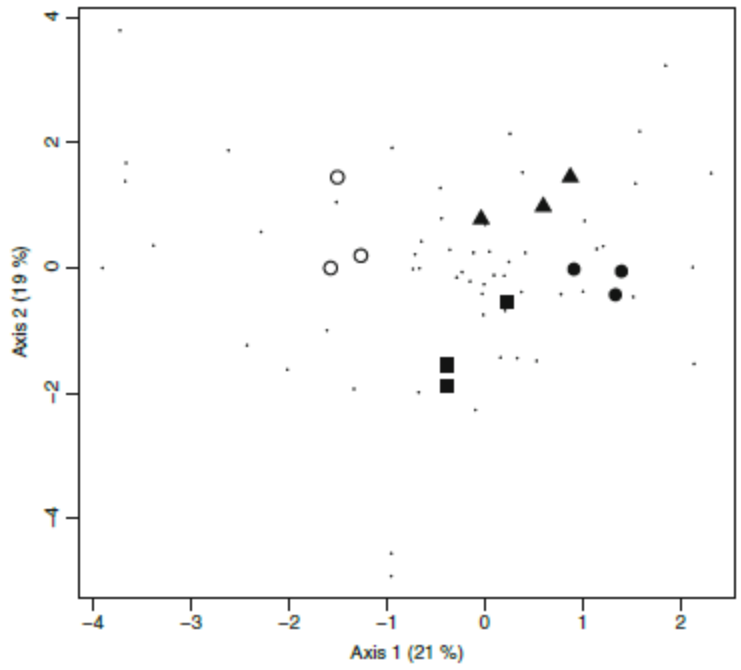

Fig. 4 Graphical representation of the results of a correspondence analysis using data on the intensity of 56 denaturing gradient gel electrophoresis (DGGE) markers in 12 sampling plots with the “dudi.coa” routine of the ade4 v1.4-14 package in R v3.0.2. Sampling plots are located in four stands subjected to different wildfire return intervals (UB, unburnt stand; B, long fire return interval stand; B1, short fire return interval stand dominated by Cistus ladanifer; B2, short fire return interval stand dominated by Erica spp., Halimium spp. and Pterospartum tridentatum. Stands: !, UB; ", B; #, B1; $, B2; ·, OUT

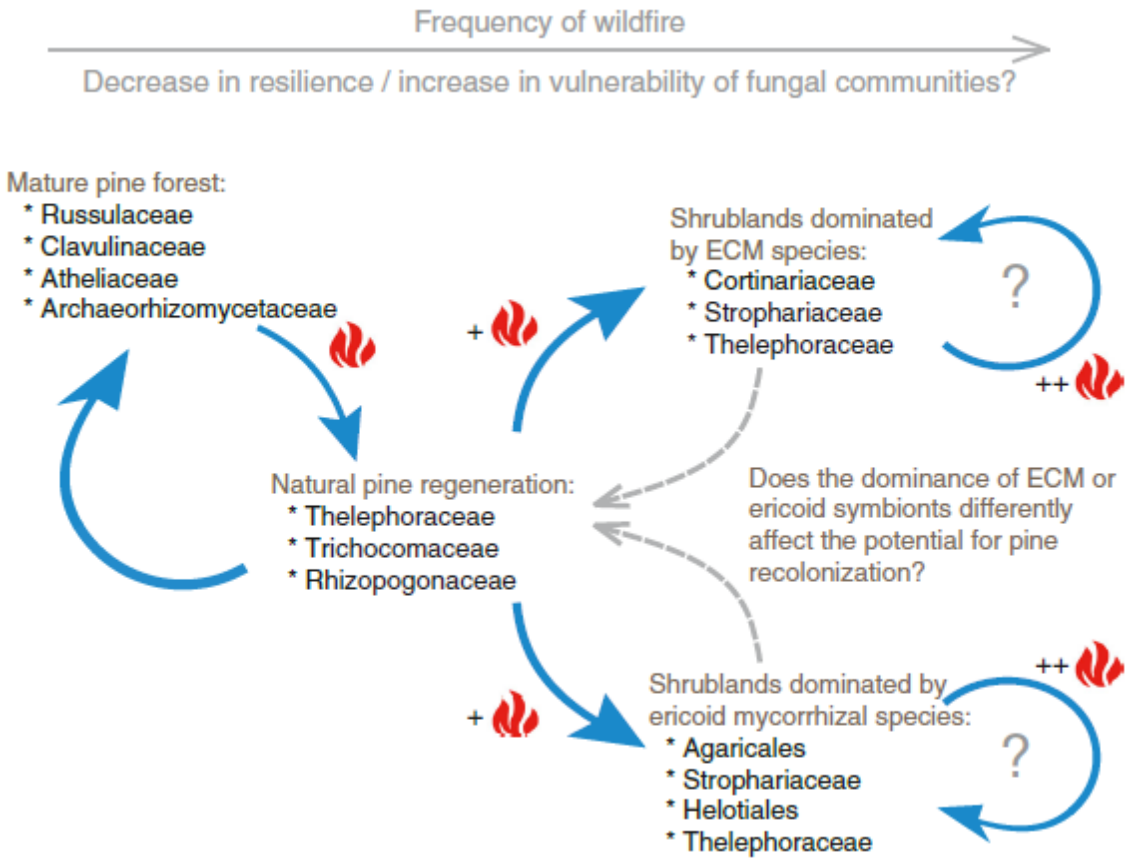

Fig. 5 Conceptual scheme of stand dynamics of maritime pine ecosystems, showing how different fire regimes change the structure of both vegetation and the associated below-ground dominant fungal taxa in central Portugal

Table 1 Overview of soil fungal diversity and richness estimators for 12 sampling plots in four forest stands subjected to different fire regimes, based on 454 pyrosequencing reads and denaturing gradient gel electrophoresis (DGGE) profiles obtained with internal transcribed spacer 1 (ITS1) amplicon libraries; diversity and richness estimators based on 454 pyrosequencing reads were calculated as averages across 50 rarefied subsamples of 4,609 sequence reads for each sampling plot

		454 pyrosequencing					DGGE	
Stand	Plot	# Sequence reads	# OTUs	*S* (SD)	Chao1 (SD)	*H'* (SD)	*S* (SD)	*H'* (SD)
UB	1	7,740	286	232 (5)	286 (17)	3.98 (0.01)	32	3.36
	2	7,034	339	283 (4)	363 (23)	4.05 (0.01)	34	3.38
	3	8,241	443	349 (7)	468 (24)	4.44 (0.02)	38	3.61
	Mean			288 (59)	372 (91)	4.16^{a} (0.25)	35 (3)	3.45 (0.14)
B	1	7,611	364	288 (6)	390 (24)	3.67 (0.02)	35	3.50
	2	8,290	278	224 (5)	286 (18)	3.00 (0.02)	30	3.25
	3	8,725	283	215 (6)	284 (22)	2.85 (0.02)	33	3.43
	Mean			242 (40)	320 (61)	3.17^{b} (0.44)	33 (3)	3.39 (0.13)
B1	1	7,014	358	296 (5)	400 (21)	3.88 (0.02)	25	3.21
	2	8,914	278	219 (5)	272 (18)	3.56 (0.02)	32	3.29
	3	7,711	306	242 (6)	326 (26)	3.48 (0.02)	32	3.40
	Mean			252 (40)	333 (64)	3.64^{ab} (0.21)	30 (4)	3.30 (0.10)
B2	1	9,269	308	253 (5)	314 (25)	4.40 (0.01)	29	3.29
	2	6,770	327	278 (5)	345 (17)	4.15 (0.01)	33	3.39
	3	4,743	280	265 (0)	346 (0)	4.54 (0.00)	38	3.60
	Mean			265 (13)	335 (18)	4.36^{a} (0.19)	33 (5)	3.43 (0.16)
	$F_{3,8}$ (*P*-value)			0.697 (0.58)	0.374 (0.77)	10.3 (0.0040)	1.025 (0.43)	0.744 (0.56)

UB, unburnt stand; B, long fire return interval stand; B1, short fire return interval stand dominated by Cistus ladanifer; B2, short fire return interval stand dominated by Erica spp., Halimium spp. and Pterospartum tridentatum. Operational taxonomic units (OTUs) were assigned at a 97 % sequence similarity level and one-way analyses of variance (ANOVA) were performed in order to assess differences in diversity and/or richness across the four stands (superscript letters reflect Tukey's test results); S, species richness; H!, Shannon's diversity; SD, standard deviation

Table 2 List of the most abundant fungal taxa (order, family and genus levels) found in soils subjected to different wildfire regimes

Order	Family	Genus	*N*	Sequence read abundance (%)			
				UB	B	B1	B2
Russulales	Russulaceae		53	**17.46**	2.69	3.05	1.09
		Russula[a]	34	**12.94**	1.95	2.54	0.95
Cantharellales			25	**8.88**	0.20	0.09	1.13
	Clavulinaceae		10	**7.85**	0.04	0.01	0.01
Atheliales	Atheliaceae		25	**8.43**	0.05	0.61	0.10
		Tylospora[a]	24	**8.42**	0.05	0.61	0.10
Archaeorhizomycetales	Archaeorhizomycetaceae	*Archaeorhizomyces*	9	**7.47**	0.07	0.09	0.11
Helotiales			46	**5.90**	0.77	0.70	**5.75**
Mortierellales			28	**4.72**	1.54	0.75	**4.15**
	Mortierellaceae		23	**4.60**	1.48	0.75	**4.04**
Thelephorales	Thelephoraceae		35	1.28	**20.97**	**9.56**	**5.45**
Eurotiales	Trichocomaceae		31	2.20	**14.22**	0.78	1.74
		Rasamsonia	11	0.03	**13.77**	0.12	0.04
Boletales			25	**4.10**	**5.53**	1.03	0.27
	Rhizopogonaceae	*Rhizopogon*[a]	13	2.20	**5.20**	0.94	0.19
Agaricales			140	**7.17**	**9.28**	**49.85**	**16.19**
	Cortinariaceae	*Cortinarius*[a]	24	0.89	0.14	**22.86**	1.34
	Strophariaceae		23	0.00	2.09	**14.94**	**7.99**
		Hebeloma[a]	18	0.00	1.98	**14.71**	**7.92**
	Amanitaceae	*Amanita*[a]	18	1.22	1.18	**4.07**	1.22
Sebacinales			16	**4.07**	0.61	3.76	**4.46**

UB, unburnt stand; B, long fire return interval stand; B1, short fire return interval stand dominated by Cistus ladanifer; B2, short fire return interval stand dominated by Erica spp., Halimium spp. and Pterospartum tridentatum). Sequence read abundance (see Table S2 for SD values) larger than 4 % are indicated in bold

a Confirmed ectomycorrhizal genus after Rinaldi et al. (2008) and Tedersoo and Smith (2013)