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Post-fire regeneration of *Pinus pinaster* Ait. forests: environmental and biological constraints

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SUMMARY

The aim of this investigation was to identify the environmental and biological constraints for the natural regeneration of maritime pine after stand-replacing wildfires and to evaluate the effects of fire frequency (measured as fire return interval) on three components of the soil fungal communities associated with maritime pine (*Pinus pinaster* Ait.): the ectomycorrhizal (ECM) resistant propagules community (RPC), the total soil fungal community and the potential for the formation of common mycorrhizal networks between pine seedlings and shrub understorey species.

Firstly, the main environmental factors responsible for maritime pine regeneration variability in north-western Italy were identified. The diversity and structure of ECM fungal communities associated with maritime pine saplings naturally regenerated after wildfire were characterized. ECM taxa were identified by direct sequencing of fungal internal transcribed spacer (ITS) regions from individual ECM root tips. The environmental factors responsible for ECM fungal species distribution were also individuated and the relation between saplings performance and ECM fungal diversity indices assessed. Pine regeneration varied throughout the study areas and was enhanced at higher elevations, in correspondence with moderate slopes, shallower soils, and a reduced cover of ericaceous shrubs and bare ground. These conditions were found in close proximity to patches of pine trees that survived the disturbance event and were previously characterized by a higher pre-fire pine biomass. Even though no correlations were found between saplings performance and ECM fungal diversity indices, common environmental factors (i.e. ericaceous shrub cover, extent of erosion, slope and soil depth) were responsible for shaping the ECM fungal distribution and for describing most of the explained regeneration variability.

Secondly, the long-term effects of wildfire frequency on the structure and composition of the total soil fungal community in an open maritime pine forest of central Portugal were evaluated. Six years after the last fire event, the structure of the soil fungal communities in areas subjected to short and long fire return intervals were analysed using 454 pyrosequencing technology and denaturing gradient gel electrophoresis, and compared to the soil fungal community structure of an unburnt control stand. Although long-term effects of wildfire on fungal species richness were not detected, wildfire and wildfire frequency had a significant effect on the structure of the soil fungal community. Wildfire induced a general shift towards the Ascomycota in the stands characterized by the same shrub cover and favoured the abundance of fungal species that preferentially inhabit mineral soils and pioneer species in all the burnt stands, while differences in the abundance of specific ECM fungal species indicated a significant effect of the post-fire vegetation cover on the soil fungal community.

Thirdly, the effects of wildfire frequency on the RPCs of the above-mentioned open pine forest were compared. Soil samples were collected in four mountain sites with different fire return intervals and used to test ECM fungal development in two hosts, *P. pinaster* and *Quercus suber* L. RPCs were characterized by direct sequencing of fungal ITS regions from individual ECM root tips. Eighteen

ECM fungal species were detected in the bioassay. The effect of the fire return interval length on individual species varied greatly: infrequent species found in the unburnt soil were completely eliminated in the short fire return interval sites (e.g. *Laccaria* spp. and *Scleroderma* spp.), some species were favoured by long fire return interval but suffered with short fire return interval (e.g. *Inocybe jacobi*), and other species positively responded to both fire regimes (e.g. *Rhizopogon roseolus*). Species richness of the ECM fungal community found on *Q. suber* was reduced by short fire return intervals. Overall changes in fire return interval length affected both RPC structure and composition.

Finally, the influence of fire frequency on the potential for common ECM networks between understorey shrub species and *P. pinaster* was investigated. ECM root tips were sampled from five shrub species belonging to the genera *Arbutus*, *Cistus* and *Halimium* and from *P. pinaster* at four stands with different fire return interval lengths. Fungal symbionts and relative hosts were identified using molecular techniques with direct sequencing of the nrDNA ITS region. Twenty eight ECM fungal species and seventeen non-ECM root inhabitants were identified. Six years after wildfire disturbance ECM species richness did not differ significantly between unburnt and burnt stands. Nine ECM fungal species were common to pine and shrubs and both their frequencies and abundances were significantly higher in the unburnt and in the low fire return interval stands when compared with the high fire return interval stands.

Fire is a major factor in the dynamics of Mediterranean forests and it is of vital importance in maintaining pine status in these ecosystems. However, changes in fire frequency can compromise the ecosystem functioning by leading to the regression of vegetation and modifying the structure and composition of soil fungal communities. This investigation revealed that highly recurrent fires have significant effects on the structure of the soil fungal communities. They also modify the structure and composition of the ECM RPCs and strongly reduced the potential for common mycorrhizal networks between understorey shrub species and pine seedlings. Therefore an increase of fire frequency could negatively affect Mediterranean pine forests by delaying the process of pine recolonization through direct and indirect effects on soil and on the above-ground plant community.

GENERAL INTRODUCTION

Fire and Mediterranean ecosystems

Several of the major biomes in the world owe their distribution, species composition, and ecological properties to the fire regime (Bond *et al.* 2005). Fire has lead to niche differentiation of Mediterranean communities favouring genetical and ecological diversity (Naveh 1975; Cowling *et al.* 1996), and has induced the development of a series of ecological traits such as resprouting ability and fire-induced germination that guarantee survival of populations under recurrent fires (Keeley 1986). The high resilience of Mediterranean ecosystems has to be attributed to the ongoing pre-historical influence of fire (Naveh, 1975). These ecosystems tend to react to wildfires with a typical autosuccession response in which pre-disturbance plant species occur amongst the early post-fire vegetation (Hanes 1971). Conifers developed a series of plant adaptive responses that promote their survival in fire-prone environment such as thick protective bark, early and large seed production, serotinous cones, and high ecological plasticity (Scarascia-Mugnozza *et al.* 2000; Fernades & Rigolot 2007).

However, changes of the fire regime in the last decades due to abandonment of agricultural lands, reduction of grazing and in the use of the forest, and increasing recreational pressure (Scarascia-Mugnozza *et al.* 2000; Moreira *et al.* 2001; Pausas 2004) may reduce ecosystem resilience, i.e. the ability to recover the pre-disturbance state (Díaz-Delgado *et al.* 2002). While Mediterranean pine forests are relatively more abundant at intermediate levels of fire recurrence, i.e. a fire every 10–40 years (Pausas 1999), an increase of fire frequency, already pictured in climate change scenarios (Pereira *et al.* 2002), may hinder its regeneration and that of other plant species, especially those without resprouting mechanisms and irregular germination (Rodrigo *et al.* 2004). This is gradually leading to the regression of vegetation and a reduction of the recovery success rate, even in absence of further disturbance events, with the progressive replacement of some Mediterranean forests by shrub-dominated communities that greatly increase the probability of fire (Pignatti 1995; Scarascia-Mugnozza *et al.* 2000; Moreira *et al.* 2001; Díaz-Delgado *et al.* 2002; Pereira *et al.* 2006).

Maritime pine

Maritime pine (*Pinus pinaster* Ait.) is a broadly distributed conifer in the western Mediterranean Basin, in Southern Europe and Africa, and along the Atlantic coast of Portugal, Spain and France over an area exceeding 4 million ha and under broad ranges of elevation, climate and soil (Alía *et al.* 1996; Alía & Martín 2003). It is an obligate seeder and populations that experience frequent fires can flower at four years and fructify between five and 12 years (Tapias *et al.* 2001). While pine crowns can keep the serotinous cones up to 40 years and seeds can remain viable for 30 years (Tapias *et al.* 2004), forest floor seed bank is scarce and transient (Fernades & Rigolot 2007). Serotiny and early flowering reflect the evader strategy of *P. pinaster* in relation to fire (Tapias *et al.* 2004). Once fire-related factors such as heat release seeds from dormancy (Trabaud 1987) seedlings' emergence and

establishment represent key-events in the life cycle of plants especially in water-limited environments, and in habitats subjected to frequent disturbances (Quintana *et al.* 2004). Ectomycorrhizal resistant propagules as well as post-disturbance interactions between early colonizing plants and plants that are established concurrently or at later stages play a determinant role in the facilitation of the regeneration following disturbance events (Pugnaire *et al.* 1996; Taylor & Bruns, 1999; Nara & Hogetsu 2004).

Fungal ectomycorrhizal symbiosis

Ectomycorrhizal (ECM) symbioses are necessary components of most plant communities on a global scale (Smith & Read 1997). ECM trees are dominant in coniferous forests in boreal and alpine regions and in many forests in temperate and Mediterranean ecosystems and occur in large areas of tropical and subtropical forests with several tree species belonging to the families of *Pinaceae*, *Betulaceae*, *Fagaceae*, and *Dipterocarpaceae* totally dependent for their performance and survival on the mycorrhizal symbiosis (Smith & Read 1997). Approximately 6000 species of ECM fungi forming highly dynamic and complex below ground communities have been described (Johnson *et al.* 2005). Their importance in the relation between biodiversity and ecosystem functioning and their potential role in controlling plant diversity and productivity is now being recognized (van der Heijden *et al.* 1998). The symbiosis benefits the micro-organism with the allocation by the host of a relevant fraction of the total amount of carbon fixed by the plant, that can reach up to 30% of seedling hosts current assimilates (Söderström 2002). On the other side, colonized root tips and hyphal network contribute relevantly to the mineralization of nutrients into available forms for plants and to their acquisition (Smith & Read 1997), and confer host protection against heavy metals, pathogens (Newsham *et al.* 1995), and drought (Nikolaou *et al.* 2003).

Plants and ECM communities' shape each others in terms of structure and diversity, and are both simultaneously affected by a series of edaphic and climatic variables. Kernaghan *et al*. (2003) suggested the existence of a positive relation between plant and ECM diversity and demonstrated that almost 40% of the variation in ECM diversity was attributable to overstorey plant diversity. On the other side, the availability of ECM fungi in systems where ECM plants are spatially dispersed in a matrix of non-ECM vegetation represents a limitation for seedlings establishment (Dickie *et al.* 2002). Diversity and composition of fungal communities are driven by mechanisms that involve interactions between ECM colonization potential, resource partitioning, species competition, and disturbance (Bruns 1995). ECM fungi show differing preferences for edaphic conditions including soil moisture, depth, presence of litter, mineral soil, wood and debris, and charcoal (Stendell *et al.* 1999) and the nature of the interactions among mycorrhizal species can be either positive or negative (Koide *et al.* 2004). Co-occurrence might occur as a consequence of physiological complementation or parasitic interaction between fungi, while avoidance might be the result of competition among species and could involve the production of inhibitory substances (Koide *et al.* 2004). The result of all these

interactions brings to a high level of diversity and spatiotemporal variability within the ECM communities (Horton & Bruns 2001).

Fire and ectomycorrhizal fungi

While in presence of low intensity fires that do not completely burn off the litter layer, ECM fungal species evenness is reduced and species richness shows very little changes (Jonsson *et al.* 1999), high intensity stand replacing fires may locally kill most ECM fungi and initiate depending on pre-fire ecosystem conditions and fire characteristic a succession process (Dahlberg 2002). The occurrence of high intensity fires is common in Mediterranean and temperate North America forests (Visser 1995; Torres & Honrubia 1997; Horton & Bruns 1998; Baar *et al.* 1999; Dahlberg 2002). In these circumstances the organic layer may be eliminated, soils may be partially sterilized by heat, and deposition of ashes can alter soil pH and availability of nutrients with potential consequences on the structure and function of the ECM communities (Grogan *et al*. 2000). Post fire ECM communities are characterized by an initial strong representation of ascomycetes that appears to dissipate within 6 years since the disturbance event (Visser, 1995). At this stage the ECM fungal community is dominated by few ruderal species that will evolve to a more complex and rich community in more mature forests (Visser, 1995).

Stendell *et al*. (1999) reported that the ECM biomass of a ponderosa pine forest affected by fire decreased significantly in the litter and organic soil horizons and most abundant pre-fire species were reduced to undetectable post-fire levels, resulting in a general increase of evenness in the post disturbance situation. Disturbance events in these cases modify the competitive environment and lead to the colonization of the affected areas by new fungal species. Martín-Pinto *et al*. (2006) evaluated the fire effects on diversity and production of sporocarps fungal communities of a maritime pine forest and found out that fire affected negatively the fungal production leading to the emergence of post-fire species such as *Pholiota carbonaria* and *Peziza violacea*. Sporocarps of species belonging to the Pezizales order are commonly found following burning of forests ecosystems (Egger & Paden 1986; Fujimura *et al.* 2005) and often dominate ECM fungal communities in early successional ecosystems and colonize seedlings naturally regenerated after fire (Baar *et al.* 1999; Grogan *et al*. 2000). Some of them are hypogeous fruiting fungi and produce thick walled spores able to survive surface fires (Tedersoo *et al.* 2006).

ECM fungi and in particular spore bank and other resistant propagules play a determinant role in the facilitation of regeneration following disturbance events (Taylor & Bruns 1999). Post fire ECM community composition has a strong resemblance to the composition of the pre-fire spore bank community that appears to be caused by survival of the soil spore bank (Baar *et al.* 1999). Species of the genus *Rhizopogon*, *Tuber*, and *Wilcoxina*, use to be abundantly present in the soil in form of spores or sclerotia that represent an important source of inoculum in post-disturbance setting (Baar *et al.*

1999; Taylor & Bruns 1999; Izzo *et al.* 2006a). They are referred as early successional fungi and they represent the most abundant colonizers of seedlings after stand-replacing fires in pine Mediterranean forests (Baar *et al.* 1999; Izzo *et al.* 2006a) in contrast with late successional fungi that colonize root tips mainly through the extension of mycelium from other colonized roots (Taylor & Bruns 1999). The ability of some of the early-stage ECM fungi to germinate and occupy a site depends indeed on the absence of competitively species (Izzo *et al.* 2006a,b).

The presence of viable ECM propagules for relatively long periods confers to the ecosystem the chance to recover after a disturbance event. The success of *P. pinaster* as a colonizer of disturbed soils may be attributed in part to its compatibility with many different ECM fungi with broad host range (Pera & Alvarez 1995). The presence of non-specific fungi in post disturbance events could help to stabilise the forest community by associating with both pre- and post-fire hosts (Perry *et al*. 1989). Plant coexistence is mediated by mycorrhizal fungi that integrate the associated plant species into common mycelial networks (Richard *et al.* 2009; van der Heijden & Horton 2009). The colonization of naturally regenerated seedlings by ECM fungi can be promoted by spores and other resistant propagules but also by resident mycelia that can remain viable on roots of trees killed by fire for several months after disturbance (Ferrier & Alexander 1985; Visser 1995). The ability of mycorrhizal propagules to maintain their viability suggests a degree of resilience that may facilitates the restoration of ecosystems biodiversity and function (Johnson *et al.* 2005).

Residual trees in recently harvested forests or in postfire events play therefore an important ecological role enhancing seedlings colonization (Perry *et al*. 1989; Cline *et al.* 2005) by retaining on their root system a unique fungal composition that can easily connect with adjacent regenerating seedlings. Another source of fungal inoculum is represented by shrubs. The overstorey of Mediterranean pine forests are commonly colonized by ECM shrub species that act as a bridge between mycorrhizal fungi and pine seedlings and play an important role in the process of seedlings establishment (Perry *et al*. 1989). The weakening of the close links between plant and soils during disturbance events and the subsequent alteration of the belowground ecosystem may lead to poor recovery of the original plant guild and to further reductions in abundance of belowground mutualists (Perry *et al*. 1989). In presence of high fire occurrence, erosion phenomena, loss of plant cover, and soil nutrients impoverishment, may be also accompanied by loss of fungal inoculum essential for the potential establishment of plant communities.

Research objectives

The aims of this research were to identify the environmental and biological constraints of maritime pine saplings naturally regenerated after a stand replacing wildfire and to evaluate the effects of fire frequency (measured as fire return interval length) on three components of the soil fungal communities associated with maritime pine: the ECM resistant propagules community (RPC), the total soil fungal community and the potential for the formation of common mycorrhizal networks between pine seedlings and shrub understorey species.

Thesis layout

This thesis is structured in four chapters.

In the first chapter the diversity and structure of the ECM fungal community associated with pine saplings naturally regenerated after a stand replacing wildfire in the north-eastern Italian Peninsula were investigated. The main environmental factors responsible for pine regeneration variability and ECM fungal species diversity were identified and the relation between sapling performance and ECM fungal diversity indices was assessed.

In the second chapter the effects of wildfire frequencies (measured as fire return interval length) on the ECM RPC of a Mediterranean open pine forest of central Portugal were evaluated. Soil samples were collected in four mountain sites with different fire return intervals and used to test ECM fungal development under controlled condition of light and temperature in two hosts, *P. pinaster* and *Quercus suber* L.

In the third chapter the long-term effects of wildfire frequency on the structure and composition of the total soil fungal community associated to an open maritime pine forest were assessed by comparing 454 pyrosequencing and denaturing gradient gel electrophoresis.

In the fourth chapter the influence of fire frequency on the potential for the formation of common ECM networks between maritime pine and five Mediterranean typically understorey species belonging to the genera *Arbutus*, *Cistus* and *Halimium* was investigated.

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CHAPTER 1

Common environmental factors explain both ectomycorrhizal species diversity and pine regeneration variability in a post-fire Mediterranean forest

Introduction

Wildfires represent a major form of disturbance of Mediterranean ecosystems and influence their dynamics and structure with changes in above and below-ground communities, functions and processes (Naveh 1975; Trabaud 1987; Neary *et al.* 1999; Certini 2005).

The success of *Pinus pinaster* Ait. as a colonizer of disturbed soils may be attributed to adaptive responses that allow survival after low intensity fires, such as thick protective bark and reproduction processes that facilitate recovery after stand replacement fire from seeds stored in serotinous cones (Fernades & Rigolot 2007). However, the occurrence of repeated high intensity fires is changing the dynamics of these pine stands with a regression of the vegetation structure and a delay of the succession processes due to the invasion of shrubs of the Mediterranean macchia. Under these unfavorable circumstances, even species that usually regenerate well after fire show irregular or low regeneration (Lloret 1998) and a better understanding of the changing regeneration processes is needed in order to promote the establishment and survival of pine seedlings. Pine regeneration is known to go on for more than two years after fire (Luis-Calabuig *et al.* 2002) and subsequent development of seedlings depends not exclusively on post-fire environmental factors (Pausas *et al.* 2004) but also on seedlings association with symbiotic ectomycorrhizal (ECM) fungi (Taylor & Bruns 1999). Pine trees are indeed ecologically obligate mycorrhizal symbionts that depend on the presence of fungal inoculum for establishment and survival (Smith & Read 1997).

The diversity and composition of ECM fungal communities are driven by mechanisms that involve the interactions between disturbance, ECM fungal colonization potential, species competition and resource partitioning (Bruns 1995). Post-fire ECM fungal communities resemble the composition of pre-fire resistant propagule communities (Baar *et al.* 1999; Taylor & Bruns 1999). However, new fungal species may colonize the areas following the disturbance event, benefitting from the release of competition induced by fire (Buscardo *et al.* 2010). Maritime pine is compatible with many different ECM fungi with broad host range (Pera & Alvarez 1995) and the presence of residual trees and shrubs after a wildfire could also play an important role by providing available fungal networks to adjacent regenerating seedlings (Perry *et al*. 1989; Cline *et al.* 2005). The understorey of Mediterranean pine forests are commonly colonized by both ECM and ericoid mycorrhizal shrubs species and the presence on shrub roots of mycorrhizal fungal species able to colonize both shrubs and pine could represent a source of inoculum for the newly regenerated seedlings, and promote their survival and establishment (Horton *et al.* 1999; Nara & Hogetsu 2004; Richard *et al.* 2009). Nevertheless, in certain conditions shrubs could also represent a threat to pine regeneration and compete with natural regenerated seedlings for light and moisture (O'Brian *et al.* 2007). The importance of post disturbance interactions between early colonizing plants and plants that are established concurrently or at later stages is well recognized (Pugnaire *et al.* 1996; Nara & Hogetsu 2004) but how environmental constraints affect

pine regeneration, ECM fungal communities and the interaction between them is poorly understood in wildfire prone forest ecosystems.

The objectives of this study were (i) to characterize the ECM fungal assemblage on 135 maritime pine saplings and individuate the environmental factors responsible for ECM fungal diversity five years after a stand replacing wildfire and (ii) to assess the variability of maritime pine regeneration in relation to ECM fungal diversity indices and a series of environmental factors including topographic variables and microsite conditions.

Materials and methods

Study site

Sampling was performed in a maritime pine open forest at S. Romolo (Imperia, Liguria), in the north-east of Italy (43º51' N, 7º44' W). In 2003, this area was affected by a severe wildfire that spread over approximately 350 ha. The vegetation covering the area previous to the disturbance event was characterized by an uneven-aged maritime pine forest (dominant trees with 35-40 cm diameter) with sporadic occurrence of broadleaved species like holly oak (*Quercus ilex* L.), downy oak (*Quercus pubescens* Willd.) and chestnut (*Castanea sativa* Mill.). The understorey was represented by typical post-fire resprouters and seeders species such as tree heather (*Erica arborea* L.), Mediterranean strawberry tree (*Arbutus unedo* L.), common heather (*Calluna vulgaris* L.), spiny broom (*Calicotome spinosa* L.), sage leaf rockrose (*Cistus salvifolius* L.), Montpellier rock-rose (*Cistus monspeliensis* L.), and hairybroom (*Cytisus villosus* Pourr.).

The rugged topography of the study area and the presence of high speed winds during the wildfire thwarted the operations of fire extinguishment. Only few patches of pine trees survived erratically on upper slope positions. Fire-killed trees were not harvested, and five years after the disturbance event understorey shrub species and naturally regenerated pine saplings were colonizing the area.

Soils, shallow to moderately deep, are classified as eutric regosols and leptosols and lay on the top portion of flysh characterized by the presence of the "Arenarie di Bordighera" formation (Sagri 1980). The region is characterized by a Mediterranean climate with cool, wet winters and warm, dry summers.

Data sampling procedure

Nine 10 x 10 m^2 plots were delimited in the burnt area in April 2008. The plots were chosen trying to encompass the variability of the area in terms of biotic and abiotic conditions. In every 10 x 10 m^2 plots three 2 x 2 m^2 subplots were randomly delimitated in an imaginary quadrate made by 25 2 x 2 m^2 subplots.

Table 1 Predictors and dependent variables used in the statistical analysis recorded at plot (P, 10 x 10 m^2) and subplot (S, 2 x 2 m^2) level.

Variable description	Units/Classes	Plot level	Mean(SE)	Min	Max
Predictors					
Elevation (ELE)	m (a.s.l.)	P	884(22)	807	975
Aspect (ASP)	0 to 2	P	0.8(0.21)	0.1	2
Slope (SLO)	%	P	22.1(2.53)	12	33
Soil depth (SoilD)	m	P	55.6(9.30)	20	100
Broadleaved tree cover (TreeC)	%	S/P	4.7(0.74)/2.1(1.06)	0	20
Broadleaved tree height (TreeH)	m	S/P	1.7(0.65)/0.4(0.13)	0	2.3
Shrub cover (ShrbC)	%	S/P	34.3(4.21)/31.7(3.40)	10	90
Shrub height (ShrbH)	m	S/P	0.7(0.04)/0.8(0.07)	0.3	1.2
Ericaceous shrub cover (EriC)	%	S	15.2(2.11)	0	43
Herbaceous species cover (HerbC)	%	S/P	5.7(4.58)/9.44(2.11)	0	20
Herbaceous species height (HerbH)	m	S/P	0.2(0.15)/0.2(0.03)	0	0.8
Ground cover (bryophytes) (GrndC)	%	S/P	0.1(0.03)/2.11(0.55)	0	0.5
Leaf litter cover (LittC)	%	S/P	49.3(3.70)/33.9(7.44)	20	90
Trunks and branches cover (TrBrC)	%	S/P	9.9(1.59)/9.9(1.7)	0.5	30
Bare ground cover (BarGC)	%	S/P	15.7(2.73)/30.0(5.00)	3	60
Pine pre-fire basal area (PreBA)	m^2/plot	P	0.3(0.15)	0.14	0.62
Distance from unburnt pine patches (DiUB)	<50 m (1), 50-100 m (2), >100 m (3)	P	1.8(0.36)	1	4
Extent of erosion (ERO)	0, 1, 2	P	1.6(0.29)	0	2
Position of unburnt pine patches (PosUB)	0 to 2	P	0.3(0.06)	0.3	0.8
Topographic position (ToPos)	gully (1), bottom slope (2), mid slope (3), upper slope (4), ridge (5)	P	2.4(0.37)	1	4
Dependent variables					
Pine saplings cover (SapC)	%	S/P	10.1(1.42)/10.9(2.81)	3	25
Pine saplings height (SapH)	m	S/P	0.5(0.04)/0.5(0.06)	0.2	1
Density of saplings with height between 1 and 40 cm (Class 1)	individuals/m^2	S	5.6(3.74)	0	12
Density of saplings with height between 41 and 100 cm (Class 2)	individuals/m^2	S	8.6(7.28)	0	27
Density of saplings with height between 101 and 200 cm (Class 3)	individuals/m^2	S	2.9(5.92)	0	28
Total sapling density (TDen)	individuals/m^2	S	17.1(12.14)	4	56

Within each 10 x 10 m^2 plot average height and percent cover of vegetation in each of several layers/growth-form categories were recorded: broadleaved trees, maritime pine saplings, shrubs, field layer (herbaceous vegetation) and ground layer (bryophytes). Percent cover of bare soil or rock, percent cover of litter and percent cover of trunks and branches were also recorded. For every 2 x 2 m^2 subplot, height and percentage cover of each of the five vegetation layers listed above were recorded as well as cover and height of ericaceous shrubs. In each subplot pine saplings were counted, measured to the nearest centimeter, and classified into three groups according to height (Table 1).

For each plot the following variables were recorded: slope, aspect (degrees), elevation, topographic position (five-point scale: from 1, gully to 5 ridge), soil depth, extent of erosion (three-point scale: from 0, no signs of erosion to 3 evident and widespread signs of erosion), distance (three-point scale: from 1, under 50 m to 3 over 100 m) and position (degrees) of the unburned maritime pine patches and pine pre-fire basal area (Table 1). Soil depth was measured using a manual soil auger. Aspect and position of the unburned pine patches were transformed to a linear scale (Beers *et al.* 1966).

To characterize the ECM fungal community associated with pine saplings five individuals were randomly sampled in every subplot resulting in a total of 135 samples. After removing litter soil samples blocks of approximately12 x 12 cm^2 surface and 20 cm depth were carefully excavated around each sapling using a spade and a sharp knife. A total of 135 samples were collected.

Pine roots were carefully washed over a 2 mm mesh sieve, analyzed under a dissecting microscope and considered to be ECM if root hair were absent, if they appeared swollen, and in presence of hyphae or hyphal sheath. For each soil sample all pine mycorrhizal roots were sorted into morphotypes based on their colour, size, texture, emanating hyphae and rhizomorphs and distinct branching morphology (Agerer 1991). The root present in 21 out of the 135 samples did not contain ECM root tips and were therefore discarded. For each seedling an average of four tips (ranging from one up to six) for each morphotype were individually sampled. A total of approximately 970 root tips were placed in Eppendorf tubes and washed in sterile water before molecular analyses.

DNA extraction, PCR and sequencing

Total DNA was extracted with the C-TAB method [100 mM Tris-HCI (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% cetyl-trimethyl-ammonium-bromide] from 220 samples. The internal transcribed spacer (ITS) region was amplified with the primer combination ITS1-F and ITS4 (White *et al.* 1990; Gardes & Bruns 1993) and succeeded for 204 samples (92.7 %). PCR was performed with ready-to-use PCR reaction mixture ImmoMix^{TM} (Bioline) in 25 µl volume with the following cycling parameters: a denaturation step at 95ºC for 7 min, followed by 35 cycles at 95ºC for 30 s, 55ºC for 30 s and 72ºC for 1 min, with a final extension step at 72ºC for 5 min. Amplification products were ran by gel electrophoresis in 1.2% agarose gel. The DNA was stained with ethidium bromide and single PCR products were purified from gel with a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). Both strands of the purified PCR products were sequenced separately by Macrogen

laboratories. DNA sequences were manually checked and edited if necessary using the BioEdit software version 7.0.9.0 (Hall 1999), and consensus sequences from the two strands of the ITS nrDNA of each isolate were compared with sequences in the UNITE (Kõljalg *et al.* 2005) and INSD (International Nucleotide Sequence Database) online nucleotides databases, using the BLASTn algorithm (Altschul *et al.* 1997). A 97% sequence similarity cut off was used for taxa identification.

Statistical analysis

Species accumulation curve with 95% confidence intervals was generated for the study site using the program EstimateS version 8 (Colwell 2006).

The data on ECM fungi were analyzed using Canonical Correspondence Analysis (CCA) (ter Braak 1986) employing species data frequency in 2 x 2 m^2 subplots. Forward selection was used to select significant explanatory variables and only those significant at the $p > 0.05$ level were included. Ordination analyses were performed using CANOCO version 4.5 (ter Braak & Smilauer 2002). Relative abundance was calculated for each morphotype on each sapling as the number of ECM root tips of that morphotype divided by the total number of ECM root tips on the considered pine sapling. Species richness (SR) and Shannon diversity index (H'; Pielou 1975) were calculated at the subplot level, and correlations between diversity indices and environmental variables, and between environmental variables themselves were analyzed using Pearson's and Spearman's rho methods with SPSS version 17.0 (SPSS Inc., Chicago, USA).

Partial least squares (PLS) regression analysis was used to examine the influence of environmental variables and ECM fungal diversity indices on regeneration variability (i.e. different growth of pine saplings and post-fire saplings density) at plot and subplot level. The number of pine saplings recorded at subplot level was transformed into pine density (individuals/m^2), which was then normalized using a logarithmic transformation. Prior to calculations, the data were centered and scaled to unit variance to give all variables the same relative importance. The significance of components for the models was determined by uncertainty tests carried out within a full cross validation. PLS was carried out using the Unscrambler 9.8 software package (CAMO).

Results

Structure and diversity of the ECM fungal community

A total of 30 taxa were detected on maritime pine saplings root system (Table 2). Three of them were considered fungal root inhabiting taxa (i.e. *Phialocephala fortinii* C.J.K. Wang and H.E. Wilcox, *Oidiodendron maius* G.L. Barron, and an uncultured *Lachnum*). Another species, *Chroogomphus rutilus* (Schaeff.) O.K. Mill., was detected on ECM root tips of both *Rhizopogon roseolus* (Corda) Th. Fr. and *Rhizopogon luteolus* Fr. and Nordholm. Even though members of the *Chroogomphus* genus

Table 2 Database matches of ITS sequences obtained from ECM fungi and fungal root inhabiting taxa(*), colonizing maritime pine saplings five years after a stand replacing wildfire.

Accession number	Type	Best blast hit	N. of colonized seedlings	BLAST expected value	% Similarity/bp
HM545718	*Amanita*	AB080776 *Amanita pantherina*	1	0.0	100%/689
HM545720	Bolete 1	DQ131609 *Boletus aestivalis*	5	0.0	99%/777
HM545722	*Chroogomphus*	UDB001529 *Chroogomphus rutilus*	20	0.0	99%/683
HM545723	*Coniochaetaceae*	EU082785 *Coniochaetaceae* sp.	1	0.0	91%/578
HM545724	*Elaphomyces*	UDB000093 *Elaphomyces muricatus*	2	0.0	99%/638
HM545725	*Hydnellum*	AY569027 *Hydnellum cyanopodium*	2	e^{-102}	96%/214
HM545727	*Lactarius* 1	AM930241 *Lactarius chrysorrheus*	4	0.0	99%/702
HM545728	*Lactarius* 2	AF249283 *Lactarius deliciosus*	3	0.0	99%/692
HM545721	Bolete 2	GU222293 *Octaviania tasmanica*	2	e^{-155}	91%/469
HM545729	*Oidiodendron*	AF062800 *Oidiodendron maius**	7	0.0	99%/528
HM545730	*Phialocephala*	EU882733 *Phialocephala fortinii**	4	0.0	99%/534
HM545731	*Rhizopogon* 1	GQ267481 *Rhizopogon luteolus*	16	0.0	99%/823
HM545732	*Rhizopogon* 2	HM036649 *Rhizopogon roseolus*	55	0.0	99%/672
HM545733	*Russula*	UDB001626 *Russula densifolia*	6	0.0	100%/663
HM545734	*Suillus*	AY898621 *Suillus bellinii*	1	0.0	99%/617
HM545735	*Thelephora*	GQ267490 *Thelephora terrestris*	3	0.0	98%/638
HM545736	*Tomentella* 1	UDB001656 *Tomentella badia*	1	0.0	99%/624
HM545737	*Tomentella* 2	UDB000219 *Tomentella ellisii*	3	0.0	99%/582
HM545740	*Tomentella* 5	AY641459 *Tomentellopsis submollis*	2	0.0	96%/681
HM545719	*Amphinema*	FJ210728 uncultured *Amphinema*	1	0.0	98%/568
HM545741	Asomycotina	EU557319 uncultured Ascomycotina	2	0.0	99%/540
HM545742	*Atheliaceae*	EU557321 uncultured Atheliaceae	1	0.0	99%/383
HM545744	uncultured ECM fungus	DQ054568 uncultured ECM fungus	1	0.0	100%/662
HM545726	*Lachnum*	FJ440910 uncultured *Lachnum**	1	0.0	98%/494
HM545747	*Tricholomataceae*	FJ816733 uncultured *Mycena*	4	0.0	97%/561
HM545745	*Sebacina*	FN393153 uncultured *Sebacina*	1	0.0	96%/624
HM545743	*Thelephoraceae* 1	AJ972894 uncultured *Thelephoraceae* 1	2	0.0	99%/474
HM545746	*Thelephoraceae* 2	FJ013055 uncultured *Thelephoraceae* 2	7	0.0	100%/686
HM545738	*Tomentella* 3	AM161537 uncultured *Tomentella* 1	1	0.0	99%/641
HM545739	*Tomentella* 4	FJ013054 uncultured *Tomentella* 2	1	0.0	96%/692

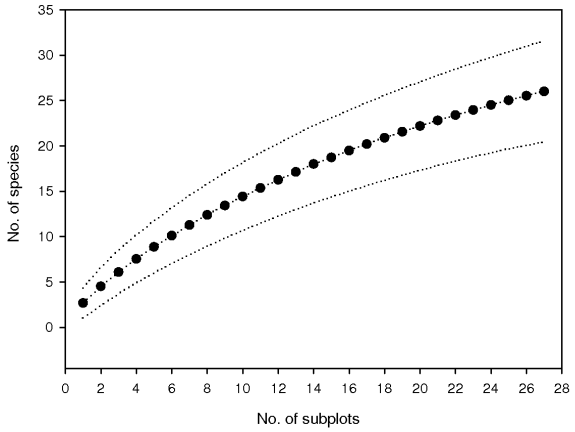

Figure 1 Species accumulation (rarefaction) curve and confidence intervals (sobs 95%) of ectomycorrhizal fungi colonizing maritime pine saplings 5 years after a stand replacing wildfire.

have been thought to be ECM with various species of pine, there are now evidences that suggest that these species act as parasites on the fungal host, the plant host, or both (Olsson *et al.* 2000; Agerer 1990). For this reason, *C. rutilus* and the other non ECM fungal root inhabiting taxa were considered to be supplementary species in the ordination analyses. The species accumulation curve increased with the number of 2 x 2 m^2 subplots without reaching a plateau (Fig. 1) indicating insufficient sampling effort. Pine saplings were colonized on average by two ECM fungal taxa (ranging from one to five).

The ECM fungal community was dominated by Basidiomycota with only three species belonging to the Ascomycota (i.e. *Coniochaetaceae* sp., *Elaphomyces muricatus* Fr. 1829, and the uncultured Ascomycotina). More than one third of the ECM fungi occurred only in a single sample. Approximately 30% of the ECM fungal species found on pine saplings belonged to the *Thelephoraceae*. *Rhizopogon* was the most frequent genus and appeared in 49.6% of the samples, followed by the uncultured *Thelephoraceae* 2 (5.6%), *Russula densifolia* Secr. ex Gillet (4.8%), and *Boletus aestivalis* (Paulet) Fr. 1838 (4%). *R. roseolus* was the most abundant species with a colonizing root rate between 50 and 100% of the total root ectomycorrhization, in half of the samples.

Environmental variables and ECM fungal community

Five variables were able to significantly explain the variation in ECM fungal species frequency (Fig. 2): broadleaved tree height (TreeH), extent of erosion (ERO), the interaction term ericaceous shrub cover (EriC) x soil depth (SoilD), trunks and branches cover (TrBrC) and slope (SLO). The first two axes (eigenvalues 0.615 and 0.489) explained respectively 10.3% and 8.2% of the total variability in the species data (total inertia 5.975; explained variability 34.66%). Axis 1 was correlated with TrBrC and TreeH and axis 2 was most closely associated with EriC x SoilD and SLO. Species that are known to be more associated with mature forests such as *Amanita pantherina* (DC.) Krombh., *Boletus aestivalis*, *Lactarius chrysorrheus* Fr. and *Suillus bellinii* (Inzenga) Watling, were found in the lower left hand side of the ordination diagram in correspondence with a decreasing erosion extent (axes 1 and 2). Species belonging to the *Thelephoraceae* presented a scattered distribution, and non ECM fungal roots inhabiting taxa were located close to the centre of ordination space, together with *Rhizopogon roseolus* and *R. luteolus*.

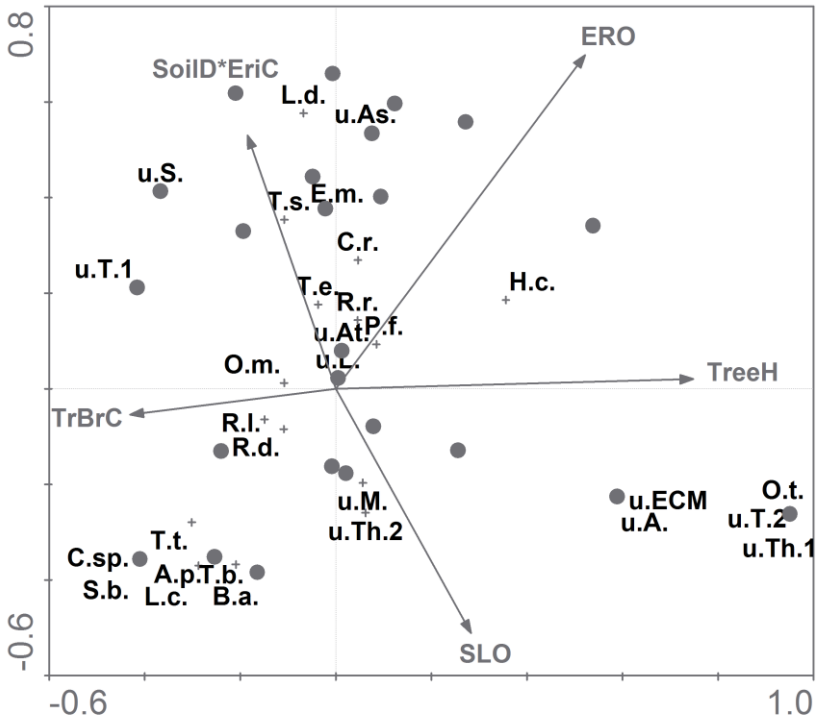

Figure 2 Results of CCA performed on frequency data of ECM fungal species colonizing maritime pine saplings 5 years after a fire event. Each circle represents a 2 x 2 m^2 subplot. Eigenvalues of axis 1and 2 are 0.615 and 0.489, respectively, and explain 34.66% of the variance of the species data. Species ('+') abbreviations are: *Amanita pantherina* (A.p.), *Boletus aestivalis* (B.a.), *Chroogomphus rutilus* (C.r.), *Coniochaetaceae* sp. (C.sp.), *Elaphomyces muricatus* (E.m.), *Hydnellum cyanopodium* (H.c.), *Lactarius chrysorrheus* (L.c.), *Lactarius deliciosus* (L.d.), *Octaviania tasmanica* (O.t.), *Oidiodendron maius* (O.m.), *Phialocephala fortinii* (P.f.), *Rhizopogon luteolus* (R.l.), *Rhizopogon roseolus* (R.r.), *Russula densifolia* (R.d.), *Suillus bellinii* (S.b.), *Thelephora terrestris* (T.t), *Tomentella badia* (T.b.), *Tomentella ellisii* (T.e.), *Tomentellopsis submollis* (T.s.), uncultured *Amphinema* (u.A.), uncultured Ascomycotina (u.As.), uncultured *Atheliaceae* (u.At.), uncultured ECM fungus (u.ECM), uncultured *Lachnum* (u.L.), uncultured *Mycena* (u.M.), uncultured *Sebacina* (u.S.), uncultured *Thelephoraceae* 1(u.Th.1), uncultured *Thelephoraceae* 2 (u.Th.2), uncultured *Tomentella* 1 (u.T.1), uncultured *Tomentella* 2 (u.T.2). Predictors abbreviations are: ericaceous shrub cover (EriC), extent of erosion (ERO), slope (SLO), soil depth (SoilD), trunks and branches cover (TrBrC), broadleaved tree height (TreeH).

Species richness (mean ± SE, 2.67 ± 0.38,) and Shannon diversity index (0.75 ± 0.12) were negatively correlated with the distance from the unburnt pine patches (DisUB), ERO, and shrub height (ShrbH), and positively correlated with pine pre-fire basal area (PreBA; Table 3). To avoid an excess of redundant data, only significant correlation between variables that were considered to be important for the purpose of the study were shown (Table 4). Elevation (ELE) was negatively correlated with SoilD, ERO, and EriC. SoilD was positively correlated with DiUB, EriC, and shrub cover (ShrbC), and was negatively correlated with PreBA. PreBA was also negatively correlated with DiUB, ERO, ShrbC, and EriC. DiUB was positively correlated with ERO, ShrbC and ShrbH.

Environmental variables/ECM fungal community and regeneration variability

Several regression analyses (PLS-R) were carried out, including the data set of explanatory variables and the ECM fungal diversity indices, and different combinations of response variables both at plot (2 x 2 m^2) and subplot (10 x 10 m^2) level.

Table 3 Pearson and Spearman correlations between ectomycorrhizal fungal diversity indices and environmental variables recorded at the subplot (2 x 2 m^2) level: distance from unburnt pine patches (DiUB), extent of erosion (ERO), pine pre-fire basal areas (PreBA), shrub height (ShrbH).

	Species richness	Shannon diversity
DiUB	-0.556**	-0.601**
ERO	-0.466*	-0.476*
PreBA	0.601**	0.629**
ShrbH	-0.389*	-0.432*

Three PLS-R performed with data recorded in the 2 x 2 m^2 subplots provided significant components: a first regression (2 components, $p < 0.05$) between pine saplings height (SapHt) and a set of four environmental variables (RMSE = 0.09; R^2 = 0.62), a second (2 components, $p < 0.05$) between the density of saplings belonging to Class 2 and a set of seven environmental variables (RMSE = 0.17; R^2 = 0.57), and a third (2 components, $p < 0.05$) between total sapling density (TDen) and a set of six environmental variables (RMSE = 0.12; R^2 = 0.72). SapHt was positively correlated with ELE and negatively correlated with slope (SLO), EriC x SoilD, and bare ground cover (BarGC; Fig. 3a); Class 2 was positively correlated with ELE and DiUB, and negatively correlated with SLO, SoilD, EriC, BarGC, and leaf litter cover (LittC; Fig. 3b), and TDen was positively correlated with ELE and negatively correlated with SLO, SoilD, ShrbH, BarGC, and LittC (Fig. 3c).

No correlations were found between sapling performance and ECM fungal diversity indices (data not shown).

Table 4 Pearson and Spearman correlations between environmental variables at the subplot (2 x 2 m^2) level: elevation (ELE), soil depth (SoilD), distance from unburnt pine patches (DiUB), extent of erosion (ERO), pine pre-fire basal areas (PreBA), ericaceous shrub cover (EriC), shrub cover (ShrbC), shrub height (ShrbH).

	ELE	SoilD	DiUB	ERO	PreBA	EriC	ShrbC
SoilD	-0.604**						
DiUB	ns	0.581**					
ERO	-0.518**	ns	0.456*				
PreBA	ns	-0.400*	-0.780**	-0.725**			
EriC	-0.401*	0.622**	ns	ns	-0.423*		
ShrbC	ns	0.775**	0.649**	ns	-0.498**	0.700**	
ShrbH	ns	ns	0.408*	ns	ns	ns	-0.409*

Discussion

Five years after the fire event, the early successional ECM fungal community that characterized the study area was dominated by *Rhizopogon* spp., which occurred abundant on the root system of half of the naturally regenerated pine saplings. Members of the genus *Rhizopogon* are known to be well distributed and persistent in soil as resistant propagules (Baar *et al.* 1999; Taylor & Bruns 1999; Izzo

et al. 2006a,b; Kjøller & Bruns 2003; Buscardo *et al.* 2010) and are considered to be weak competitors often favored by fire competition release (Bruns *et al.* 2002; Kennedy *et al*. 2007). Almost one third of the ECM fungi colonizing pine saplings root system belonged to the *Thelephoraceae*, that showed a general scattered distribution, while a group of more typical late seral fungi (i.e. *Amanita pantherina, Boletus aestivalis*, *Lactarius chrysorrheus*, and *Russula densifolia*) were found exclusively in uneroded soils.

Pine regeneration in the study area was variable both in terms of density and height ranging from 1 to 14 individuals/m^2 with saplings belonging mainly to Class 2 (2.1 individuals/m^2), and mean values (4.27 individuals/m^2) not very different from the 5-years-old *Pinus halepensis* Mill. woodland in SE Spain (3.6 individuals/m^2; de las Heras *et al.* 2002). Pine regeneration was enhanced in the study areas at higher elevations, in correspondence with shallower soils, and a reduced cover of ericaceous shrubs and bare ground. These areas were in close proximity to patches of pine trees that survived the disturbance event and were previously characterized by a higher pre-fire pine biomass (measured as pre-fire basal area). In these circumstances pine trees may have provided a larger canopy seed bank as well as microclimatic conditions that improved seedling establishment.

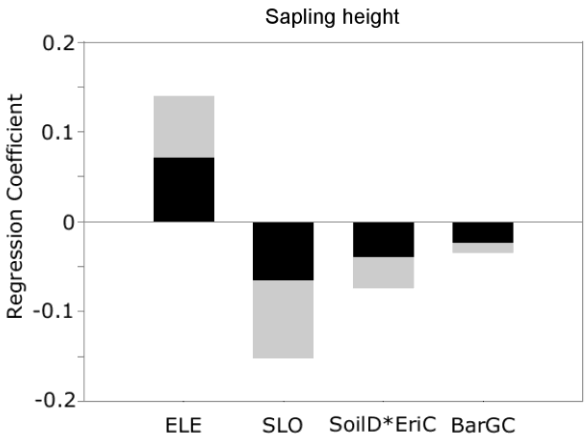

Figure 3a Significant predictor variables resulting from a partial least squares regression (PLS-R) analysis carried out with pine sapling height at the subplot level as response variable (two significant components, PC1, PC2). The weights of each predictor variable in the two PLS-R components are indicated in black (PC1) and grey (PC2). RMSE (root mean square error of prediction from full cross validation) = 0.09; R^2 = 0.62. Predictors abbreviations are: elevation (ELE), slope (SLO), soil depth (SoilD), ericaceus shrub cover (EriC), bare ground cover (BarGC).

Furthermore, newly regenerated seedlings might have benefitted from an existing ECM fungal network able to simultaneously colonize and interconnect them with mature pine trees.

Although k-selected ECM fungi were found on pine saplings in proximity to residual pine trees patches, they are known to colonize as well understorey species belonging to the genus *Cistus* (Comandini *et al.* 2006). These species are unable to compete with early seral fungi for colonization of new roots, unless if associated with other living roots (Fleming 1984), or until the size of vegetation patches containing plant hosts is about 5 m^2 (Nara *et al.* 2003). Although data on fire severity were not available for the study site it can be presumed that this was less pronounced in the proximity of residual pine trees.

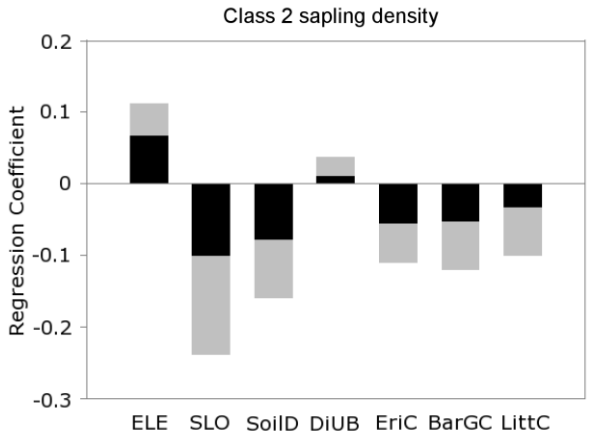

Figure 3b Significant predictor variables resulting from a partial least squares regression (PLS-R) analysis carried out with Class 2 sapling density as response variable (two significant components, PC1, PC2). The weights of each predictor variable in the two PLS-R components are indicated in black (PC1) and grey (PC2). RMSE (root mean square error of prediction from full cross validation) = 0.17; R^2 = 0.57. Predictors abbreviations are: elevation (ELE), slope (SLO), soil depth (SoilD), distance from unburnt pine patches (DiUB), ericaceus shrub cover (EriC), bare ground cover (BarGC), leaf litter cover (LittC).

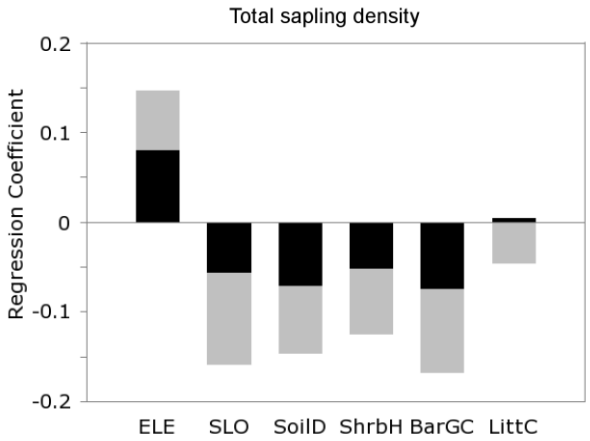

Figure 3c Significant predictor variables resulting from a partial least squares regression (PLS-R) analysis carried out with total sapling density as response variable (two significant components, PC1, PC2). The weights of each predictor variable in the two PLS-R components are indicated in black (PC1) and grey (PC2). RMSE (root mean square error of prediction from full cross validation) = 0.12; R^2 = 0.72. Predictors abbreviations are: elevation (ELE), slope (SLO), soil depth (SoilD), shrub height (ShrbH), bare ground cover (BarGC), leaf litter cover (LittC).

In these circumstances late seral ECM fungi could have survived on pine trees and/or shrubs providing an ECM fungal network to newly regenerated pine seedlings. The number of ECM fungi detected on maritime pine saplings in the present study was comparable to those found on naturally regenerated seedlings of other pine species (Visser 1995; Horton *et al*. 1998; Stendell *et al.* 1999; Grogan *et al.* 2000; Smith *et al*. 2004; Rincón & Pueyo 2010). The proximity to patches of pine trees that survived the fire event and a high pre-fire pine basal area appeared to promote both ECM fungal species richness and diversity, which were instead negatively affected by higher degrees of erosion and higher shrub height. Although it has been argued that the association with several ECM fungi should enhance the productivity of young seedlings (Jones *et al*. 1997), no correlations were found between saplings performance and ECM fungal diversity indices in this study. However, knowledge of diversity in ECM fungi functioning within communities is generally poor and more research is needed to better understand the effects of ECM fungal diversity over seedling regeneration (Jones *et al*. 2003).

The understorey of the study area is mainly dominated by a patchy distribution of heather and rockrose shrubs. In Mediterranean and Boreal forests, where *Ericaceae* and ECM trees occurs in mixed communities, ericoid mycorrhizal endophytes associate as well with ECM root tips (Bergero *et al.* 2000; Vrålstad *et al.* 2000).

The fungal roots inhabitant taxa found in this study on pine saplings are also common inhabitant of ericaceous shrub roots (Bougoure *et al.* 2007; Tedersoo *et al.* 2009), they form mostly non-specific associations with many plant hosts (Chambers *et al.* 2008), and their occurrence on ECM plants may have an important nutritional significance, either as true mycorrhizal fungi or as root-associated saprotrophic fungi (Bergero *et al.* 2000). However, there was a negative interaction between pine saplings (both in terms of height and density) and ericaceous shrubs. Shrubs can compete with naturally regenerated pine seedlings for light and moisture (O'Brian *et al.* 2007), and can affect as well their ECM symbionts. Conifer seedlings are often nutrient stressed and ECM fungal colonization may be reduced in sites that become dominated by ericoid mycorrhizal plants (Handley 1963). Walker *et al*. (1999) found that total ECM fungal colonization of *Tsuga canadensis* (L.) Carrière seedlings was reduced in thickets of *Rhododendron maximum* L., and that this decline was correlated with a decreased productivity of the seedlings. The tendency towards domination by ericoid mycorrhizal shrubs after fire could lead as well to the ericoid mycorrhizal invasion of previously ECM dominated fungal communities and consequently delay the succession processes. In the present study, shrubs performance and competition between shrubs and saplings were enhanced in areas characterized by a reduced pre-fire pine basal area, in correspondence with deeper soils. In these conditions the understorey was dominated by *Erica arborea*. This species is a typical post-fire resprouter characterized by populations that are relatively stable for long periods of time, with new seedlings recruitment and consequent population expansion favored by long fire return intervals (De Lillis 1995). Its release by fire from competition with pine for light and growing space (O'Brian *et al.* 2007), increased its capacity to spread out at expenses of newly regenerated pine seedlings in low density pre-fire pine areas. Analogous results were found in another Mediterranean ecosystems where the dominance of *E. arborea* reduced dramatically the survival of *Quercus ilex* seedlings during the summer following germination (Richard *et al.* 2009), and the high percentages of shrub cover decreased the probability of *Pinus radiata* D. Don seedling presence (O'Brian *et al.* 2007).

More than 30% of the ECM fungal species found on pine saplings in the present study are known to colonize different species of the genus *Cistus* (reviewed by Comandini *et al.* 2006). However, the potential bridge mediated by *Cistus* spp. between pre-fire ECM fungal communities and emerging seedlings, defined by Perry *et al*. (1989) as bootstrapping, could have been masked in the present study by *E. arborea*. The large presence of *E. arborea* in some areas together with an increased extent of erosion, and soil depth seem to be the main factors responsible for shaping the ECM fungal distribution, and for describing with elevation and slope, most of the explained regeneration variability.

Common environmental factors (i.e. ericaceous shrub cover, extent of erosion, slope and soil depth) were responsible for shaping the ECM fungal distribution and for describing most of the explained regeneration variability. The proportion of variability that remains unexplained could be due to an inadequate sampling effort or the lack of information on other abiotic factors, such as fire

characteristics and soil properties. Nevertheless, the results presented in this study are relevant to improve post-fire restoration operations by allowing the identification of areas where poor pine regeneration can be expected. Management to promote maritime pine in declining natural stands should focus on reducing the cover of *E. arborea* and providing seed sources in areas with lower pre-fire pine biomass.

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CHAPTER 2

Long-term effects of wildfire frequency on the soil fungal community of a pine forest assessed by 454 pyrosequencing and by DGGE

Introduction

In contrast to most prescribed fires, which promote renovation of the dominant vegetation through the elimination of undesired species and a transient increase of pH and available nutrients, stand-replacing wildfires have several negative effects on the soil (Certini 2005). Organic matter is removed, soil structure and porosity deteriorate, nutrients are lost through volatilization, leaching and erosion, and the structure of the soil microbial community is altered (Neary *et al.* 1999).

Mediterranean ecosystems tend to react to wildfires with a typical autosuccession response in which pre-disturbance plant species occur amongst the early post-fire vegetation (Hanes 1971). However, an increase of fire frequency may hinder the regeneration of particular plant species, especially those without resprouting mechanisms and irregular germination, and alter the functioning of the ecosystem (Rodrigo *et al.* 2004). This may lead to the regression of vegetation and a reduction of the recovery success rate, even in absence of further disturbance events, as became apparent in the last decades by the progressive replacement of Mediterranean forests by shrub-dominated communities (Pignatti 1995).

Because of a strong physiological link, some fungal functional groups (e.g. ectomycorrhizal (ECM) fungi) follow the distribution of their host plants and their succession patterns integrate with the floristic succession of forested stands (Twieg *et al.* 2007). Moreover, as most plant communities are dominated by species that are considered "ecologically obligate" ECM symbionts (Smith & Read 1997), the availability of ECM inoculum, in particular spore bank and other resistant propagules, is important for tree seedling establishment and thus for the recovery of the ecosystem from wildfires (Taylor & Bruns 1999).

Multiple studies have documented the impact of single wildfire events or repeated prescribed burning on fungal communities, in particular on plant fungal symbionts (Visser 1995; Horton *et al.* 1998; Grogan *et al.* 2000; Bastias *et al*. 2006; Rincón & Pueyo 2010). However, no comparisons have been made between stands with different wildfire history. Because recurrent wildfires are common in Mediterranean ecosystems and information about the impact of recurrent wildfires on soil fungal communities is lacking, this study aimed at characterizing the influence of wildfire frequency (measured as fire return interval length) on the structure and composition of the post-fire soil fungal communities in areas where the vegetation has changed from a mature open maritime pine forest to a transitional phase with shrubs and young pine trees (stand with long fire return interval) or to shrublands (stands with short fire return intervals).

Material and methods

Study sites

The study area is situated in Central Portugal between Alvito da Beira (39º48' N, 7º49' W, altitude 500-600 m) and Isna de Oleiros (39º51' N, 7º51' W, altitude 750-850 m), a region characterized by a Mediterranean climate, with hot dry summers and cool wet winters, a lithosol soil (a stony soil lacking horizon and structure development) and a plant community dominated by *Pinus pinaster* Ait.

For the present study four sampling stands on three replicated sites at least 300 m apart were selected. The four stands were characterized by different wildfire histories that represent the current fire return interval lengths in this region (Pereira *et al*. 2006). Unburnt pine forest (UB) was not affected by wildfire in the last 40 years, the long fire return interval areas (B) has an average fire return interval of approximately 24 years with the last wildfire event in 2003, and the short fire return interval areas (B1 and B2) have an average of fire return of 7.6 years and were affected by fire in 1992 and 2003. While long fire return interval length allowed the typical autosuccession response of Mediterranean vegetation ecosystems, short fire return interval length hindered pine regeneration.

UB was characterized by a 40 years old open uneven-aged maritime pine forest (dominant trees with trunk diameters of 40-45 cm) with an understory shrub community dominated by *Erica* spp., *Halimium* spp. and *Pterospartum tridentatum* (L.) Wk. & Lge. and a soil with an incipient organic layer. B showed vigorous natural pine regeneration with the same shrubby species present in UB. B1 and B2 were located approximately 5 km from UB/B and were characterized by an open uneven-aged pine forest with a shrubby understory before 1992 but the repeated fires have transformed the vegetation to a shrubland dominated by either *Cistus ladanifer* L. (B1) or *P. tridentatum*, *Erica* spp. and *Halimium* spp. (B2).

Soil sampling and DNA extraction

In June 2009, nine independent soil samples were collected, after removing litter, at each site at a depth of approximately 20 cm using a shovel. Soil samples were subsequently pooled per site, homogenized and cleared from debris using a 2 mm sieve. Two 50 mL subsamples for each site were stored at 4 °C for DNA extraction. Soils were analysed for organic matter (OM), pH (H_2O), P_2O_5, K_2O, Ca^{2+}, Mg^{2+} and total N at the soil laboratory of the Coimbra Higher School of Agriculture.

Three independent DNA extractions were carried out for each of the 24 soil subsamples using 0.4 g of pooled soil and the UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's instructions with an extra incubation step at 70 °C for 10 minutes after addition of the Inhibitor Removal Solution and subsequent vortexing for 5 minutes.

Pyrosequencing

Amplicon libraries for pyrosequencing were prepared by polymerase chain reaction (PCR) amplification of the variable region of the fungal internal transcribed spacer 1 (ITS1) with the fungus-

specific oligonucleotide primer ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3'; Gardes & 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'-GCCTCCCTCGCGCCATCAG-3') extended with an 8-bp error-correcting barcode sequence (Hamady *et al.* 2008), and with a Roche 454 B sequencing adapter (5'-GCCTTGCCAGCCCGCTCAG- 3'; see Table S1). PCR amplification of each of the 72 samples was performed in 50 µL reactions, containing 10 ng template DNA, 200 nM of each of the forward and the reverse primers, 200 µM 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 and purified with the High Pure PCR Product Purification Kit (Roche) and 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.

Denaturing gradient gel electrophoresis

Fungal DNA regions, 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 samples in 25 µL reactions, containing 1 µL template DNA, 400 nM of each of the forward and the reverse primers, 200 µM of each dNTP, 0.5 U DFS-Taq DNA polymerase (Bioron) and 100 µg 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'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3'; Muyzer *et al.* 1993) at the 5'-end to prevent complete denaturing, 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 15 µL 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 site, 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 region with the primer combination ITS1F and ITS2 in 9 known ECM species found previously in the study area (Buscardo *et al.* 2010). DGGE was performed in 1× TAE buffer at 20 V for 15 min, followed by 16 h at 70 V and 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).

Data analysis

Pyrosequencing reads were quality trimmed and subsequently filtered by removing reads with remaining unresolved nucleotides, reads without a valid primer sequence or DNA tag and reads with partial ITS1 sequences shorter than 100 bp. The sequences that met these quality control requirements were assigned to samples based on their 8-bp barcode (see Table S1) and grouped into operational taxonomic units (OTUs) based on ITS1 sequence similarity using CD-hit v4.0 (Li & 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), once independently for each of the sets of sequences from the twelve different sites and once for the total set of sequences obtained from the four stands.

OTU frequencies (p_i) in each of the twelve sites were calculated as the weighted averages of the two OTU frequencies in the corresponding amplicon libraries generated separately from the same pooled soil sample and reproducibility of the pyrosequencing approach was assessed by pairwise comparison of the OTU frequencies in the replicate amplicon libraries. The overall OTU richness (S) was calculated for each site as the number of different OTUs found across the replicate amplicon libraries. In addition, the extrapolative richness estimator Chao1 (Chao *et al.* 2005), Shannon's diversity ($H' = -\sum p_i \ln(p_i)$) and rarefaction curves were generated for each site considering exclusively non-single tons data using the vegan v1.17-3 package in R v2.11.1 (R Development Core Team 2007). Averages of the different diversity and richness indices were calculated for each stand and significance of the differences in diversity or richness across the sampling stands was assessed by one-way analysis of variance (ANOVA).

454 pyrosequencing data sets read abundance can be biased between species comparisons by innate sequence structure and limit therefore the utility of such comparison (Amend *et al.* 2010). For this reason read abundance comparisons were complemented by a correspondence analysis performed with data on presence/absence of the non singleton OTUs. The OTUs were identified in the total set of sequences using the dudi.coa routine of the ade4 v1.4-14 package in R v2.11.1 in order to visualize similarities in fungal species composition among the twelve sampled sites. The OTUs were taxonomically identified by BLASTn comparison (Altschul *et al.* 1997) of their longest representative sequence with all sequences in the non-redundant GenBank database using the BLAST Network Service (NCBI, National Center for Biotechnology Service; http://www.ncbi.nlm.nih.gov/BLAST),

and assignment at the genus level was made in case of ≥ 90 % sequence similarity with ≥ 80 % coverage and at the species level in case of ≥ 97 % sequence similarity with ≥ 90 % coverage (Hughes *et al.* 2009). 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, followed by the Bonferroni-Holm's procedure for controlling experiment wise error rates for multiple independent tests (Holm, 1979).

Digital images of DGGE gels were analyzed with GelCompar II v4.0 (Applied Maths) and subsequent statistical analyses were performed with SPSS v17.0 (SPSS Inc.) and CANOCO v4.5 (Microcomputer Power). Species richness (*S*) was calculated as the total number of bands in a sample and Shannon's diversity (*H'*) was calculated by assigning each band in a sample to one of five possible intensity classes. The potential effects of different fire return interval lengths on species diversity were tested using one-way ANOVA, followed by Tukey's multiple comparison tests, and correspondence analysis using band presence/absence data was performed to visualize the fungal species composition of the different stands.

DNA sequences from excised DGGE bands were manually checked and, if necessary, edited using BioEdit v7.0.9.0 (Hall 1999). Sequences were then compared with the non-redundant GenBank database using the BLAST Network Service, with a 97 % sequence similarity threshold for assignment of OTUs at the species level.

Differences in soil properties between the stands were tested with one-way ANOVA and Tukey's multiple comparison tests in R v2.11.1.

Results

Soil properties

Amounts of organic matter, Ca^{2+} and Mg^{2+} differed among soils from the stands subjected to fire (B, B1 and B2) and soil from the control stand not subjected to fire (UB), with significantly (*P*-value < 0.05) lower values in the stands subjected to fire (Table 1). Total N was higher in soils obtained from stands UB and B compared to soils from both stands with short fire return intervals (B1 and B2), but significant (*P*-value < 0.05) differences were only observed between the control stand UB and B1/B2.

Pyrosequencing

A total of 97,900 partial ITS1 sequences, varying in length between 101 and 438 bp (average = 259 bp), were retained after quality control of the pyrosequencing reads and the number of retained sequences per amplicon library varied between 2373 and 5036 (average = 4047).

Combining the replicate amplicon libraries per site and grouping the sequences based on similarity using a threshold of 97 % revealed between 279 and 568 OTUs (average = 373), including singletons, and between 212 and 386 non-singleton OTUs (average = 266) while Shannon's diversity varied

between 3.20 and 4.62 per site (average = 4.04). Relatively high (average Pearson correlation coefficient = 0.89) and significant (*P*-value < 0.05) levels of correlation between the OTU frequencies in replicate amplicon libraries were thereby found, indicating good reproducibility of the pyrosequencing approach. The rarefaction curves show that the number of OTUs increased with the number of sequences without reaching a plateau for most sites (Fig. 1) and the Chao1 extrapolative richness estimator predicted maximum numbers of non-singletons OTUs, defined at 97 % sequence similarity, per site ranging from 348 to 766 (average = 490). As can be seen from Table 2, which provides an overview of the different diversity and richness indices for each of the four sampling stands included in this study, a significant difference (*P*-value < 0.05) among the four sampling stands was only found for Shannon's diversity, and multiple comparison testing showed that this was due to a significantly (*P*-value < 0.05) lower diversity in stand B compared to stands UB and B2.

Table 1 Soil properties in the four stands subjected to different fire 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*.Values are averaged data (n = 3) with standard deviation, SD, within parentheses. Letters in superscript indicate statistical differences between stands as determined by Tukey HSD (P < 0.05).

Stand	OM (SD)	pH (SD)	P_2O_5 (SD)	K_2O (SD)	Ca^{2+} (SD)	Mg^{2+} (SD)	N (SD)
UB	18.85 (4.84)^{a}	4.2 (0.17)^{a}	28.33 (5.51)	105.67 (13.01)	1.66 (0.34)^{a}	0.95 (0.28)^{a}	0.4 (0.03)^{a}
B	8.86 (1.22)^{b}	4.4 (0.10)^{ab}	25.67 (10.07)	73.67 (7.02)	0.38 (0.14)^{b}	0.31 (0.04)^{b}	0.3 (0.04)^{ab}
B1	5.82 (0.85)^{b}	4.43 (0.06)^{ab}	16 (1.73)	92.33 (25.5)	0.58 (0.12)^{b}	0.29 (0.02)^{b}	0.21 (0.03)^{b}
B2	7.96 (2.22)^{b}	4.57 (0.06)^{b}	16.67 (4.04)	100 (30)	0.43 (0.08)^{b}	0.25 (0.02)^{b}	0.24 (0.06)^{b}

A total of 2365 OTUs, including 670 singletons (28 %), were identified by clustering the sequences at 97 % similarity across the sampling sites (longest read sequence of each OTU is available at GenBank, accession numbers HQ124333 - HQ126697).

The twelve sampled sites clustered more or less together according to their stand of origin in a correspondence analysis based on the site presence/absence of 1695 non-singletons OTUs: the first axis in the graphical representation of the correspondence analysis results clearly separates the sites of the control stand (UB) from the sites of the stands subjected to fire (B, B1 and B2) while the second axis distinguishes the sites of the stands subjected to fire according to their respective stands of origin (Fig. 2). Of the 300 most abundant OTUs, which represent 85 % of the total number of sequence reads, 283 (94 %) gave significant hits in a BLASTn analysis using the longest read sequence of each

OTU as query sequences and phylum or lower level assignment was possible for 226 OTUs (75 %; Table S2). One hundred and ten OTUs (37 % of OTUs; 35 % of the sequence reads) were assigned to the Ascomycota, 106 (35 % of OTUs; 45 % of the sequence reads) to the Basidiomycota, 7 (2 % of OTUs; 3 % of the sequence reads) to the Zygomycota and 3 (1 % of OTUs; 0.5 % of the sequence reads) to the Chytridiomycota.

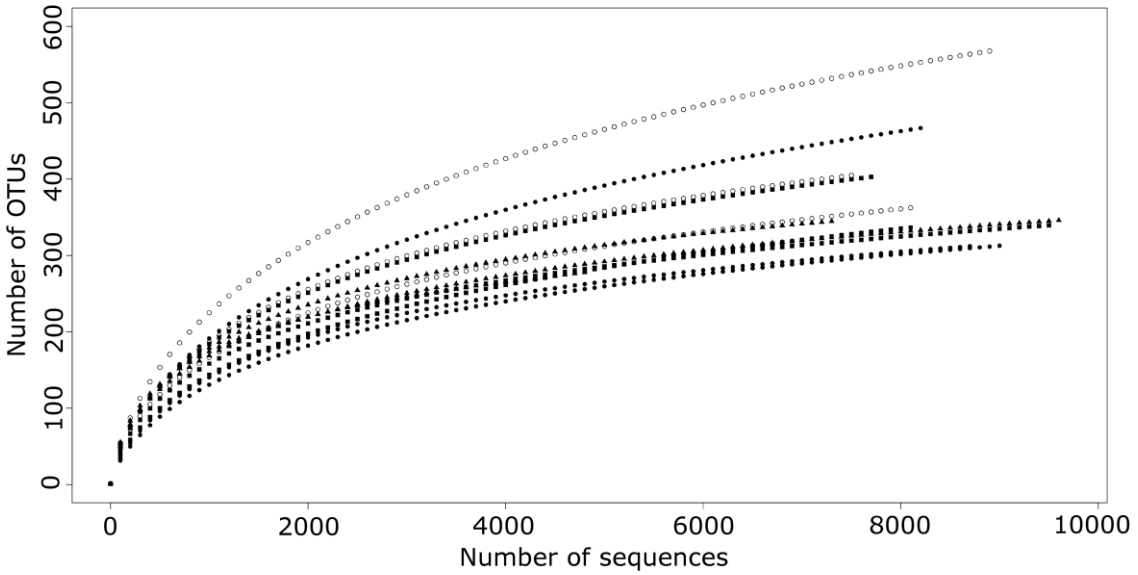

Figure 1 Rarefaction curves for each of the 12 plots included in this study, located in four sites subjected to different fire return intervals (UB, unburnt site; B, long fire return interval site; B1, short fire return interval site dominated by *Cistus ladanifer*; B2, short fire return interval site dominated by *Erica* spp., *Halimium* spp. and *Pterospartum tridentatum*), illustrating the dependence of the number of operational taxonomic units (OTUs) assigned at 97 % sequence similarity on the number of internal transcribed spacer 1 (ITS1) sequences obtained by pyrosequencing of ITS 1 amplicon libraries generated from soil-extracted DNA; sites: ○, UB; ●, B; ■, B1; ▲, B2.

Table 2 Overview of fungal diversity and richness estimators for four stands subjected to different fire return intervals, based on 454 pyrosequencing results. 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 non-parametric Kruskal-Wallis tests were performed in order to assess differences in diversity and/or richness across the four stands; S, species richness; H', Shannon's diversity; SD, standard deviation.

	S			
Site	Total (SD)	Non-singleton (SD)	Chao1 (SD)	*H*' (SD)
UB	445 (108)	309 (68)	597 (147)	4.35 (0.24)
B	364 (89)	251 (49)	501 (148)	3.51 (0.52)
B1	360 (37)	254 (29)	472 (49)	3.88 (0.22)
B2	323 (38)	248 (31)	390 (37)	4.43 (0.08)
$F_{3,8}$ (*P*-value)	1.412 (0.309)	1.171 (0.380)	1.845 (0.217)	5.849 (0.021)

The identified OTUs were distributed across 24 fungal orders, the most frequently encountered being the Agaricales (33 OTUs; 19.1 % of the sequence reads), the Thelephorales (12 OTUs; 9.9 % of the sequence reads) and the Russulales (14 OTUs, 6.0 % of the sequence reads), and 34 families, the most common being the *Thelephoraceae* (12 OTUs; 9.9 % of the sequence reads), the *Cortinariaceae* (12 OTUs; 8.3 % of the sequence reads) and the *Bolbitiaceae* (9 OTUs; 7.5 % of the sequence reads). Genus and species level assignments were possible for respectively 126 (42 %) and 42 (14 %) OTUs, with the most commonly encountered genera being *Thelephora* (9 OTUs; 9.5 % of the sequence reads), *Hebeloma* (9 OTUs; 7.5 % of the sequence reads) and *Cortinarius* (5 OTUs; 6.9 % of the sequence reads), and the most common species being *Thelephora terrestris* (7.9 % of the sequence reads), *Hebeloma cistophilum* (3.3 % of the sequence reads) and *Russula drimeia* (1.4 % of the sequence reads).

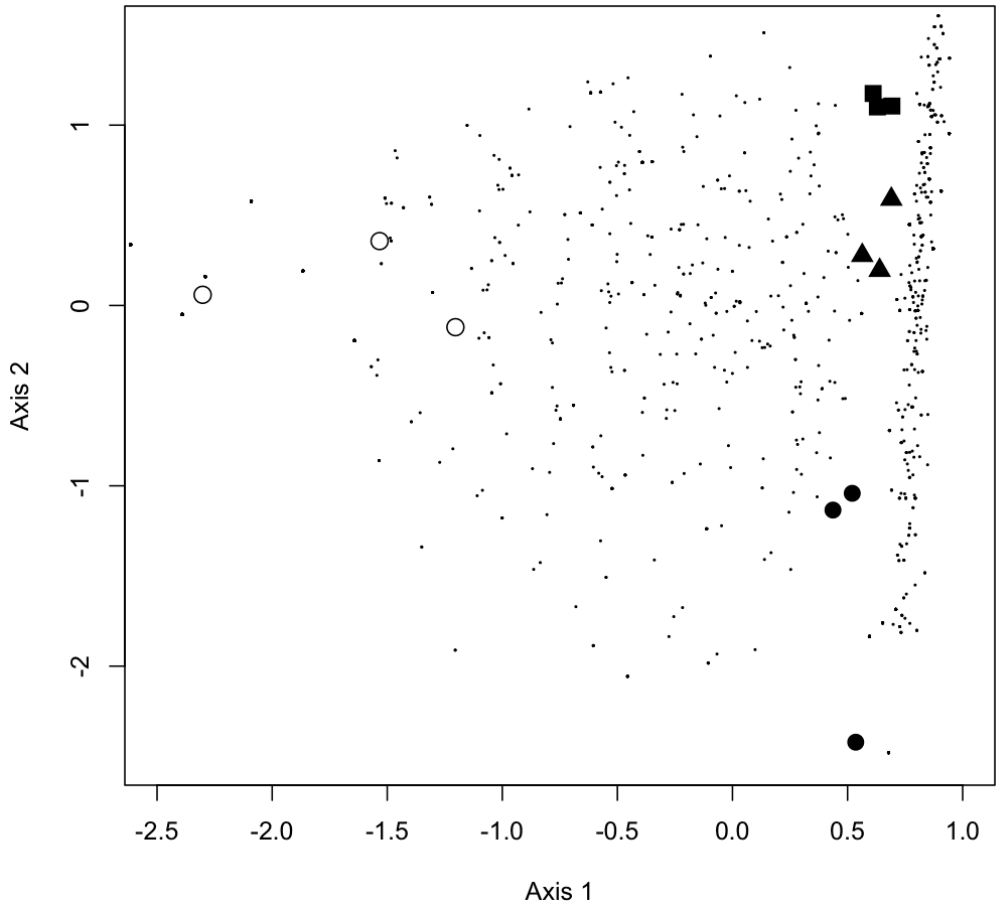

Figure 2 Results of a correspondence analysis based on non-singleton fungal operational taxonomic unit (OTU, assigned at 97 % sequence similarity) frequency data in twelve sampled plots, located in four sites subjected to different fire return intervals (UB, unburnt site; B, long fire return interval site; B1, short fire return interval site dominated by *Cistus ladanifer*; B2, short fire return interval site dominated by *Erica* spp., *Halimium* spp. and *Pterospartum tridentatum*), using the dudi.coa routine of the ade4 v1.4-14 package in R v2.11.1; sites: ○, UB; ■, B; ●, B1; ▲, B2; •, OTU.

One-way ANOVA indicated 6 families, 5 genera and 3 species to be distributed significantly different (*P*-value < 0.05) across the four sampling stands (Table S2): at the family level, *Monoblepharidaceae* and *Atheliaceae* occurred at higher frequency in the control stand compared to the stands subjected to fire, while *Cucurbitariaceae*, *Helotiaceae*, *Cortinariaceae* and *Rhizopogonaceae* were more frequent in at least one of the stands subjected to fire. At the genus level, *Cladosporium*, *Rhizoscyphus*, *Cortinarius*, *Gymnopilus* and *Rhizopogon* occurred at significantly higher frequencies in at least one of the stands subjected to fire compared to the control stand. At the species level, *Cladophialophora minutissima* and *Rhizoscyphus ericae*, occurred at higher frequencies in at least one of the stands subjected to fire, while *Amanita spissa* occurred more frequently in the control stand.

Denaturing gradient gel electrophoresis

DGGE analysis generated complex banding patterns for all sites in the four sampling stands (Figures S1, S2 and S3), with the number of bands per site varying between 29 and 32 and a total of 56 different bands across all sites. Banding patterns were found to be highly reproducible between independent DGGE runs (on average, 90 % of the bands were found to be reproducible). Clearly different banding patterns were obtained for the four sampling stands, with 26 bands (46 %) occurring in all four stands and 7 bands (13 %) occurring exclusively in a single stand. Species richness varied between 29 and 32 per stand (average of 30), while Shannon's diversity varied between 3.52 and 3.79 (average of 3.62), and no significant differences in diversity were found across the four stands (Table 3). Correspondence analysis based on band presence/absence data revealed clustering of the different sites according to their stand of origin (Fig. 3).

Table 3 Overview of fungal diversity and richness estimators for four stands subjected to different fire return intervals, based on DGGE results. 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*. Differences in diversity and/or richness across the four stands were tested using one-way ANOVA; S, species richness; H', Shannon's diversity; SD, standard deviation.

	S (SD)	*H*' (SD)
UB	31(5)	3.79(0.16)
B	29(2)	3.52(0.20)
B1	29(3)	3.61(0.61)
B2	32(5)	3.56(0.45)
F (*P*-value)	2.053(0.139)	1.329(0.293)

After band excision and reamplification, sequences were obtained for 34 different bands and 26 of these (76 %) gave significant hits in a BLASTn analysis (Table S3). DNA sequences of bands at the same position in the DGGE profiles of different stands showed highest homologies to sequences of the same fungal species, but in several cases bands at different positions in the DGGE profile were found to be derived from the same fungal species. Fungal species composition was found to be different in the four stands, with the number of species occurring exclusively in a single stand varying between 0 and 5 (average of 2).

Five species occurred only in the unburnt stand and four were exclusive to the burnt stands. Some species were ubiquitous, but their abundances differed between stands. For instance, *Russula drimeia* and *Lactarius* sp. were more abundant in the unburnt stand compared to the burnt stands, while *Thelephora terrestris*, *Cortinarius* sp., *Cenococcum geophilum* and *Hebeloma cistophilum* were more abundant in at least one of the three burnt stands (see Table S3).

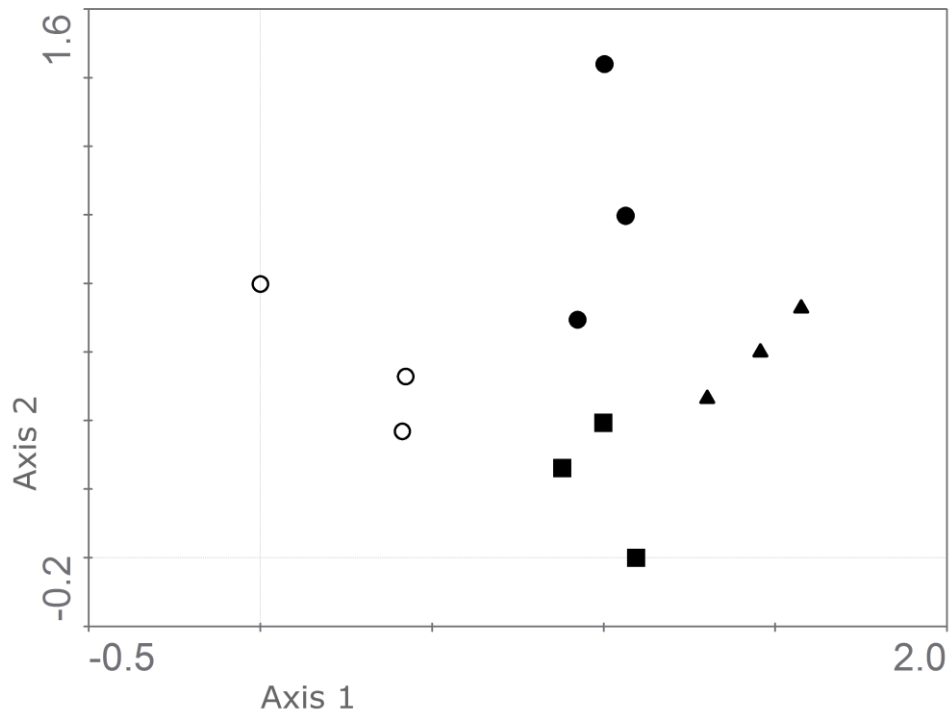

Figure 3 Results of a correspondence analysis based on DGGE bands presence/absence data in twelve sampled plots, located in four sites subjected to different fire return intervals (UB, unburnt site; B, long fire return interval site; B1, short fire return interval site dominated by *Cistus ladanifer*; B2, short fire return interval site dominated by *Erica* spp., *Halimium* spp. and *Pterospartum tridentatum*), using CANOCO; sites: ○, UB; ■, B; ●, B1; ▲, B2.

Discussion

In this study, the long-term effects of wildfire and of fire return interval length on the soil fungal diversity of an open maritime pine forest were evaluated using two methods for the assessment of microbial diversity: DGGE, an established PCR-based method, and 454 pyrosequencing, a high-throughput next-generation sequencing technology. Soil samples were collected from four different stands located in a region where the plant community is dominated by *Pinus pinaster*: an unburnt control stand, a stand with long wildfire return intervals and two stands with short wildfire return intervals. Natural pine regeneration was prevented by the short fire return interval, and the inclusion of soil samples from two stands subjected to high fire frequencies with different post-fire shrub covers allowed an evaluation of the influence of vegetation composition on the post-fire soil fungal diversity.

Soil nutrient composition of the burned stands was in line with previous reports, with reduced organic C and N and inorganic ions compared to the unburnt stand (see Knicker 2007 for a review).

The effects of wildfire disturbance on the belowground microbial community and the resulting effects on ecosystem sustainability are complex. Soil fungal communities can be killed directly by soil heating, but can also be affected by fire induced changes on physical and chemical soil properties and on the above-ground vegetation (Neary *et al.* 1999; Certini 2005). Vegetation dynamics in Mediterranean ecosystems are largely determined by fire regime (Trabaud 1994), variations in which can lead to a complete loss of vegetation cover by depleting resprouter bud banks and seeder seed banks (Zedler *et al.* 1983) or to moderate changes in the relative abundance of species with different life history traits (Lloret *et al.* 2003). While the effects of different fire regimes on the above-ground vegetation structure are well-characterized, studies of the effects of fire on the forest soil fungal community have mainly focused on single disturbance events and often involve prescribed fires and/or specific functional groups (Tuininga & Dighton 2004; Hart *et al.* 2005; Bastias *et al*. 2006; Anderson *et al*. 2007). This study represents the first attempt to investigate the effects of different wildfire regimes on the whole soil fungal community.

High levels of fungal species richness were detected in the four study stands, with 454 pyrosequencing-based estimates about ten-fold higher than DGGE-based estimates after exclusion of non-singletons OTUs (Dickie 2010; Kunin *et al.* 2010; Medinger *et al.* 2010; Tedersoo *et al.* 2010) and comparable to those reported for oak and beech plantations (Buée *et al.* 2009). Species richness appeared unaffected by wildfire occurrence or regime. Recovery from possible initial reductions of species richness, which are common after stand replacing fires (Certini 2005), was likely in the disturbed stands due to the relatively long time since the last wildfires, which allowed extensive (re)colonization by fungal species. However, fire regime had a significant effect on the structure of the soil fungal communities. Ordination analysis using 454 pyrosequencing-based OTUs presence/absence clearly separated the control stand and stands subjected to wildfire along the first axis and disturbed stands according to wildfire regime along the second axis. The soil fungal community structure of stand B2 appeared more or less intermediate between stand B, which was characterized by the same post-fire shrub cover, and stand B1, which was characterized by the same wildfire return interval length. Thus, an effect of both fire regime and of post-fire vegetation on the soil fungal community was observed. Differences in fungal community structures between unburnt and burnt stands were also observed analyzing DGGE banding patterns but further differences among stands B, B1 and B2 were less pronounced.

The abundance of Basidiomycota *versus* Ascomycota varied considerably between the control and the disturbed stands. In the stands characterized by the same shrub cover (UB, B and B2), wildfire disturbance induced a shift in the soil fungal community towards the Ascomycota with an abundance ratio Basidiomycota/Ascomycota three times higher in the unburnt stand UB compared to stands B and B2. Short-lived shifts in dominance from basidiomycetous to ascomycetous fungi following fire

have been described in ECM fungal communities (Visser 1995; Torres & Honrubia 1997; Baar *et al.* 1999; Grogan *et al*. 2000) and are believed to be due to changes in soil environment and shifts in the amount or type of inoculum. Visser (1995) reported that the initial strong representation of ascomycetes within post-fire ECM communities begins to disperse within six years after a wildfire occurred. In our study areas, where the same amount of time passed since the last disturbance event, a pronounced shift towards ascomycetous fungi was still found in two of the burned stands (B and B2) and this was mainly attributable to non-ECM fungi. The short fire return interval stand B1, characterized by a shrub cover dominated by *Cistus* spp. had a ratio Basidiomycota/Ascomycota more similar to the stand UB and comparable to the results obtained in undisturbed temperate forest soils (O'Brian *et al.* 2005; Buée *et al.* 2009), with three ECM genera (i.e. *Cortinarius*, *Hebeloma* and *Thelephora*) representing more than 50 % of the OTUs abundance. In a study conducted in the same areas, it was observed that *Hebeloma* sp. and *Thelephora* sp. dominated as well the ECM root tips of the dominant shrubs in stand B1 (Buscardo *et al*. unpublished). These results indicate long-term effects of wildfire on the soil fungal community through changes in the vegetation cover, unrelated to alterations of soil properties. Although our knowledge of *Cistus* spp. ECM communities is limited (Comandini *et al.* 2006), the similarities between the ECM fungi found in B1 and the fruit-bodies survey of an unburnt *C. ladanifer* shrubland in north-western Spain (Martín-Pinto *et al.* 2006) suggest a fast post disturbance succession in B1 towards a mature rockrose fungal community that could be attributable to the shorter lifespan of these shrubs compared to pines (Roy & Sonie 1992; de las Heras *et al.* 2002).

ECM fungal species represented the most abundant functional ecological group in all stands, with approximately 80 % of the sequence reads assigned at the genus level in UB and B, 50 % in B2 and 90 % in B1. Scarcity of saprotrophic species was expected in the burnt soils where the decrease of the C:N ratio could reduce their competitiveness and favour their replacement by mycorrhizal fungi that do not depend on litter-derived energy (Lindahl *et al.* 2007) and are less affected by environmental stress (Villeneuve *et al.* 1989). However, more saprotrophic fungi that develop on thick litter and humus were expected in the unburnt forest floor in UB. Different factors could have changed this outcome, such as a seasonal effect which leads to an increase in abundance of ECM fungi during the growing season (Jumpponen *et al.* 2010) or an underrepresentation of decaying fungi in environmental studies and public sequence databases (Ryberg *et al.* 2009).

The unburnt pine forest was dominated by fungal genera that are favoured by the presence of litter and humus, such as *Russula*, *Lactarius* and *Tylospora* (Gardes & Bruns 1996; Horton & Bruns 1998; Stendell *et al.* 1999; Taylor & Bruns 1999) while the genera *Hebeloma* and *Thelephora*, which group several pioneer species that benefit from the release of competition induced by fire (Baar *et al.* 1999; Smith *et al*. 2004; Buscardo *et al.* 2010), dominated in the stands subjected to fire. Comparable results were obtained in a study conducted in conjunction with the present one examining the ECM root tips of maritime pine and understorey shrub species (Buscardo *et al*. unpublished).

The post-fire vegetation cover can have a significant effect on the soil fungal communities, specifically on the mycorrhizal fungal composition which is directly influenced by factors such as host specificity in the recruitment of symbiotic partners (Anderson *et al*. 2003). This is probably reflected by the high abundance of the genus *Cortinarius* in stand B1, several species of which are *Cistus*-specific (Comandini *et al.* 2006), and the relatively high abundance of *Helotiaceae* in stand B2, especially species belonging to the *Rizoscyphus ericae* aggregate which frequently form ericoid mycorrhizas (Vrålstad *et al.* 2002).

Using both DGGE and 454 pyrosequencing-based profiling of the soil fungal community, illustrated the technical superiority of the second generation sequencing technology. The 454 pyrosequencing-based approach revealed much higher levels of species richness and allowed a more extensive characterization of the soil fungal community structures, providing clear evidence of the effects of wildfire on the soil fungal community structure. Analogies between the results obtained from the comparisons with 454 pyrosequencing read abundance data sets and those obtained in other two studies examining the ECM resistant propagules community and the ECM root tips fungal community in the study areas (Buscardo *et al.* 2010; Buscardo *et al*. unpublished) revealed that read abundance data may enclose valuable information for the understanding of soil fungal communities. Ammend *et al*. (2010) demonstrated that 454 pyrosequencing read abundance can vary among species that were known to be present in equal quantity of spores and they suggested that quantitative biases may originate from spore DNA extraction efficiency. By comparing the the structures of ECM resistant propagules community (Buscardo *et al.* 2010) and the ECM root tips fungal community (Buscardo *et al*. unpublished) of the study area is reasonable to believe that soil inoculum in the current study was mainly dominated by fungal hyphae which presence could have reduced the above mentioned biases.

PCR-based methods, such as DGGE, often fail to detect the less abundant members of the community (Anderson & Cairney 2004) and obtaining DNA sequence information often requires repeated rounds of excision and PCR reamplification. However, DGGE still provides a means for a rapid visual identification of shifts in the structure of the soil fungal community.

To conclude, both methods illustrate the long-term effects of wildfire and wildfire return interval length on the soil fungal community structure. While species richness appeared not to be affected by fire disturbance, the community structure was clearly affected by the different fire regimes with shifts in the fungal species composition that indicate the importance of direct and indirect effects – through alterations of the soil properties and of the post-fire vegetation structure – of fire.

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Supplementary information

Table S1 Overview of modified ITS1F and ITS2 oligonucleotide primers used to prepare amplicon libraries for 454 pyrosequencing by PCR amplification of the variable region of fungal ITS1 in DNA samples extracted from soil subjected to different fire frequency regimes.

Soil DNA sample	Modified ITS1F oligonucleotide primer (5' -> 3') Fusion product of *Roche pyrosequencing primer A*, **8-bp barcode**, CA-linker and ITS1F primer	Modified ITS2 oligonucleotide primer (5' -> 3') Fusion product of *Roche pyrosequencing primer B*, TC-linker and ITS2 primer
UB-1a	*GCCTCCCTCGCGCCATCAG*-**AACCAACC**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
UB-1b	*GCCTCCCTCGCGCCATCAG*-**AACCAAGG**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
UB-2a	*GCCTCCCTCGCGCCATCAG*-**AACCATCG**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
UB-2b	*GCCTCCCTCGCGCCATCAG*-**AACCATGC**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
UB-3a	*GCCTCCCTCGCGCCATCAG*-**AACCGCAT**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
UB-3b	*GCCTCCCTCGCGCCATCAG*-**AACCGCTA**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B-1a	*GCCTCCCTCGCGCCATCAG*-**AACCGGAA**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B-1b	*GCCTCCCTCGCGCCATCAG*-**AACCGGTT**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B-2a	*GCCTCCCTCGCGCCATCAG*-**AACCTACG**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B-2b	*GCCTCCCTCGCGCCATCAG*-**AACCTAGC**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B-3a	*GCCTCCCTCGCGCCATCAG*-**AACCTTCC**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B-3b	*GCCTCCCTCGCGCCATCAG*-**AACCTTGG**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B1-1a	*GCCTCCCTCGCGCCATCAG*-**AACGAACG**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B1-1b	*GCCTCCCTCGCGCCATCAG*-**AACGAAGC**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B1-2a	*GCCTCCCTCGCGCCATCAG*-**AACGATCC**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B1-2b	*GCCTCCCTCGCGCCATCAG*-**AACGATGG**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B1-3a	*GCCTCCCTCGCGCCATCAG*-**AACGCCAT**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B1-3b	*GCCTCCCTCGCGCCATCAG*-**AACGCCTA**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B2-1a	*GCCTCCCTCGCGCCATCAG*-**AACGCGAA**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B2-1b	*GCCTCCCTCGCGCCATCAG*-**AACGCGTT**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B2-2a	*GCCTCCCTCGCGCCATCAG*-**AACGGCAA**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B2-2b	*GCCTCCCTCGCGCCATCAG*-**AACGGCTT**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B2-3a	*GCCTCCCTCGCGCCATCAG*-**AACGTACC**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC
B2-3b	*GCCTCCCTCGCGCCATCAG*-**AACGTAGG**-CA-CTTGGTCATTTAGAGGAAGTAA	*GCCTTGCCAGCCCGCTCAG*-TC-GCTGCGTTCTTCATCGATGC

Table S2 Overview and abundances of taxonomic groups of soil fungi identified by 454 pyrosequencing of the internal transcribed spacer 1 region in DNA extracted from soil samples obtained from an unburnt control site (UB) and three sites subjected to fire, one with long fire return intervals (B) and two with short fire return intervals (B1, dominated by *Cistus ladanifer*; B2, dominated by *Erica* spp., *Halimium* spp. and *Pterospartum tridentatum* and B2). Differences in abundances across the four sites were assessed by one-way analysis of variance followed by by the Bonferroni-Holm's procedure for controlling experimentwise error rates for multiple independent tests (ns, not significant).

							Taxon frequency (%)						
Phylum	Class	Order	Family	Genus	Species	# OTUs	UB (SD)	B (SD)	B1 (SD)	B2 (SD)	Across sites	$F_{3,8}$	*P*-value
Ascomycota						110	23.9 (3.2)	49.8 (15.4)	22.5 (1.3)	40.1 (12.1)	35.0	5.291	ns
	Dothideomycetes					19	1.3 (0.7)	1.4 (0.5)	4.6 (2.4)	3.6 (2.4)	2.8	2.637	ns
		Capnodiales				4	0.8 (0.6)	0.8 (0.5)	0.1 (0.1)	0.8 (0.2)	0.6	2.131	ns
			Mycosphaerellaceae			3	0.8 (0.6)	0.7 (0.5)	0.1 (0.1)	0.6 (0.2)	0.5	1.883	ns
				Cladosporium		1	0.0 (0.0)	0.1 (0.1)	0.1 (0.1)	0.6 (0.2)	0.2	32.771	< 0.05
			Inc. sed.			-	-	-	-	-	-	-	-
				Capnobotryella		1	0.0 (0.0)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.1	2.307	ns
		Dothideales				1	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.2 (0.2)	0.1	3.525	ns
			Dothioraceae			1	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.2 (0.2)	0.1	3.525	ns
				Aureobasidium		1	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.2 (0.2)	0.1	3.525	ns
		Pleosporales				8	0.1 (0.1)	0.5 (0.2)	0.9 (0.5)	1.4 (1.3)	0.8	1.939	ns
			Cucurbitariaceae			1	0.0 (0.0)	0.2 (0.1)	0.0 (0.0)	0.1 (0.1)	0.1	71.963	< 0.05
			Pleosporaceae			1	0.1 (0.1)	0.1 (0.1)	0.2 (0.2)	0.0 (0.0)	0.1	2.076	ns
				Drechslera		1	0.1 (0.1)	0.1 (0.1)	0.2 (0.2)	0.0 (0.0)	0.1	2.076	ns
					D. erythrospila	1	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.0 (0.0)	0.1	2.065	ns
			Venturiaceae			2	0.0 (0.0)	0.1 (0.1)	0.2 (0.1)	0.2 (0.1)	0.1	3.059	ns
				Venturia		2	0.0 (0.0)	0.1 (0.1)	0.2 (0.1)	0.2 (0.1)	0.1	3.059	ns
			Inc. sed.			-	-	-	-	-	-	-	-
				Camarosporium		1	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.3 (0.4)	0.1	2.508	ns
					C. leucadendri	1	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.3 (0.3)	0.1	2.446	ns
				Phoma		2	0.1 (0.1)	0.1 (0.1)	0.6 (0.3)	0.6 (0.7)	0.3	2.201	ns
					P. herbarum	1	0.1 (0.1)	0.1 (0.1)	0.0 (0.0)	0.1 (0.1)	0.1	2.097	ns
		Inc. sed.				-	-	-	-	-	-	-	-
			Inc. sed.			-	-	-	-	-	-	-	-
				Cenococcum		4	0.3 (0.3)	0.0 (0.0)	3.4 (2.4)	0.1 (0.1)	1.0	5.701	ns
					C. geophilum	1	0.0 (0.0)	0.0 (0.0)	2.6 (2.0)	0.1 (0.1)	0.7	4.685	ns
	Eurotiomycetes					30	5.6 (1.5)	21 (17)	5.6 (1.3)	9.0 (2.9)	10.9	2.111	ns
		Chaetothyriales				22	3.8 (1.3)	1.1 (0.8)	4.1 (1.6)	5.9 (1.2)	3.7	7.655	ns
			Herpotrichiellaceae			22	3.8 (1.3)	1.1 (0.8)	4.1 (1.6)	5.9 (1.2)	3.7	7.655	ns
				Cladophialophora		6	1.7 (1.1)	0.1 (0.1)	1.2 (1.0)	0.0 (0.0)	0.8	3.905	ns
					C. chaetospira	1	0.3 (0.2)	0.0 (0.0)	0.2 (0.2)	0.0 (0.0)	0.1	3.513	ns
					C. minutissima	2	0.0 (0.0)	0.1 (0.1)	0.6 (0.2)	0.0 (0.0)	0.2	13.160	< 0.05
				Phialophora		2	0.6 (0.5)	0.5 (0.4)	0.5 (0.2)	0.3 (0.2)	0.5	0.444	ns
		Eurotiales				6	1.8 (0.7)	14.4 (17.5)	0.7 (0.2)	1.4 (0.9)	5.1	1.690	ns
			Trichocomaceae			6	1.8 (0.7)	14.4 (17.5)	0.7 (0.2)	1.4 (0.9)	5.1	1.690	ns
				Eurotium		1	0.5 (0.4)	0.1 (0.1)	0.4 (0.1)	1.1 (0.9)	0.5	2.698	ns
				Penicillium		2	1.4 (1.0)	0.2 (0.1)	0.1 (0.1)	0.2 (0.1)	0.4	4.817	ns
					P. spinulosum	1	0.9 (0.5)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.3	7.190	ns

							Taxon frequency (%)						
Phylum	Class	Order	Family	Genus	Species	# OTUs	UB (SD)	B (SD)	B1 (SD)	B2 (SD)	Across sites	$F_{3,8}$	*P*-value
		Inc. sed.				-	-	-	-	-	-	-	-
			Inc. sed.			-	-	-	-	-	-	-	-
				Calyptrozyma		2	0.0 (0.0)	5.4 (1.9)	0.9 (0.4)	1.8 (1.2)	2.2	12.509	ns
	Lecanoromycetes					1	0.1 (0.1)	0.0 (0.0)	0.1 (0.2)	0.1 (0.1)	0.1	0.963	ns
		Agyriales				1	0.1 (0.1)	0.0 (0.0)	0.1 (0.2)	0.1 (0.1)	0.1	0.963	ns
			Agyriaceae			1	0.1 (0.1)	0.0 (0.0)	0.1 (0.2)	0.1 (0.1)	0.1	0.963	ns
	Leotiomycetes					24	5.7 (1.6)	4.7 (1.8)	3.2 (0.8)	14.7 (4.1)	6.9	13.775	< 0.05
		Helotiales				18	5.6 (1.7)	3.2 (0.9)	2.5 (0.5)	11.5 (4.1)	5.5	9.691	ns
			Dermateaceae			3	3.2 (2.3)	0.7 (0.4)	0.4 (0.4)	1.2 (0.9)	1.4	2.869	ns
			Helotiaceae			8	1.4 (0.4)	0.5 (0.5)	0.5 (0.3)	7.0 (2.2)	2.1	22.043	< 0.05
				Rhizoscyphus		5	0.4 (0.4)	0.5 (0.5)	0.3 (0.2)	5.9 (1.7)	1.6	26.807	< 0.05
					R. ericae	2	0.1 (0.1)	0.4 (0.4)	0.3 (0.2)	4.4 (1.4)	1.1	24.181	< 0.05
			Inc. sed.			-	-	-	-	-	-	-	-
				Leptodontidium		2	0.3 (0.2)	0.1 (0.1)	0.8 (0.5)	1.5 (1.2)	0.7	3.184	ns
		Inc. sed.				-	-	-	-	-	-	-	-
			Inc. sed.			-	-	-	-	-	-	-	-
				Leohumicola		4	0.1 (0.1)	0.9 (0.7)	0.1 (0.1)	1.2 (0.3)	0.5	8.387	ns
					L. verrucosa	1	0.1 (0.1)	0.3 (0.3)	0.0 (0.0)	0.8 (0.4)	0.2	6.046	ns
	Pezizomycetes					8	0.6 (0.7)	3.6 (0.8)	2.9 (1.1)	2.5 (1.3)	2.5	5.083	ns
		Pezizales				8	0.6 (0.7)	3.6 (0.8)	2.9 (1.1)	2.5 (1.3)	2.5	5.083	ns
			Pezizaceae			4	0.1 (0.1)	3.3 (1.1)	0.1 (0.1)	2.5 (1.4)	1.5	10.547	ns
			Pyronemataceae			2	0.0 (0.0)	0.3 (0.5)	1.9 (1.5)	0.1 (0.1)	0.6	3.662	ns
				Wilcoxina		2	0.0 (0.0)	0.3 (0.5)	1.9 (1.5)	0.1 (0.1)	0.6	3.662	ns
			Tuberaceae			2	0.5 (0.7)	0.0 (0.0)	1.0 (1.2)	0.1 (0.1)	0.4	1.326	ns
				Tuber		2	0.5 (0.7)	0.0 (0.0)	1.0 (1.2)	0.1 (0.1)	0.4	1.326	ns
	Sordariomycetes					6	1.3 (1.4)	0.2 (0.2)	1.6 (1.2)	0.5 (0.2)	0.9	1.518	ns
		Hypocreales				1	0.2 (0.1)	0.1 (0.1)	0.1 (0.2)	0.1 (0.1)	0.1	0.765	ns
			Hypocreaceae			1	0.2 (0.1)	0.1 (0.1)	0.1 (0.2)	0.1 (0.1)	0.1	0.765	ns
				Trichoderma		1	0.2 (0.1)	0.1 (0.1)	0.1 (0.2)	0.1 (0.1)	0.1	0.765	ns
		Microascales				1	0.2 (0.1)	0.0 (0.0)	0.1 (0.1)	0.1 (0.2)	0.1	2.028	ns
			Inc. sed.			-	-	-	-	-	-	-	-
				Sporendocladia		1	0.2 (0.1)	0.0 (0.0)	0.1 (0.1)	0.1 (0.2)	0.1	2.028	ns
		Phyllachorales				2	0.9 (1.4)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.2	1.317	ns
			Magnaporthaceae			2	0.9 (1.4)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.2	1.317	ns
		Inc. sed.				-	-	-	-	-	-	-	-
			Apiosporaceae			1	0.0 (0.0)	0.1 (0.2)	0.1 (0.1)	0.1 (0.1)	0.1	0.956	ns
Basidiomycota						106	64.5 (8.0)	41.2 (13.6)	70.2 (2.53)	34.2 (10.8)	52.3	9.908	< 0.05
	Agaricomycetes					82	56.3 (10.6)	36.3 (8.0)	65.0 (4.0)	25.1 (7.0)	45.8	16.556	< 0.05
		Agaricales				33	6.6 (3.6)	5.9 (7.9)	49.8 (4.8)	12.9 (11.2)	19.1	23.498	< 0.05
			Bolbitiaceae			9	0.0 (0.0)	2.7 (4.6)	18.9 (7.6)	8.5 (12.2)	7.5	3.692	ns
				Hebeloma		9	0.0 (0.0)	2.7 (4.6)	18.9 (7.6)	8.5 (12.2)	7.5	3.692	ns
					H. cistophilum	6	0.0 (0.0)	0.1 (0.1)	8.1 (4.9)	5.3 (8.3)	3.3	2.093	ns
			Cortinariaceae			12	1.1 (0.6)	2.0 (3.1)	26.8 (8.6)	1.5 (1.3)	8.3	22.457	< 0.05

							Taxon frequency (%)						
Phylum	Class	Order	Family	Genus	Species	# OTUs	UB (SD)	B (SD)	B1 (SD)	B2 (SD)	Across sites	$F_{3,8}$	*P*-value
				Cortinarius		5	0.3 (0.4)	0.1 (0.2)	23.9 (8.9)	1.5 (1.3)	6.9	19.831	< 0.05
				Dermocybe		1	0.5 (0.8)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.1	1.090	ns
				Gymnopilus		1	0.0 (0.0)	0.1 (0.1)	0.2 (0.1)	0.0 (0.0)	0.1	31.201	< 0.05
				Inocybe		5	0.3 (0.1)	1.8 (2.9)	2.7 (1.2)	0.1 (0.1)	1.3	1.921	ns
					I. praetervisa	1	0.1 (0.1)	0.1 (0.1)	0.7 (0.8)	0.1 (0.1)	0.2	2.208	ns
			Hydnangiaceae			1	0.8 (1.3)	0.0 (0.0)	0.3 (0.4)	0.0 (0.0)	0.2	0.804	ns
				Laccaria		1	0.8 (1.3)	0.0 (0.0)	0.3 (0.4)	0.0 (0.0)	0.2	0.804	ns
			Pluteaceae			3	1.2 (0.4)	1.3 (1.1)	3.9 (3.7)	1.0 (0.9)	1.9	1.458	ns
				Amanita		3	1.2 (0.4)	1.3 (1.1)	3.9 (3.7)	1.0 (0.9)	1.9	1.458	ns
					A. spissa	1	0.7 (0.2)	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)	0.2	32.965	< 0.05
					A. torrendii	1	0.1 (0.1)	0.7 (1.2)	3.4 (3.2)	0.8 (0.8)	1.3	2.156	ns
			Tricholomataceae			6	3.4 (3.0)	0.0 (0.0)	0.1 (0.1)	0.8 (1.4)	0.9	2.874	ns
				Tricholoma		6	3.4 (3.0)	0.0 (0.0)	0.1 (0.1)	0.8 (1.4)	0.9	2.874	ns
					T. flavovirens	3	0.6 (1.0)	0.0 (0.0)	0.0 (0.0)	0.2 (0.4)	0.2	0.879	ns
					T. portentosum	1	1.5 (2.6)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.4	1.000	ns
					T. saponaceum	1	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.5 (0.9)	0.1	1.000	ns
					T. ustale	1	0.7 (1.3)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.2	0.985	ns
		Boletales				8	4.3 (2.0)	5.9 (0.8)	1.6 (2.0)	0.2 (0.3)	3.1	9.022	ns
			Boletaceae			1	1.9 (2.9)	0.3 (0.2)	0.0 (0.0)	0.0 (0.0)	0.6	1.178	ns
				Xerocomus		1	1.9 (2.9)	0.3 (0.2)	0.0 (0.0)	0.0 (0.0)	0.6	1.178	ns
					X. badius	1	1.5 (2.3)	0.2 (0.2)	0.0 (0.0)	0.0 (0.0)	0.5	1.182	ns
			Rhizopogonaceae			5	2.4 (1.6)	5.6 (1.0)	0.8 (0.7)	0.2 (0.3)	2.3	17.685	< 0.05
				Rhizopogon		5	2.4 (1.6)	5.6 (1.0)	0.8 (0.7)	0.2 (0.3)	2.3	17.685	< 0.05
					R. evadens	1	1.1 (1.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.3	1.000	ns
					R. luteolus	1	0.4 (0.4)	2.9 (1.4)	0.1 (0.1)	0.1 (0.1)	0.9	10.387	ns
					R. rubescens	2	0.0 (0.0)	0.1 (0.1)	0.7 (0.5)	0.2 (0.2)	0.2	3.067	ns
		Cantharellales				3	9.2 (2.2)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	2.2	53.873	< 0.05
			Cantharellaceae			1	1.0 (1.8)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.2	0.992	ns
				Craterellus		1	1.0 (1.8)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.2	0.992	ns
					C. tubaeformis	1	0.8 (1.5)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.2	0.991	ns
			Clavulinaceae			1	0.8 (1.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.2	1.219	ns
		Ceratobasidiales				2	0.0 (0.0)	0.1 (0.1)	0.1 (0.1)	0.8 (0.8)	0.2	2.720	ns
			Ceratobasidiaceae			2	0.0 (0.0)	0.1 (0.1)	0.1 (0.1)	0.8 (0.8)	0.2	2.720	ns
				Ceratobasidium		2	0.0 (0.0)	0.1 (0.1)	0.1 (0.1)	0.8 (0.8)	0.2	2.720	ns
		Polyporales				6	9.5 (4.5)	0.1 (0.1)	0.6 (0.9)	0.1 (0.2)	2.6	11.939	ns
			Atheliaceae			5	9.2 (4.1)	0.1 (0.1)	0.6 (0.9)	0.1 (0.2)	2.5	13.723	ns
				Tylospora		5	9.2 (4.1)	0.1 (0.1)	0.6 (0.9)	0.1 (0.2)	2.5	13.723	ns
			Corticiaceae			1	0.3 (0.5)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1	1.000	ns
		Russulales				14	18.8 (15.2)	2.5 (2.8)	2.7 (2.4)	1.5 (1.8)	6.0	3.364	ns
			Russulaceae			13	18.8 (15.2)	2.5 (2.8)	2.7 (2.4)	1.2 (2.0)	5.9	3.393	ns
				Lactarius		1	4.8 (7.7)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.2	1.199	ns
				Russula		12	14.0 (16.7)	2.5 (2.8)	2.8 (2.4)	1.2 (2.0)	4.7	1.445	ns
					R. albonigra	1	0.2 (0.3)	0.0 (0.0)	0.5 (0.7)	0.8 (1.4)	0.3	0.649	ns

							Taxon frequency (%)						
Phylum	Class	Order	Family	Genus	Species	# OTUs	UB (SD)	B (SD)	B1 (SD)	B2 (SD)	Across sites	$F_{3,8}$	*P*-value
					R. drimeia	1	3.6 (4.9)	1.6 (2.4)	0.5 (0.9)	0.0 (0.0)	1.4	0.953	ns
					R. gracilis	1	0.0 (0.0)	0.0 (0.0)	0.5 (0.9)	0.0 (0.0)	0.2	1.000	ns
		Thelephorales				12	1.3 (2.0)	21.3 (2.6)	9.8 (8.8)	6.0 (4.2)	9.9	8.344	ns
			Thelephoraceae			12	1.3 (2.0)	21.3 (2.6)	9.8 (8.8)	6.0 (4.2)	9.9	8.344	ns
				Pseudotomentella		1	0.6 (1.1)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.1	0.995	ns
				Thelephora		9	0.1 (0.2)	21.3 (2.6)	9.4 (9.0)	6.0 (4.2)	9.5	9.114	ns
					T. terrestris	7	0.1 (0.1)	18.2 (2.8)	8.0 (7.8)	5.0 (3.5)	8.0	8.718	ns
				Tomentella		2	0.5 (0.9)	0.0 (0.0)	0.3 (0.3)	0.0 (0.0)	0.2	0.906	ns
	Microbotryomycetes					1	1.3 (0.8)	0.1 (0.2)	0.1 (0.1)	0.0 (0.0)	0.3	7.087	ns
		Sporidiobolales				1	1.3 (0.8)	0.1 (0.2)	0.1 (0.1)	0.0 (0.0)	0.3	7.087	ns
	Tremellomycetes					11	5.6 (3.5)	1.1 (0.8)	4.2 (0.8)	3.8 (4.2)	3.6	1.367	ns
		Filobasidiales				3	1.1 (1.1)	0.4 (0.2)	0.2 (0.1)	0.2 (0.2)	0.5	2.005	ns
			Filobasidiaceae			3	1.1 (1.1)	0.4 (0.2)	0.2 (0.1)	0.2 (0.2)	0.5	2.005	ns
				Cryptococcus		3	1.1 (1.1)	0.4 (0.2)	0.2 (0.1)	0.2 (0.2)	0.5	2.005	ns
		Tremellales				8	4.4 (3.2)	0.7 (0.6)	4.0 (0.8)	3.6 (4.3)	3.1	1.183	ns
			Sebacinaceae			7	4.1 (3.3)	0.7 (0.6)	3.8 (0.9)	3.6 (4.3)	3.0	1.011	ns
				Sebacina		5	4.1 (3.3)	0.2 (0.4)	3.8 (0.9)	0.1 (0.1)	2.1	4.943	ns
Chytridiomycota						3	1.3 (0.4)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.3	26.382	< 0.05
	Monoblepharidomycetes					3	1.3 (0.4)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.3	26.382	< 0.05
		Monoblepharidales				3	1.3 (0.4)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.3	26.382	< 0.05
			Monoblepharidaceae			3	1.3 (0.4)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.3	26.382	< 0.05
Zygomycota						7	4.7 (2.3)	1.3 (0.8)	0.9 (0.5)	4.2 (1.4)	2.7	5.668	ns
	Zygomycetes					6	4.5 (1.9)	1.2 (0.7)	0.8 (0.5)	4.2 (1.4)	2.6	6.900	ns
		Mortierellales				5	4.4 (1.9)	1.2 (0.7)	0.8 (0.5)	4.0 (1.4)	2.5	6.900	ns
			Mortierellaceae			5	4.4 (1.9)	1.2 (0.7)	0.8 (0.5)	4.0 (1.4)	2.5	6.900	ns
				Mortierella		5	4.4 (1.9)	1.2 (0.7)	0.8 (0.5)	4.0 (1.4)	2.5	6.900	ns
		Mucorales				1	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.2 (0.1)	0.1	4.231	ns

Table S3 Database matches of partial ITS sequences obtained from excised DGGE bands from DNA samples extracted from soil subjected to different fire return intervals regimes and sequences from GenBank. Letters A, B and C that precede band position in the first column refer to Figures S1, S2 and S3 respectively.

Sequenced band	*Sequence length (bp)*	*Accession number*	*Best blast hit*	*BLAST expected value*	*% Similarity/Overlap(bp)*
A1a	162	HQ201731	EU557320 *Russula drimeia*	8e-77	99/163
A1b	216	HQ201732	HM164559 uncultured fungus	2e-88	98/193
A3a	227	HQ201728	HM164559 uncultured fungus	9e-93	98/204
A3b	240	HQ201729	GU566277 *Penicillium corylophilum*	2e-114	99/239
A3c	191	HQ201730	FJ624264 *Neosartorya fischeri*	5e-55	97/135
A5a	231	HQ201721	GQ925387 *Amanita torrendii*	2e-115	100/229
A5b	204	HQ201722	EU668911 uncultured *Cortinarius*	8e-98	99/203
A5c	267	HQ201723	DQ422029 *Russula albonigra*	2e-125	100/247
A5d	230	HQ201726	FJ554320 uncultured Sordariomycetes	6e-75	91/227
A6a	242	HQ201724	DQ422029 *Russula albonigra*	1e-111	100/222
A6b	206	HQ201725	FJ554320 uncultured Sordariomycetes	2e-63	90/203
A6c	230	HQ201727	FJ554320 uncultured Sordariomycetes	6e-75	91/227
A7a	228	HQ201718	HM164559 uncultured fungus	9e-93	97/204
A7b	297	HQ201720	EU570177 *Hebeloma cistophilum*	2e-144	100/281
A8a	265	HQ201719	FJ553753 uncultured Sebacinales	2e-115	96/264
B1a	219	HQ201733	GQ925387 *Amanita torrendii*	5e-110	100/219
B1b	287	HQ201738	FJ816731 uncultured *Lactarius*	3e-128	100/252
B3a	249	HQ201734	GU067737 *Ceratobasidium* sp.	2e-104	97/238
B3b	260	HQ201735	HM146851 uncultured *Russula*	5e-131	99/260
B5a	239	HQ201737	HM146865 uncultured Sebacinales	2e-114	99/234
B7a	195	HQ201736	FJ475667 uncultured Dothideomycetes	5e-85	97/196
B8a	246	HQ201739	HM146865 uncultured Sebacinales	3e-118	99/238
B8b	207	HQ201740	AB278185 *Phialemonium dimorphosporum*	2e-94	99/204
B8c	212	HQ201742	FJ475785 uncultured Ascomycota	3e-92	97/211
B8d	207	HQ201741	FJ475667 uncultured Dothideomycetes	4e-91	97/207
C1a	147	HQ201743	FJ236031 *Xerocomus badius*	3e-70	100/147
C3a	226	HQ201744	HM164559 uncultured fungus	9e-93	98/204
C3b	204	HQ201745	FJ624264 *Neosartorya fischeri*	5e-55	97/135
C5a	227	HQ201746	EU668911 uncultured *Cortinarius*	2e-109	99/227
C5b	296	HQ201747	EU570177 *Hebeloma cistophilum*	2e-144	100/281
C7a	248	HQ201749	HM036626 uncultured fungus	5e-101	99/206
C7b	209	HQ201750	AF393723 *Cladosporium staurophorum*	7e-79	93/211
C8a	269	HQ201748	GU931704 *Thelephora terrestris*	5e-136	99/269
C8b	209	HQ201751	AF393723 *Cladosporium staurophorum*	7e-79	93/211

Figure S1 DGGE profiles of partial fungal ITS sequences amplified from pooled DNA samples from the first plot of each of the four sites subjected to different fire return intervals (UB, unburnt site; B, long fire return interval site; B1, short fire return interval site dominated by *Cistus ladanifer*; B2, short fire return interval site dominated by *Erica* spp., *Halimium* spp. and *Pterospartum tridentatum*), with each plot represented by two replicates (UB: 1,2; B: 3,4; B1: 5,6; B2: 7,8). The ladder was obtained by pooling the DNA fragments resulting from a PCR amplification of known ECM species (L.b., *Laccaria bicolour*; I.j., *Inocybe jacobi*, W.m., *Wilcoxina mikolae*, R.r., *Rhizopogon roseolus*, T.t., *Thelephora terrestris*, T.e., *Tomentella ellisi*, L.h., *Lactarius hepaticus*, C.g., *Cenococcum geophilum*; S.p., *Scleroderma polyrrhizum*). Bands marked with letters indicate bands that were excised and sequenced.

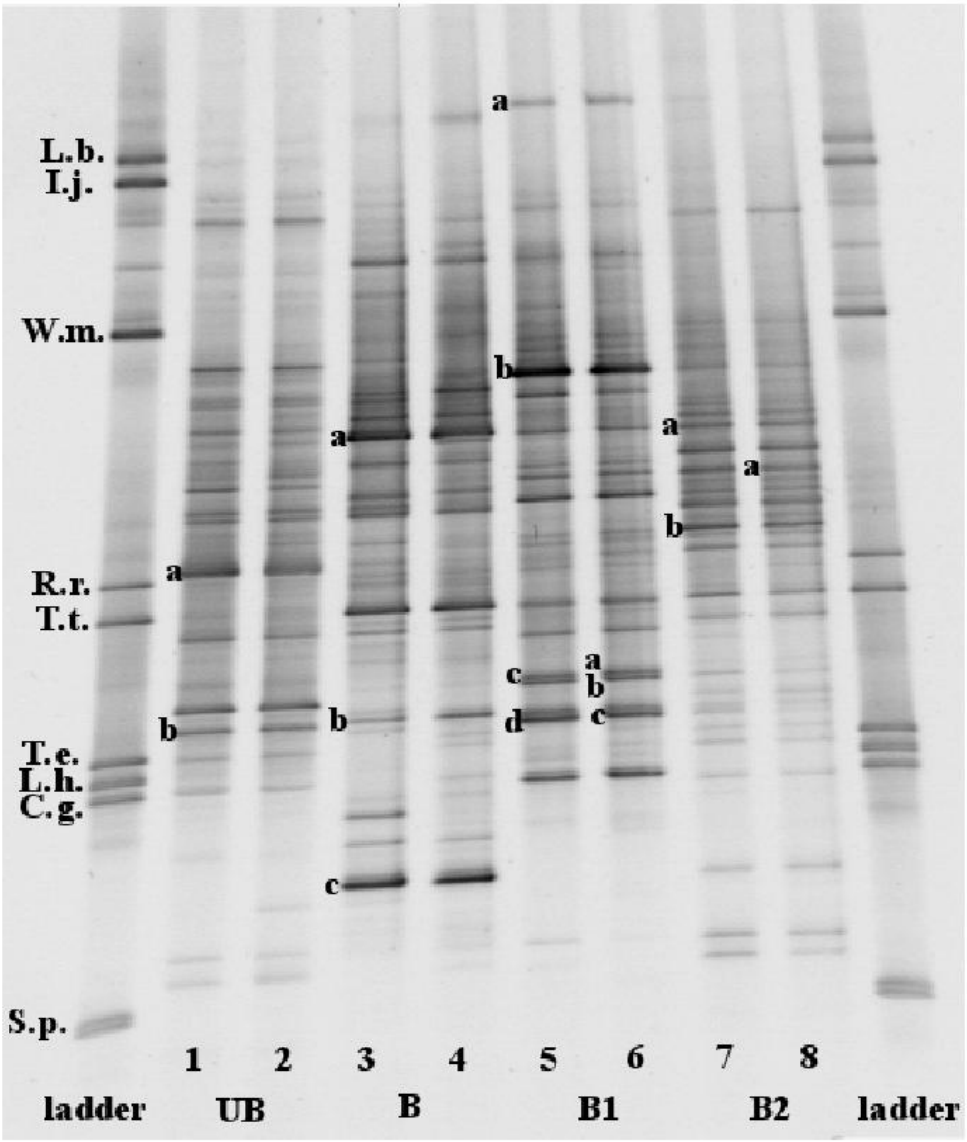


Figure S2 DGGE profiles of partial fungal ITS sequences amplified from pooled DNA samples from the second plot of each of the four sites subjected to different fire return intervals (UB, unburnt site; B, long fire return interval site; B1, short fire return interval site dominated by *Cistus ladanifer*; B2, short fire return interval site dominated by *Erica* spp., *Halimium* spp. and *Pterospartum tridentatum*), with each plot represented by two replicates (UB: 1,2; B: 3,4; B1: 5,6; B2: 7,8). The ladder was obtained by pooling the DNA fragments resulting from a PCR amplification of known ECM species (L.b., *Laccaria bicolour*; I.j., *Inocybe jacobi*, W.m., *Wilcoxina mikolae*, R.r., *Rhizopogon roseolus*, T.t., *Thelephora terrestris*, T.e., *Tomentella ellisi*, L.h., *Lactarius hepaticus*, C.g., *Cenococcum geophilum*; S.p., *Scleroderma polyrrhizum*). Bands marked with letters indicate bands that were excised and sequenced.

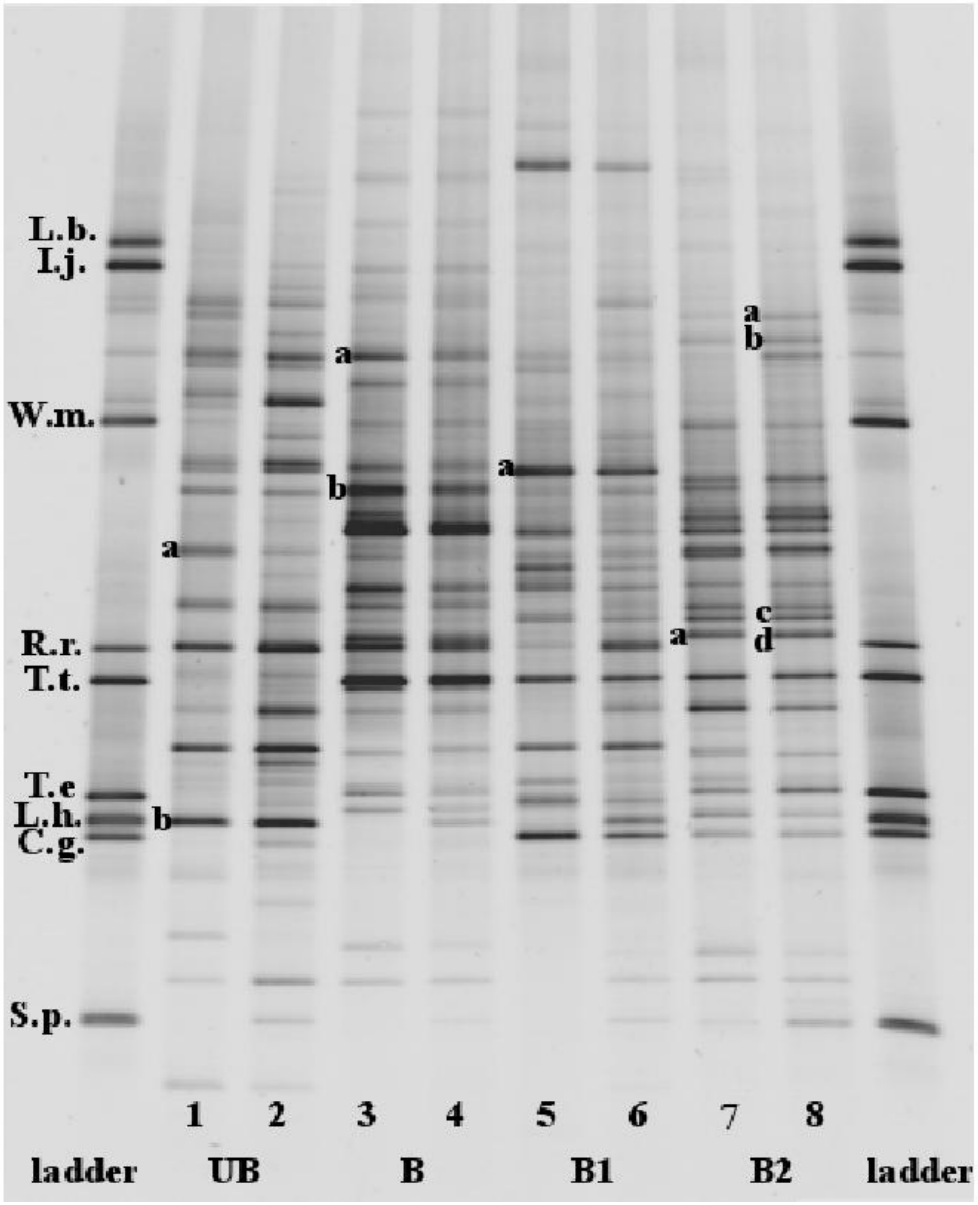
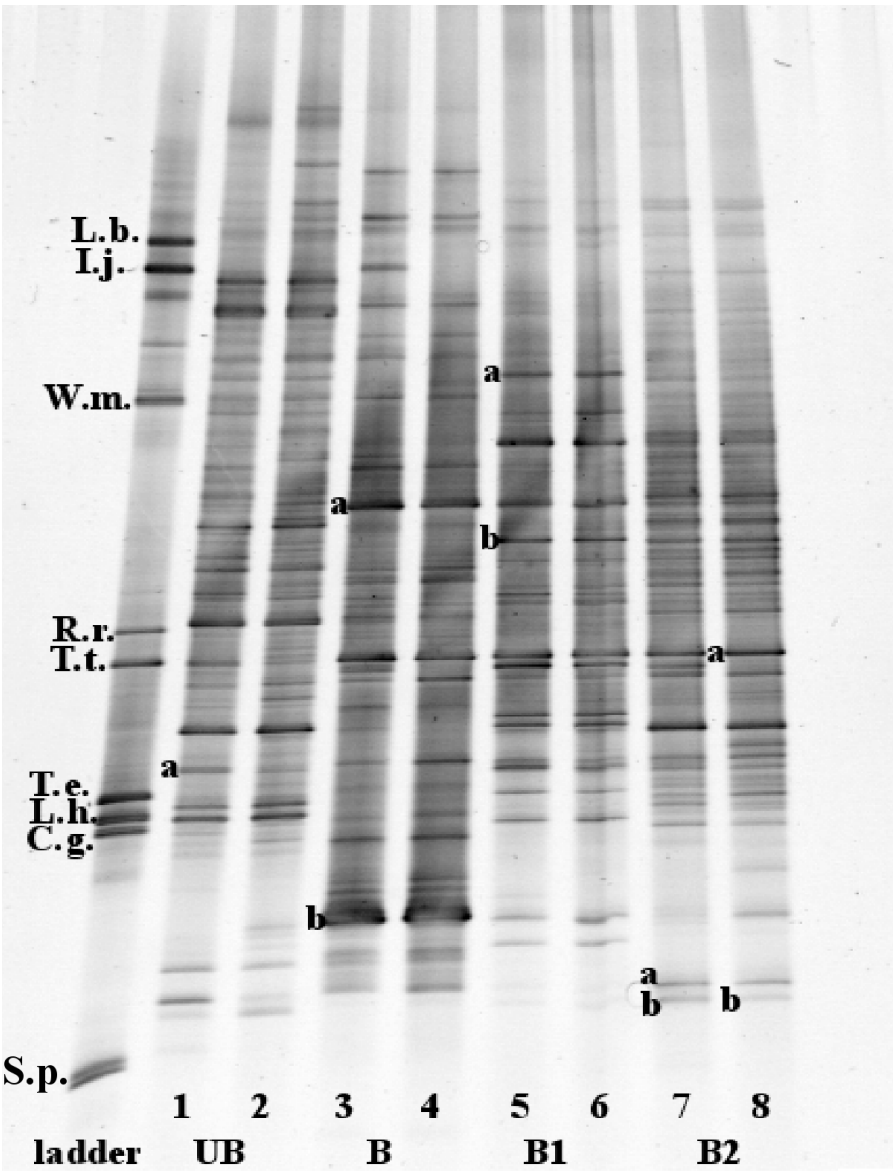


Figure S3 DGGE profiles of partial fungal ITS sequences amplified from pooled DNA samples from the third plot of each of the four sites subjected to different fire return intervals (UB, unburnt site; B, long fire return interval site; B1, short fire return interval site dominated by *Cistus ladanifer*; B2, short fire return interval site dominated by *Erica* spp., *Halimium* spp. and *Pterospartum tridentatum*), with each plot represented by two replicates (UB: 1,2; B: 3,4; B1: 5,6; B2: 7,8). The ladder was obtained by pooling the DNA fragments resulting from a PCR amplification of known ECM species (L.b., *Laccaria bicolour*; I.j., *Inocybe jacobi*, W.m., *Wilcoxina mikolae*, R.r., *Rhizopogon roseolus*, T.t., *Thelephora terrestris*, T.e., *Tomentella ellisi*, L.h., *Lactarius hepaticus*, C.g., *Cenococcum geophilum*; S.p., *Scleroderma polyrrhizum*). Bands marked with letters indicate bands that were excised and sequenced.



CHAPTER 3

Impact of wildfire return interval on the ectomycorrhizal resistant propagules communities of a Mediterranean open forest

Introduction

The increase in fire frequency and intensity of wildfires in the Mediterranean basin can compromise the resilience of ecosystems in highly disturbed areas, modifying landscape and vegetation structure (Pausas 2004) and altering ecosystem functioning (Rodrigo *et al.* 2004). Many Mediterranean trees, like pines and cork oaks, show adaptations to fire disturbance, such as thick bark or cork layers, resprouting ability, serotiny and fire-induced seed germination. In spite of this, frequent fires can change the vegetation from pine forests to shrub dominated areas because the regenerating trees are unable to reach reproductive maturity between successive fires (Vázquez & Moreno 2001). Pine and oak trees are also ecologically obligate ectomycorrhizal (ECM) symbionts that depend on the presence of fungal inoculum for establishment and survival. However, high-intensity fires may affect the function of ECM communities and lead to the local extinction of ECM fungi by eliminating the organic soil layer, partially heat-sterilizing the soil, and altering the soil pH and the availability of nutrients through the deposition of ashes (Grogan *et al*. 2000; Dahlberg 2002).

The complexity of ECM communities tends to be reduced after a stand replacing fire, with stable pre-fire communities composed of relatively large numbers of species being replaced by more homogeneous and simple post-fire communities composed of relatively few and previously rare species (e.g. species of the order Pezizales and the genus *Rhizopogon*; Visser 1995; Stendell *et al.* 1999; Baar *et al.* 1999; Grogan *et al*. 2000; Smith *et al*. 2004; Fujimura *et al.* 2005). Because of the survival of fungal species through spores or other resistant propagules, the composition of the post-fire ECM communities has a strong resemblance to the composition of the pre-fire resistant propagules community (RPC) (Baar *et al.* 1999; Taylor & Bruns 1999). New fungal species may also colonize the affected areas after the disturbance event, benefitting from the release of competition induced by fire. Nevertheless, the spore bank and other resistant propagules of ECM fungi, play an important role in succession processes facilitating regeneration following disturbance events (Taylor & Bruns 1999). Fungal resistant propagules can successfully colonize poor soil regions, resist periodic droughts, severe temperatures, and other natural stresses (Gupta *et al.* 2000), and their absence may delay the colonization of disturbed areas by ECM trees (Dickie & Reich 2005). Therefore, understanding the effect of fire frequency on ECM fungi is crucial to understand the dynamics of secondary succession after fire.

Several studies have examined the effects of different fire factors, such as soil drying and heating, on the RPC, and revealed that different ECM fungi have differential responses to these fire-related factors. For example, the abundance of *R. olivaceotinctus* A.H. Sm. increased significantly in dried soil (Baar *et al.* 1999) and after heat treatment at 70-75ºC (Izzo *et al.* 2006a; Peay *et al.* 2009), while *Wilcoxina* sp., *Cenococcum geophilum* Fr. (Izzo *et al.* 2006a), *Laccaria proxima* (Boud.) Pat. and *Tomentella subliacina* (Ellis & Holw.) Wakef. (Peay *et al.* 2009) showed a decrease in abundance in heat-treated soils. However, the changes induced by fire on the RPC also depend on fire regime and

while there are studies that evaluate the effects of fire occurrence and intensity on the RPC, little information appears to exist about the effects of fire frequency on whole ECM communities (Hart *et al.* 2005; Tuininga & Dighton 2004; Bastias *et al.* 2006; Anderson *et al.* 2007).

The main objective of this study was to reveal possible effects of fire frequency (measured as fire return interval) on the composition of the RPC in areas where the vegetation has changed from a mature maritime pine open forest to a transitional phase with shrubs and young pine trees (long fire return interval) or to shrublands (short fire return interval). It was hypothesized (1) that short fire return intervals and the subsequent alterations of the vegetation structure from pine forest to shrubland had a greater impact on the structure and composition of the RPC than long fire return intervals and (2) that this effect of fire return interval length on the composition of the ECM RPC community limits the availability of compatible symbiotic partners for different host plants.

Materials and Methods

Study sites and sampling

Sampling was conducted in June 2007 in a mountainous area in Central Portugal between: Alvito da Beira (39º48' N, 7º49' W, altitude 500-600 m) and Isna de Oleiros (39º51' N, 7º51' W, altitude 750-850 m). The region is characterized by a Mediterranean climate with hot dry summers and cool wet winters and has a lithosol soil (a stony soil lacking horizon and structure development). The whole region is covered mainly by *Pinus pinaster* Ait. and contains areas in close proximity to each other with differing fire histories. In this study it will be referred to 'short fire return interval' to describe the situation in which two fire events are close enough together in time to hinder pine regeneration, and 'long fire return interval' when the time period elapsed between two successive fire events allows the typical autosuccession response of Mediterranean vegetation ecosystems.

Four sampling sites were selected in this area: an unburnt control site (UB), a site with 'long fire return interval' (B) and two sites with 'short fire return interval' (B1 and B2) covered by different dominant shrubs. The unburnt site (UB) was characterized by an uneven-aged-maritime pine open forest (dominant trees with 40-45 cm diameter), unaffected by fire in the preceding 40 y. This site had an understorey shrub community dominated by *Erica* spp., *Halimium* spp. and *Pterospartum tridentatum* (L.) Willk. in Willk. & Lange and was characterized by soil with an incipient organic layer. Site B was separated from UB by a road and was covered by the same maritime pine open forest previous to the disturbance event in 2003. Five years after the fire it showed a vigorous natural pine regeneration associated with the same shrubby species present in site UB. Sites B1 and B2 have been affected by two high-intensity fires in 1992 and 2003. Before the first fire in 1992, the area was characterized by an uneven-aged pine open forest with a shrubby understorey. The last fire in 2003 changed the vegetation cover radically from a maritime pine young forest to a shrubby area with a vegetation cover dominated either by *Cistus ladanifer* L. (B1) or by *P. tridentatum*, *Erica* spp. and

Halimium spp. (B2). The inclusion of a second site affected by high fire frequency with a different shrub cover allowed us to evaluate the possible influence of vegetation composition on the resistant propagules inoculum present in these arrested stages of succession.

In June 2007, three 10 x 10 m^2 plots were delimited along a belt of approximately 600 m, spaced equally in each of the four sampling sites. Within each plot, nine soil samples were taken at a depth of approximately 20 cm using a shovel. The environmental conditions at the time of sampling, high temperatures and summer drought, likely eliminated non-resistant fungal species. In order to ensure that the soil contained only resistant propagules, soil samples were further air-dried for 4 weeks. Since it is known that the RPC represents a quite homogeneous and predictable inoculum source for seedlings in comparison to the highly diverse and patchy field root community (Izzo *et al.* 2006a) soils belonging to the same site were pooled, sieved through a 2 mm sieve, and stored at 4ºC for a month. Pooling soils is a common practice in studies for the assessment of the RPC (Taylor & Bruns 1999; Kjøller & Bruns 2003; Izzo *et al.* 2006b).

Preparation of the bioassays

Bioassays were performed with maritime pine (*Pinus pinaster*) and cork oak (*Quercus suber* L.) seedlings. *Pinus pinaster* represents the dominant species in the area and is known to be an early colonizer after disturbance events. *Quercus suber* is occasionally present in the region and it occurs in the unburnt forest as naturally regenerated seedlings.

In August 2007 the soil of each site was mixed with autoclaved coarse sand to help improve drainage (1:1) and this mixture was used to set up the bioassay. To check for exogenous contamination, negative controls were prepared with autoclaved coarse sand and autoclaved soil from each site. Soil-sand mixtures were put in 300 ml pots previously disinfected in 10% bleach for 30 min and soaked in tap water for another 30 min. Pine seeds were surface-sterilized in 80% bleach and soaked in running water for 48 h. Oak acorns were immersed in tap water for 2 d. Afterwards, the acorns that presented a 3 mm germinated radicle were selected, surface-sterilized in 80% bleach, and soaked in running tap water for 10 min. For each host, 15 replicate pots and 15 control pots were planted for each site (240 pots in total). Three seeds (for *P. pinaster*) or an acorn (for *Q. suber*) were sown in each pot. After emergence of pine seedlings, only one was allowed to develop. The experiment was conducted in a growth chamber under controlled conditions, 14 h/10 h of light/darkness at 24ºC/18ºC. Seedlings were watered twice a week with tap water. To avoid cross-contamination between treatments, pots were grouped by site and single trays were separated from each other with unbending plastic sheets.

Characterization of the seedling ECM community

Pine seedlings were harvested after 6 m in February 2008. Since oak seedling root systems developed slower than those of pine, oak seedlings were left to grow for 6 additional months. Roots of all seedlings were gently washed and screened under a dissecting microscope to quantify mycorrhization.

Mycorrhizal root tips were morphotyped under a dissecting microscope and the percentage of root colonization was recorded for each morphotype. On each seedling 1-5 morphotypes were found. Depending on the amount of material 1 to 6 single root tips for each morphotype were collected and conserved in separate eppendorf tubes in 300 µl CTAB buffer, which was removed before the molecular analysis. Total DNA was extracted with a modification of the Qiagen DNeasy® Plant Mini kit protocol (QIAGEN, Valencia, CA, USA). Root tips were ground with eppendorf pestles in AP1 buffer and incubated overnight at 60ºC prior to DNA extraction. The internal transcribed spacer (ITS) region was amplified with the primer combination ITS1-F and ITS4 (White *et al.* 1990; Gardes & Bruns 1993) that targets both Ascomycotina and Basidiomycotina. Polymerase chain reactions (PCRs) were performed with PuReTaq™ Ready-To-Go™ PCR Beads (GE Healthcare UK Limited, Buckinghamshire, UK) in 25 µl volume with the following cycling parameters: an initial denaturation step at 94ºC for 5 min, followed by five cycles of denaturation at 94ºC for 30 s, annealing at 54ºC for 30 s and extension at 72ºC for 1 min and 30 s, followed by 33 cycles of denaturation at 94ºC for 30 s, annealing at 48ºC for 30 s and extension at 72ºC for 1 min and 30s, with a final extension step at 72ºC for 10 min. Amplification products were separated by gel electrophoresis in 2% agarose gel in 1 x TAE buffer and visualized using SYBR green. Prior to sequencing, single PCR products were purified from gel using QIAquick gel PCR purification kit (QIAGEN). Both strands of the purified PCR products were sequenced separately using the primers mentioned above at Secugen S.L. (Madrid, Spain).

Sequencher™ 4.2 (Gene Codes Corporations, Ann Arbor, MI, USA) was used to identify the consensus sequence from the two strands of the ITS nrDNA of each isolate. Using the BLASTn algorithm (Altschul *et al.* 1997), the consensus sequences were compared with sequences in the UNITE (Kõljalg *et al.* 2005) and INSD (International Nucleotide Sequence Database) online nucleotides databases. The new consensus sequences have been lodged in the EMLN-EBI database with the accession numbers indicated in Table 1.

Data analysis

Correspondence analyses (CAs) were performed to analyze the ECM assemblage on seedlings using CANOCO version 4.5 (Microcomputer Power, Ithaca, NY, USA). ECM presence/absence data were used considering each seedling as an individual sample.

The potential effects of fire return interval on the percentage of seedling ECM colonization were tested using one-way analysis of variance (ANOVA), followed by Tukey HSD test. Changes in the ECM species colonizing seedlings in the four treatments were tested using both frequency, based on the presence/absence of morphotypes on the seedlings, and abundance (colonization of the ECM species i on the seedling j multiplied by total ectomycorrhization of the seedling j divided by 100). Since both principles of normality and variance homogeneity could not be assumed for the data set, non-parametric Kruskal-Wallis and Mann-Whitney U-tests were used to compare species frequency

and abundance in the four sites, followed by the Bonferroni-Holm's procedure (Holm 1979) for controlling experimentwise error rates for multiple independent tests. Species that occurred too infrequently to apply statistical tests were left out.

The following diversity indices were calculated for each site: species richness, Shannon diversity index (Pielou 1975) and the evenness index (Smith & Wilson 1996).

Non-parametric Kruskal-Wallis and Mann-Whitney U-tests were used to test for differences in the diversity indices between different treatments. Statistical testing was carried out using SPSS version 17.0 (SPSS Inc., Chicago, USA).

Results

Of the initial 240 seedlings, 190 (96 pine seedlings and 94 oak seedlings) survived until the end of the experiment. ECM colonization was not detected on control seedlings grown in sterile soil. A total of 251 root tips were sampled. Two hundred and six were analyzed with molecular techniques and of these 181 (88%) were successfully identified from sequence data. Due to the high frequency of root samples colonized by *Cenococcum geophilum* and the fact that this species is morphologically easily identified, molecular identification was performed for 14 of the 59 samples colonized by this fungal species. Using a 97% sequence similarity cut off 18 species of ECM fungi were identified (Table 1).

Two samples revealed the presence of a non-ECM fungal root inhabiting species, *Hamigera avellana* Stolk & Samson. The most common ECM fungal species were *C. geophilum*, *Thelephora terrestris* Ehrh., *Tomentella ellisii* (Sacc.) Jülich & Stalpers, *Inocybe jacobi* Kühner found in both host trees, and two *Rhizopogon* species on maritime pine (Table 2).

Both short and long fire return intervals affected the ECM colonization of pine and oak seedlings, in terms of species composition, frequency and abundance.

The CA showed changes that could be attributable to fire interval length although interactions with other unknown factors cannot be excluded. ECM assemblages of all seedlings were clearly separated into two groups that corresponded to the two different hosts (Fig. 1).

In addition, the results of the CA indicated a difference in the ECM assemblages found on the seedlings of both host species between the long fire return interval site (B) and the short fire return interval sites (B1 and B2): the individual seedlings clustered separately and the variation among the seedlings corresponding to site B appears smaller than the variation among the seedlings corresponding to sites B1 and B2. Pine and oak seedlings grown on soil from site B were grouped mainly on the upper left hand side of the ordination diagram and showed a reduced number of host specific ECM species when compared with the unburnt site and a greater similarity of ECM assemblages in the two hosts.

Table 1 Database matches of ITS sequences obtained from ectomycorrhizal fungi colonizing pine (P) and oak (O) seedlings grown in soils obtained from four sites with different fire regimes. Blast expected value was equal to 0.0 for all the alignments.

Accession number	Morphotype	Host	Best blast hit	% similarity/bp
GQ205351	*Cadophora* sp.	Q	AF486119 *Cadophora finlandica*	98%/839
GQ205352	*Cenococcum geophilum*	P	EU285479 *Cenococcum geophilum*	99%/511
GQ205369	*Cenococcum geophilum*	Q	AY394919 *Cenococcum geophilum*	98%/577
GQ205353	*Inocybe* sp.	P	AM882710 *Inocybe jacobi*	100%/505
GQ205370	*Inocybe* sp.	Q	AM882710 *Inocybe jacobi*	100%/505
GQ205354	*Laccaria* sp.1	Q	GQ994982 *Laccaria proxima* 1*	98%/704
GQ205355	*Laccaria* sp.2	Q	GQ267477 *Laccaria proxima* 2*	97%/709
GQ205356	*Lactarius* sp.	Q	UDB000861 *Lactarius hepaticus*	100%/699
GQ205357	*Rhizopogon* sp.1	P	GQ267481 *Rhizopogon luteolus*	99%/832
GQ205358	*Rhizopogon* sp.2	P	EU379678 *Rhizopogon roseolus*	99%/608
GQ205360	*Scleroderma* sp.1	Q	EU784412 *Scleroderma cepa*	99%/715
GQ205359	*Scleroderma* sp.2	Q	EU718117 *Scleroderma laeve*	99%/755
GQ205361	*Scleroderma* sp.3	Q	EU718123 *Scleroderma polyrhizum*	98%/507
GQ205362	*Thelephoraceae* 1	P	GQ267490 *Thelephora terrestris*	99%/695
GQ205371	*Thelephoraceae* 1	Q	GQ267490 *Thelephora terrestris*	99%/695
GQ205372	*Thelephoraceae* 2	P	UDB000219 *Tomentella ellisii*	100%/581
GQ205363	*Thelephoraceae* 2	Q	UDB000219 *Tomentella ellisii*	100%/581
GQ205364	Ascomycotina	P	EU046087 uncult. Ascomycotina	99%/512
GQ205365	*Cortinarius* sp.	P	AY634134 uncult. *Cortinarius*	99%/629
GQ205366	*Atheliaceae*	P	AB456677 uncult. *Tylospora*	99%/618
GQ205368	unc. ECM	P	EF484935 uncult. ECM	99%/589
GQ205367	*Wilcoxina* sp.	P	GQ267499 *Wilcoxina mikolae*	98%/636

* High sequence similarity with *Laccaria bicolor* and *L. laccata* prohibits species level determination.

ECM species colonizing Pinus pinaster

ECM colonization of pine seedlings did not differ significantly between treatments and ranged from 55 ± 7.34 % (mean ± S.E.) in the unburnt soil to 64.2 ± 9.94 % in the site B2 (Table 2). No significant differences were found in ECM richness, diversity or evenness among treatments (see Table 2).

While *Cenococcum geophilum* and both *Rhizopogon* species were present on pine seedlings grown in all four sampled soils, species like *Inocybe jacobi* and *Thelephora terrestris* were found only on the pine seedlings grown in the burnt soils. The frequency and abundance of *Rhizopogon luteolus* Fr. & Nordholm was significantly lower on seedlings grown in soil from sites B1 and B2 when compared with seedlings grown in soil from sites UB and B (Fig. 2a,b; Table 3). A complete opposite pattern was found for *Rhizopogon roseolus* (Corda) Th. Fr. that was favoured by fire, and even more by its recurrence. *Inocybe jacobi* showed a statistically significant increase between UB and B sites, and a decrease from B to B2. Significant differences in the frequency and abundance of *C. geophilum* were observed only between UB and B sites with a decrease of the species after the disturbance event.

Table 2 Occurrence, ECM root colonization (%), species richness, Shannon diversity, and evenness of ECM species on oak (Q) and pine (P) seedlings grown in soils obtained from 4 sites with different fire regimes: UB, unburnt site; B, long fire return interval site; B1, short fire return interval site dominated by *Cistus ladanifer*; B2, short fire return interval site dominated by ericaceous shrubs and *Pterospartum tridentatum*. Letters in superscript indicate statistical differences between sites as determined by the Mann-Whitney test ($p < 0.05$).

Species	P-UB	P-B	P-B1	P-B2	Q-UB	Q-B	Q-B1	Q-B2
Cadophora finlandica					1			
Cenococcum geophilum	11	4	6	6	9	6	11	6
Inocybe jacobi		7	2	1	1	12		
Laccaria proxima 1					1			
Laccaria proxima 2						1		
Lactarius hepaticus					1			
Rhizopogon luteolus	10	10	4	3				
Rhizopogon roseolus	1	4	7	9				
Scleroderma cepa					1			
Scleroderma leave					1			
Scleroderma polyrhizum					2			
Thelephora terrestris		10	2	4	10	6		4
Tomentella ellisii			2				11	8
uncult. Ascomycotina	6			1				
uncult. *Cortinarius*			1					
uncult. ECM fungus				1				
uncult. *Tylospora*	1							
Wilcoxina mikolae	1		9	3				
Root colonization (S.E.)	55 (7.34)	59.1 (6.10)	60 (9.51)	64.2 (9.94)	42.5 (6.9)^{ab}	38.1 (6.0)^{ab}	59.1 (6.8)^{a}	25.5 (6.9)^{b}
Species richness (S.E.)	2.50 (0.167)	3.09 (0.315)	2.57 (0.369)	2.22 (0.278)	2.60 (0.267)^{a}	1.92 (0.211)^{ab}	2.00 (0.00)^{b}	1.64 (0.152)^{b}
Shannon diversity (S.E.)	0.45 (0.112)	0.44 (0.091)	0.40 (0.087)	0.32 (0.102)	0.52 (0.119)^{a}	0.38 (0.082)^{a}	0.24 (0.058)^{a}	0.06 (0.025)^{b}
Evenness (S.E.)	0.45 (0.096)	0.41 (0.079)	0.39 (0.072)	0.32 (0.095)	0.44 (0.114)^{a}	0.37 (0.105)^{ab}	0.35 (0.084)^{a}	0.08 (0.035)^{b}
Total n. of seedlings	12	12	12	12	11	13	11	11

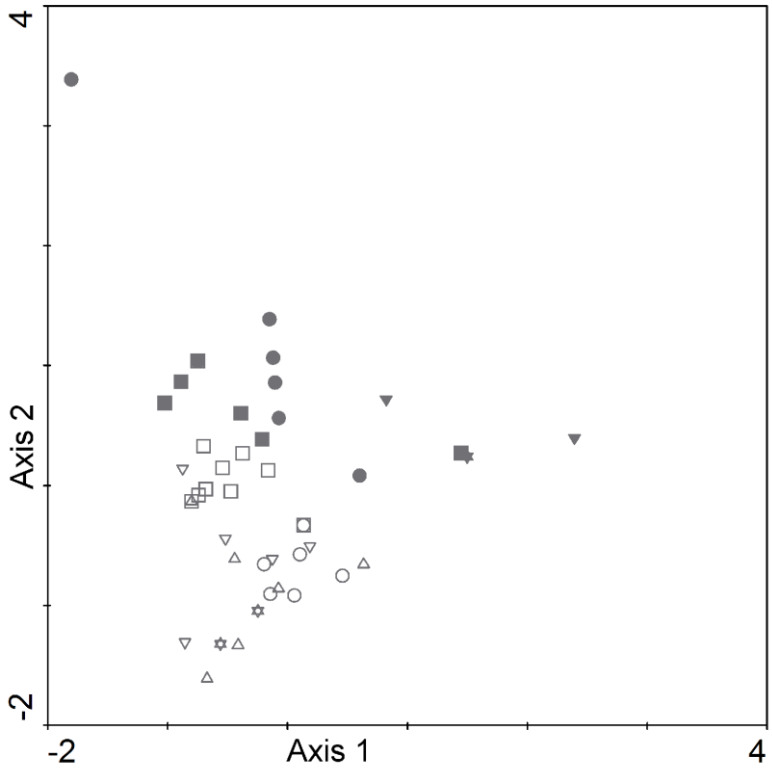


Figure 1 Results of CA performed on presence/absence data of ECM species colonizing *Pinus pinaster* (open symbols) and *Quercus suber* seedlings (closed symbols) grown in soils obtained from four sites with a different fire regime. Each point represents a single seedling. Eigenvalues of axis 1 and 2 are 0.631 and 0.591, respectively, and explain 21 % of the total variation in the data. ○ and ●, unburnt site (UB); □ and ■, long fire return interval site (B); Δ and ▲, short fire return interval site dominated by *Cistus ladanifer* (B1); ∇ and ▼, short fire return interval site dominated by ericaceous shrubs and *Pterospartum tridentatum* (B2).

The frequency and abundance of *T. terrestris* were positively affected by long fire return interval, increasing from UB to B, but negatively affected in the short fire return interval sites. The uncultured Ascomycotina was found only in UB and at low frequencies in B2. *Wilcoxina mikolae* Chin S. Yang & H.E. Wilcox frequency was higher in B1 when compared to UB and B.

The ECM communities of pine seedlings grown in UB and B soils were different although there was a slight overlap between both groups (Fig. 3a). The samples from sites B1 and B2 were mainly grouped together in the left hand side of the ordination plot in correspondence with *W. mikolae* and *R. roseolus*, and showed a greater similarity with the samples from site B than with samples from the unburnt site.

ECM species colonizing Quercus suber

Oak root colonization ranged between 25.5 ± 6.9 % on seedlings grown in soil from site B2 and 59.1 ± 6.8 % on seedlings grown in soil from B1 (Table 2), with a significant difference (*P*-value < 0.05) only between B1 and B2. The number of ECM species found on oak seedlings decreased in soils from burnt sites, both with short and long fire return intervals (Table 2).

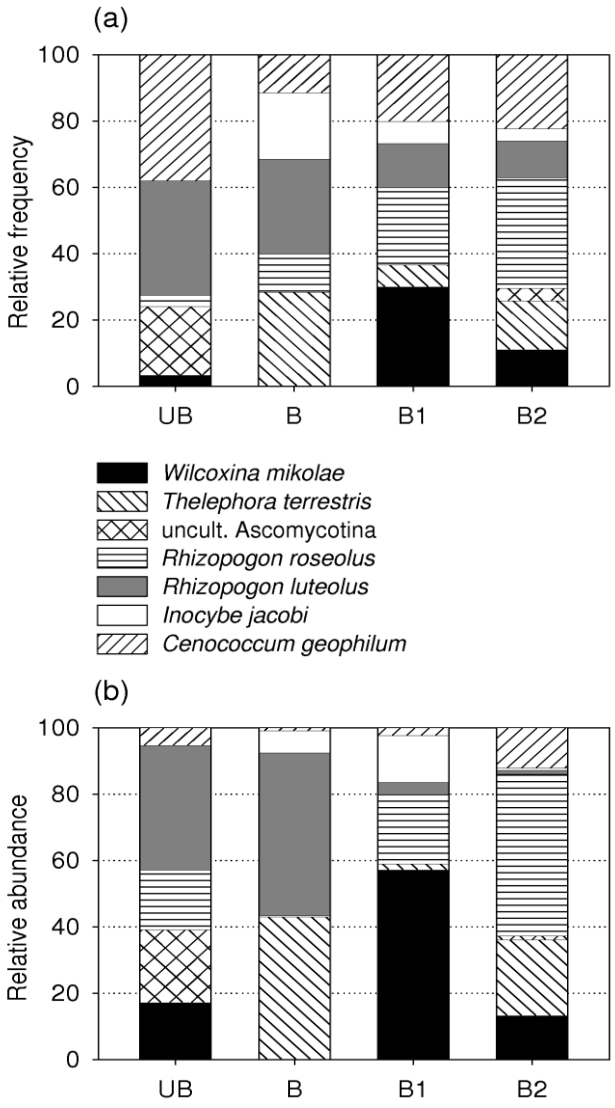


Figure 2 Relative frequency (a) and abundance (b) of different taxa of ECM fungi colonizing pine seedlings grown in soil obtained from an unburnt site (UB), soil obtained from a long fire return interval site (B), soil obtained from a short fire return interval site dominated by *Cistus ladanifer* (B1), and soil obtained from a short fire return interval site dominated by ericaceous shrubs and *Pterospartum tridentatum* (B2).

ECM species richness on oak seedlings was severely affected by short fire return interval decreasing from 2.60 ± 0.267 in the unburnt soil to lower values in short fire return interval sites (B1: 2.00 ± 0.000, *P*-value = 0.050; B2: 1.64 ± 0.152, *P*-value = 0.010). Shannon diversity and evenness were significantly lower (*P*-value < 0.05) on soil B2 than in the remaining treatments (Table 2).

Cenococcum geophilum was the only species colonizing oak seedlings grown in soils of all four sites. Its frequency increased from UB and B to B1, and was higher in B1 than in B2 (Fig. 4a). Six species, *Cadophora finlandica* (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew, *Laccaria* sp. 2, *Lactarius hepaticus* Plowr., *Scleroderma cepa* Pers., *Scleroderma leave* Lloyd and *Scleroderma polyrhizum* (J.F. Gmel.) Pers. were present exclusively in the unburnt soils. *Laccaria* sp. 2 occurred only in the B site. However, due to the high similarity of its ITS sequences with that of *Laccaria* sp. 1 these two isolates might belong to the same species. *Tomentella ellisii* was completely absent in UB and B sites. *Inocybe jacobi* was favoured by a long fire return interval (site B) but its abundance and frequency decreased significantly with a short fire return interval in both B1 and B2 (Fig. 4a,b; Table 3). *Thelephora terrestris* was the most abundant species on oak root tips grown in the unburnt soil and decreased in abundance and frequency in soil from all burnt sites (Fig. 4a,b). The ECM assemblages on oak seedlings clustered into two distinct groups. These corresponded to seedlings grown in soil from the site B and to the seedlings growing in all the remaining plots, at lower and higher values of axis 2 respectively (Fig. 3b). All oak seedlings grown on soil B1 were colonized by *C. geophilum* and *T. ellisii*, which results in a single symbol in the diagram. The CA placed the samples from oak seedlings grown on soil B2 scattered along axis 1 and overlapped with B1 and UB.

Table 3 Results of a non-parametric Kruskal-Wallis test that compares the frequencies and abundances of ECM fungi colonizing pine and oak seedlings grown in 4 different types of soil: soil obtained from an unburnt site (UB), soil obtained from a site (B) with a long fire return interval and two soils obtained from two different sites (B1 and B2) with short fire return intervals.

		Frequency		Abundance	
		χ^2	*P-value*	χ^2	*P-value*
PINE	*Cenococcum geophilum*	8.87	0.031	7.61	0.050
	Inocybe jacobi	14.35	0.002	12.08	0.007
	Rhizopogon luteolus	14.18	0.003	12.68	0.005
	Rhizopogon roseolus	12.19	0.007	9.26	0.026
	Thelephora terrestris	20.56	0.000	16.57	0.001
	uncult. Ascomycotina	16.21	0.001	16.81	0.000
	Wilcoxina mikolae	20.14	0.000	11.02	0.012
OAK	*Cenococcum geophilum*	9.91	0.019	16.71	0.001
	Inocybe jacobi	36.16	0.000	35.55	0.000
	Thelephora terrestris	18.39	0.000	26.75	0.000
	Tomentella ellisii	36.20	0.000	34.07	0.000

Discussion

Fire events usually affect the ECM community directly, and the post-fire ECM community often resembles the pre-fire spore bank to a large extent (Baar *et al.* 1999). However, new fungal species may colonize the affected areas after the disturbance event, benefitting from the release of competition induced by fire. The composition of the ECM communities detected on bioassay seedlings in the present study might therefore be the result of direct effects of fire combined with an indirect posterior spore germination phenomenon. Nevertheless, our results show that the direct and indirect effects of fire affect RPC's and individual ECM fungal species. Although relatively few taxa characterized the resistant ECM propagules communities of the four study areas, species composition and diversity of the RPC's were clearly shaped differently by short and long fire return intervals. The effect of the fire return interval length on individual species varied thereby greatly: infrequent species found in the unburnt soil were completely eliminated in the short fire return interval sites, some species were favoured by long fire return interval but suffered with short fire return interval, and other species positively responded to both fire regimes.

Pine and oak trees have an obligate ecological association with ECM fungi and depend on the presence of fungal inoculum for establishment and survival (Smith & Read 1997). Previous studies on the effects of fire on the RPC associated with other coniferous trees in North America have found similar results to the ones here (Baar *et al.* 1999; Taylor & Bruns 1999; Izzo *et al*. 2006a,b). However, information about the RPC colonizing broadleaved hosts after fire is lacking. Host specific differences were observed in the RPC of the bioassays with only four species shared by *Pinus pinaster* and *Quercus suber*. The ECM assemblages on seedlings belonging to different sites varied depending on the host tree and showed greater similarities for oak than for pine within each site. Also, species richness and diversity were not affected by fire return interval length on pine bioassays, but decreased

on oak bioassays. Oak seedlings grown in soil from short fire return interval sites were colonized only by three non-species specific ECM fungi, while pine was associated with ten ECM fungal species, four of these being in common with oak. These results suggest that recurrent fires can have a more detrimental effect on oak trees than on pine trees. While the presence of ECM fungi in soils could facilitate pine recolonization of disturbed areas, potentially contributing to succession from shrubby areas to pine forest, the loss of suitable oak ECM inoculum in short fire return interval sites, could influence seedlings establishment and performance negatively and therefore hinder the regeneration of *Q. suber*.

Fire had a negative effect on *R. luteolus*, which decreased progressively with fire frequency. *Rhizopogon roseolus*, on the contrary, responded positively to the disturbance event increasing both in frequency and abundance in the burnt sites. Species of the genus *Rhizopogon* are known to be abundant in soil as fire-resistant spores or sclerotia that represent an important source of inoculum after disturbances (Baar *et al.* 1999; Taylor & Bruns 1999; Izzo *et al.*, 2006a,b; Kjøller & Bruns 2003).

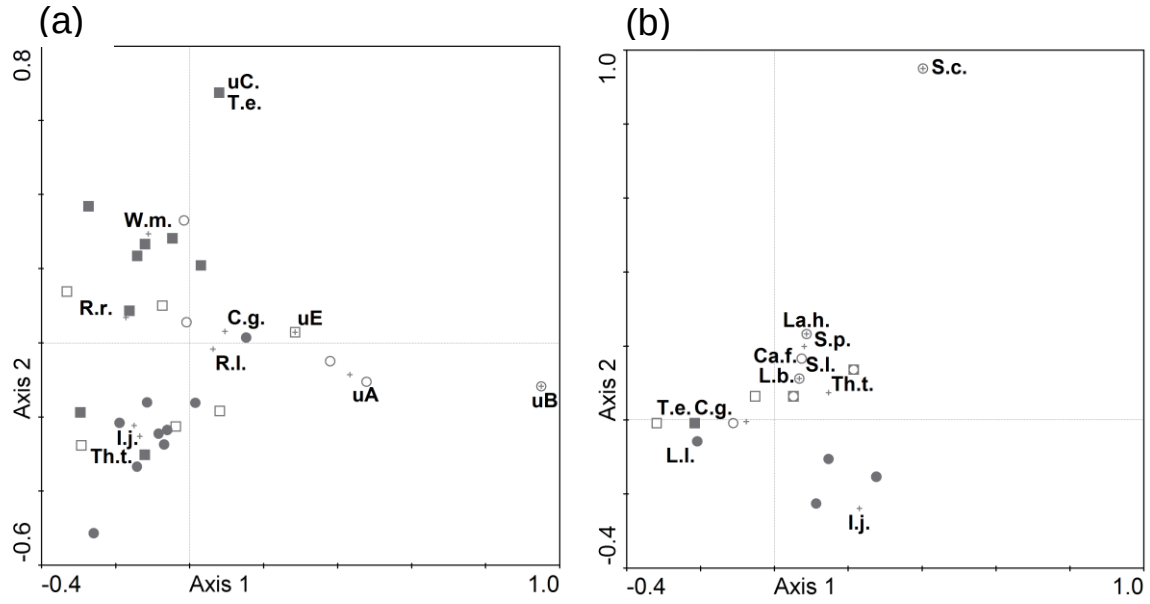


Figure 3 Results of CAs performed on presence/absence data of ECM species colonizing *Pinus pinaster* (a) and *Quercus suber* (b) seedlings grown in soils obtained from four sites with different fire regimes. Each point represents a single seedling. Eigenvalues of axis 1 and 2 are 0.519-0.491 (a) and 0.682-0.539 (b) respectively, and explain 37.8% (a) and 33.4% (b) of the variance of species data. ○, unburnt site (UB), ●, long fire return interval site (B), ■, short fire return interval site dominated by *Cistus ladanifer* (B1), □, short fire return interval site dominated by ericaceous shrubs and *Pterospartum tridentatum* (B2). Species ('+') abbreviations are: *Cadophora finlandica* (Ca.f.), *Cenococcum geophilum* (C.g.), *Inocybe jacobi* (I.j.), *Laccaria proxima* 1 (L.p1), *Laccaria proxima* 2 (L.p2), *Lactarius hepaticus* (La.h.), *Rhizopogon luteolus* (R.l.), *Rhizopogon roseolus* (R.r.), *Scleroderma cepa* (S.c.), *Sleroderma leave* (S.l.), *Scleroderma polyrhizum* (S.p.), *Thelephora terrestris* (Th.t.), *Tomentella ellisii* (T.e.), uncultured Ascomycotina (uA), uncultured *Cortinarius* (uC.), uncultured ECM (uE), uncultured *Tylospora* (uT.), *Wilcoxina mikolae* (W.m.).

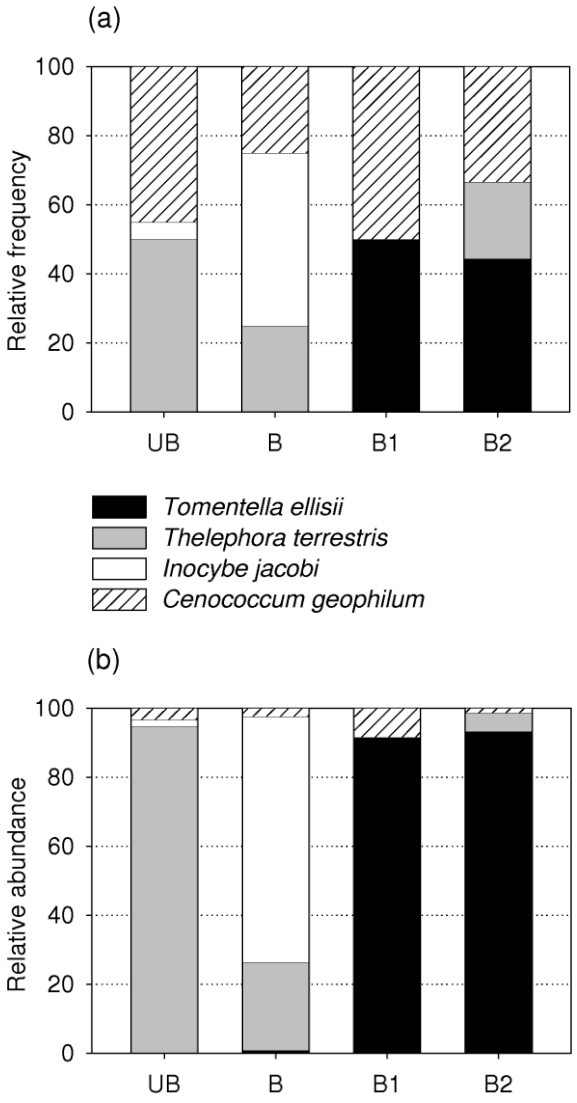


Figure 4 Relative frequency (a) and abundance (b) of ECM taxa colonizing oak seedlings grown in soil obtained from an unburnt site (UB), a long fire return interval site (B), a short fire return interval site dominated by *Cistus ladanifer* (B1), and a short fire return interval site dominated by ericaceous shrubs and *Pterospartum tridentatum* (B2).

Post-disturbance seedling colonization is favoured by a combination of traits characteristic of this genus of hypogeous fungi, such as frequent fruiting, efficient dispersal and long-lived spores (Molina *et al*. 1999).

Competition between the *Rhizopogon* species found in this study is strongly influenced by the local environment and can be shaped by a series of factors like dispersal limitation and nutrient availability (Kennedy *et al*. 2007). The different effect of repeated fires on these species suggests that *R. roseolus* has adaptive traits able to grant it the capacity to stand recurrent stressful conditions such as heat and long-term soil drying.

Inocybe jacobi colonized both pine and oak seedlings and showed a response to the fire event comparable to *R. roseolus*, being apparently favoured by a long fire return interval. Nevertheless, a phenomenon of post-fire colonization by external spores cannot be excluded. Since unburnt and long fire return interval sites are close to each other, post-fire colonization could have been expected to have occurred in both sites. However, changes in biotic and abiotic variables after the fire event could have created potential niches for airborn spores that favoured their settlement exclusively in the disturbed area. The scarce presence of *I. jacobi* in the short fire return interval sites could also be attributed to the negative effect of short fire return interval on this species or to a lower rate of colonization by airborne spores compared to the long fire return interval site. Biotic interactions such as competition and host specificity might also have an effect on the analyzed colonization of bioassay seedlings by different ECM species. For example, *Tomentella ellisii* was more abundant on oak than on pine seedlings in sites B1 and B2.

The number of ECM colonizing species in the soil from short fire return interval sites was higher for pine than oak seedlings, and their interactions could have led to a process of competition that affected *T. ellisii* negatively. Similar processes were described for *R. occidentalis* Zeller & C. W. Dodge and *T. subliacina* by Lilleskov & Bruns (2003), who showed that *R. occidentalis* rapidly colonizes seedling roots from spores after a fire event, which leads to an early peak in the abundance of this species that is later reduced by competition with *T. subliacina*.

The overstorey composition of the short fire return interval sites could also explain quantitative differences in the ECM communities. The survival on shrub roots of mycorrhizal fungal species able to colonize both shrubs and pine trees potentially represents a source of inoculum available for pine seedlings and plays an important role in the process of seedling establishment. The bridge between mycorrhizal fungi and pine seedlings mediated by shrubs is a known example of a bootstrapping connection in ecology (Perry *et al*. 1989) and offers an important chance for ecosystem recovery (Horton *et al.* 1999; Richard *et al.* 2009). Species like *Cenococcum geophilum* and *T. ellisii* on oak, and *W. mikolae* on pine bioassays, were more frequent and abundant in the *Cistus ladanifer* dominated site (B1) than in the site dominated by *Erica* spp. and *Pterospartum tridentatum* (B2). However, due to the very low number of species colonizing oak seedlings in these sites, a small oscillation in the abundance or frequency of one of them in one direction can shift the frequency and abundance of the others in the opposite direction, highlighting differences that cannot be definitely attributed to the overstorey composition. The RPC composition was quite similar in the two sites, and presented the highest number of species colonizing pine seedlings stressing the importance of shrubby areas as sources of ECM inoculum, independently, in this specific case, of the overstorey composition.

Resistant propagules of species of the genus *Laccaria*, *Scleroderma* and *Lactarius* were found solely in soil from unburnt and long fire return interval sites and occurred exclusively associated with oak seedlings. These species are usually observed fruiting in disturbed sites with young trees and shrubs (Danielson 1984; Comandini *et al.* 2006). Their absence in the short fire return interval sites and a single presence in the long fire return interval site could reveal sensitivity to the harsher environmental conditions of the disturbed sites and in particular to the short fire return interval sites, characterized by patches of vegetation growing on a skeletal soil lacking an organic layer. This hypothesis is supported by the fact that oak occurred as natural regenerated seedlings punctually distributed, only in the mature unburnt pine stand. The low soil moisture of the short fire return interval sites, which are more exposed to the effects of sun and wind than the other sites, could possibly have affected oak trees and their symbionts negatively (Kennedy & Peay 2007), hindering oak colonization and favouring more ruderal ECM species associated with pine.

The results obtained in this study indicate that changes in fire return interval affect both RPC structure and composition. Although I am aware of the fact that the direct effects of fire on ECM communities could be masked by changes in host species composition and successional stage, changes in vegetation

structure and composition in the study areas are driven by different fire regimes and shape the ECM community.

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CHAPTER 4

Is the potential for the formation of common mycorrhizal networks influenced by fire frequency?

Introduction

Post-disturbance interaction between early colonizing plants and plants that are established concurrently or at later stages play a key role in plant community (Pugnaire *et al.* 1996; Nara & Hogetsu 2004). The coexistence of plant species is mediated by mycorrhizal fungi that integrate the associated plant species into common mycelial networks (Richard *et al.* 2009; van der Heijden & Horton 2009), which allow nutrient or water sharing between linked individuals (Finlay & Read 1986a,b; Simard *et al.* 1997b), mediate plant competition (Perry *et al*. 1989; Kytöviita *et al.* 2003), prevent nutrient leaching and facilitate seedling establishment (van der Heijden & Horton 2009).

Most mycorrhizal fungi are not host specific and can simultaneously colonize multiple plant species among different canopy trees and between canopy trees and understorey plants (Visser 1995; Horton & Bruns 1998; Horton *et al.* 1999; Cullings *et al.* 2000; Kennedy *et al*. 2003; Ishida *et al.* 2007; Smith *et al.* 2009). Understorey shrubs could therefore play a major role in the recovery of the ecosystem after a disturbance event acting as a bridge between the pre-fire ectomycorrhizal (ECM) communities and emerging seedlings, a mechanism defined as bootstrapping (Perry *et al*. 1989). It is demonstrated that shrubs associated with ECM fungi (*Arbutus* spp. and *Helianthemum* spp.) favour the colonization of neighboring tree seedlings (*Quercus* spp.) in shrubland-forest successions (Dickie *et al.* 2004; Richard *et al.* 2005). Also *Arctostaphylos uva-ursi* L. Spreng., an understorey plant in jack pine mature forests regenerates rapidly by sprouting following wildfire and associates with many of the ECM fungi present on *Pinus banksiana* Lamb. (Visser 1995; Horton *et al.* 1999). The weakening of the close links between plant and soil during disturbance events, and the subsequent alteration of the belowground ecosystem may lead to poor recovery of the original plant guild and to further reductions in abundance of belowground mutualists (Perry *et al*. 1989). The increase of wildfire frequency in Mediterranean ecosystems may therefore compromise the functioning of the ecosystem by leading to the regression of vegetation (Pignatti 1995; Pausas *et al*. 2004; Rodrigo *et al.* 2004), modifying the structure and composition of soil fungal communities (Buscardo *et al*. unpublished) and altering the level of ECM host specificity (Horton & Bruns 1998).

The aim of this study was to determine the level of host specificity of ECM species associated with maritime pine and five understory shrub species. The objectives were (i) to characterize the ECM fungal communities associated with those species in four areas with distinct fire frequency (measured as fire return interval length) and (ii) to determine the influence of fire frequency on the potential for common ECM networks between the five understorey shrub species and *Pinus pinaster* Ait. Molecular methods were used to identify the ECM fungi colonizing the roots of maritime pine and shrub species in these areas. It was hypothesized that recurrent wildfires and consequent changes of vegetation structure reduce the number of shared symbionts between understorey shrub species and maritime pine.

Materials and methods

Study sites

The study area is located in Central Portugal between Alvito da Beira (39º48'N, 7º49'W, altitude 500-600 m) and Isna de Oleiros (39º51'N, 7º51'W, altitude 750-850 m). The region characterized by a Mediterranean climate with hot dry summers and cool wet winters, a lithosol soil and a plant community dominated prior to fire disturbance by *Pinus pinaster*.

Four sampling stands with different fire histories that represent the current fire return interval lengths in this region (Pereira *et al.* 2006) were selected in the study area: an unburnt control stand (UB) that was not affected by wildfire in the last 40 years, a long fire return interval stand (B) characterized by an average fire return interval of approximately 24 years and last affected by wildfire in 2003, and two short fire return interval stands (B1 and B2) with an average of fire return of 7.6 years affected by fire in 1992 and 2003. While long fire return interval length allowed the typical autosuccession response of Mediterranean vegetation ecosystems, short fire return interval length hindered pine regeneration.

UB was characterized by an open uneven-aged maritime pine forest (dominant trees with trunk diameters of 40-45 cm) with an understory shrub community dominated by *Erica* spp., *Halimium* spp. and *Pterospartum tridentatum* (L.) Willk. Stand B showed vigorous natural pine regeneration with the same shrubby species present in stand UB. Stands B1 and B2 were located approximately 5 km from UB/B. The last fire in 2003 changed the vegetation cover radically from a young maritime pine forest to a shrubby area with a vegetation cover dominated by either *Cistus* spp. (B1) with a scattered distribution of *Arbutus unedo* L., or by *P. tridentatum*, *Erica* spp. and *Halimium* spp. (B2). Stands B2, B and UB represent three temporal stages of post disturbance succession. The inclusion of a second stand affected by high fire frequency with a different shrub cover (B1) allowed us to evaluate the possible influence of vegetation composition on the ECM fungi present in these arrested stages of succession.

Root sampling and DNA extraction

In November 2009 soil samples were collected at each stand along a transect of approximately 600 m at the base of different shrub species (Table 1). In every stand, samples were collected from eight individuals of each plant species at a depth of approximately 20 cm using a shovel, resulting in a total of 80 samples.

Roots were extracted from each soil sample in the laboratory, gently washed and examined for mycorrhizal infection under a dissecting microscope. The roots present in 10 out of the 80 samples did not contain ECM root tips and were discarded. All mycorrhizal root tips from the remaining 70 samples were morphotyped and the percentage of root colonization was recorded for each morphotype. Single ECM root tips, one to six of each morphotype per soil core were subjected to DNA extraction and PCR amplification of the nrDNA internal transcribed spacer (ITS) region as

described in Buscardo *et al*. (2010). DNA was extracted from 265 ECM samples from which 333 PCRs were performed to amplify the fungal DNA. Single PCR products were purified from agarose gel using QIAquick gel PCR purification kit (QIAGEN) and sequenced at Macrogen laboratories (South Korea). DNA sequences were manually checked and edited if necessary using the BioEdit software version 7.0.9.0 (Hall 1999). The fungal sequences were compared with UNITE (Kõljalg *et al.* 2005) and INSD (International Nucleotide Sequence Database) online nucleotides databases using the BLASTn algorithm (Altschul *et al.* 1997) and submitted to the EMLN-EBI database with the accession numbers indicated in Tables 2 and S1. Because the possibility of host misidentifications was high due to the presence of very closely related shrub species, an overestimation of the potential for mycorrhizal networks was avoided by identification of the host with molecular methods. The *trn*L (UAA) intron region was amplified in all 265 root samples using the *trn*L-c and *trn*L-d primers (Taberlet *et al.* 1991) with the same PCR conditions used for fungal ITS region. The obtained sequences were compared with those available in GenBank by BLAST searches in the NCBI web page (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A different sequences alignment was elaborated manually for each of the occurring plant families using the program Se-Al v.2.0a11 (Rambaut 2002). To identify taxa within *Cistaceae*, the sequences were first analyzed by parsimony bootstrap analysis (Felsenstein 1985), implementing 10,000 replicates of the fast heuristic search (Mort *et al.* 2000) in PAUP* 4.0b10 (Swofford 2002) and using *Shorea* (*Dipterocarpaceae*) as outgroup. The identification of the *Cistus* clades was straightforward comparing our sequences with those deposited in GenBank. However, since for *Halimium* spp. there are not enough sequences of the trnL intron to identify our two clades, the complete trnL-trnF region, including the trnL intron and trnL-F spacer, was amplified from representatives of the two clades using the primers trnL-c and trnF-f (Taberlet *et al.* 1991) and the same PCR conditions previously described. The sequences of the trnL-F spacer obtained were included in an alignment with sequences of all the species of *Halimium* from the Iberian Peninsula together with four species of *Cistus* and *Hudsonia* that was selected as the outgroup. The matrix was analyzed by parsimony bootstraping as previously described.

If roots belonging to multiple hosts were present in a soil sample, only fungal species that colonized the expected host were taken into consideration for analyses. In 9 out of the 70 screened soil samples, the root tips belonged to a plant species other than the expected host and were therefore considered as additional samples for that host (for example, a sample from *Arbutus unedo* L. containing more than 95% of ECM roots from *Cistus ladanifer* L. was assigned to *C. ladanifer* but not to *A. unedo*).

Data analysis

Rarefaction curves were generated for each stand and each host using the program EstimateS, version 8 (Colwell 2006).

Both frequency and abundance of the different ECM species on each host were used to test for differences in the ECM community in the four stands. Frequency was estimated from the

presence/absence data matrix of each fungal species. Relative abundance was calculated for each stand as the number of root tips colonized by a taxon divided by the total number of ECM root tips identified at that stand. Non-parametric Kruskal-Wallis and Mann-Whitney U-tests were used to compare species frequency and abundance in the four stands, followed by the Bonferroni-Holm's procedure (Holm 1979) for controlling experiment wise error rates for multiple independent tests. Species that occurred too infrequently to apply statistical tests were left out. Non-parametric Kruskal-Wallis followed by Mann-Whitney U-tests were also used to assess the relative frequency and abundance of the ECM taxa shared by maritime pine and understorey shrub species across the four stands. Statistical testing was carried out using SPSS version 17.0 (SPSS Inc., Chicago, USA).
Detrended Correspondence analyses (DCAs) were performed to analyze the ECM assemblage on different hosts in the four sampling stands using data on ECM abundance in each sample. These analyses were performed with CANOCO version 4.5 (Microcomputer Power, Ithaca, NY, USA).

Results

ECM community structure

Using a 97 % sequence similarity cutoff, 45 fungal species were identified from the 333 PCRs performed on plant root samples. Most sequences (62.2%) corresponded to ECM species but 17 non-ECM root inhabitants fungi (37.8% of all fungal species) were also found (Table S1). A total of 28 ECM species belonging to 18 different genera was identified (Table 2). Basidiomycetes and ascomycetes accounted for 76.9 % and 23.1 % of the identified species, respectively.

Table 1 Plant species sampled in four stands with 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 ericaceous shrubs, *Halimium* spp. and *Pterospartum tridentatum*.

Species sampled	UB	B	B1	B2
Pinus pinaster Ait.	x	x		
Halimium ocymoides (Lam.) Willk.	x	x		x
Halimium lasianthum (Lam.) Spach *subsp. alyssoides* (Lam.) Greuter & Burdet		x		x
Arbutus unedo L.			x	
Cistus ladanifer L.			x	
Cistus laurifolius L.			x	

Rarefaction curves increased with the number of samples without reaching a plateau for all stands and for all hosts species within each stand except for *Halimium lasianthum* (Lam.) Spach *subsp. alyssoides* (Lam.) Greuter & Burdet and *Pinus pinaster* (B) of which the rarefaction curve tended to level off (Fig 1). The species accumulation curves had strongly overlapping confidence intervals (not shown) suggesting no substantial difference in ECM diversity among stands and plant hosts with the exception

of stand B2 (between stands) and *H. lasianthum* subsp. *alyssoides* (between hosts). However, since the number of samples with ECM root tips belonging to *H. lasianthum* subsp. *alyssoides* in B2 was reduced (n=3) any speculation regarding species richness differences between B2 and the other stands should be approached with caution. Approximately 36% of the species occurred only in one sample. *Cadophora finlandica* (C.J.K. Wang & H. E. Wilcox) T.C. Harr. & McNew colonized exclusively the shrubs of stand B1 while *Tomentellopsis* sp. and *Tomentella terrestris* (Berk. & Broome) M.J. Larsen were found just in the unburnt stand (Table 2).

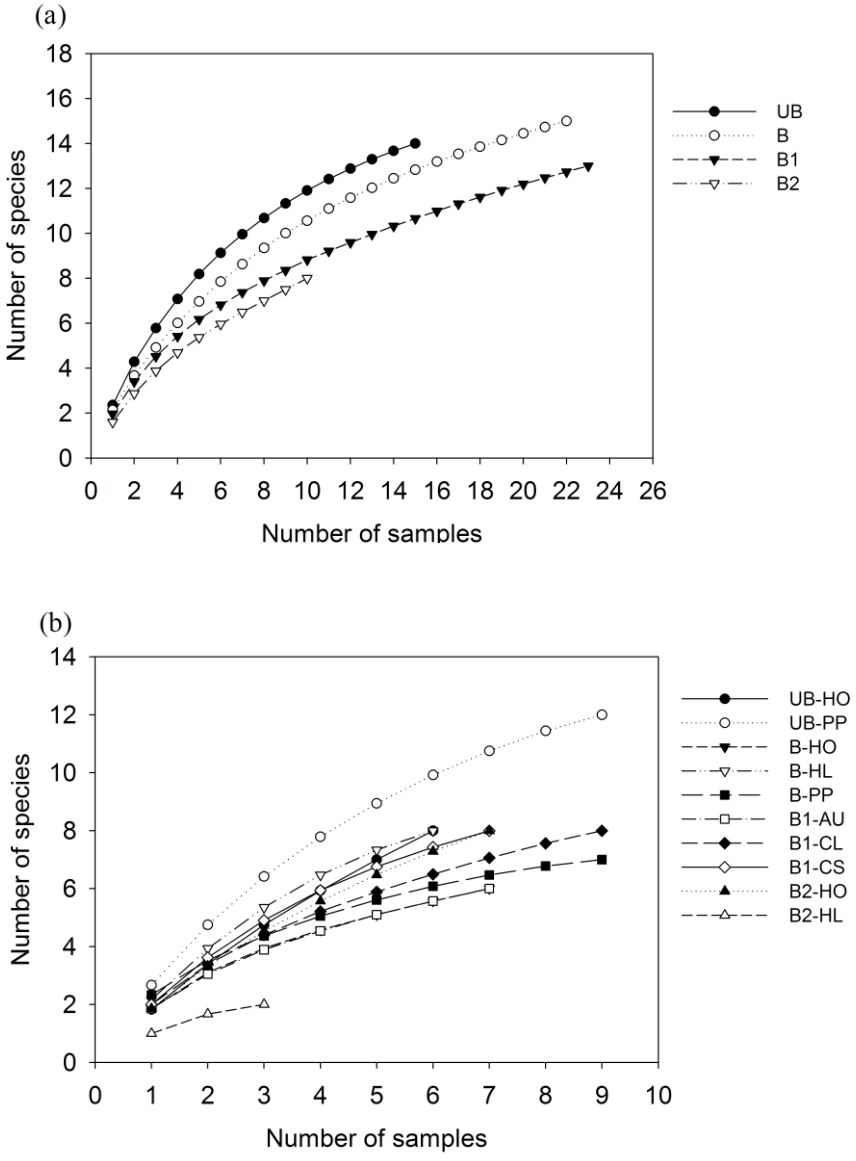

Figure 1 Rarefaction curves of ECM fungal species in four stands subjected to different fire return intervals (a) and on different host species present in the four stands (b). UB, unburnt stand; B, long fire return interval stand; B1, short fire return interval stand dominated by *Cistus ladanifer*; B2, short fire return interval stand dominated by ericaceous shrubs, *Halimium* spp. and *Pterospartum tridentatum*; PP, *Pinus pinaster*; HO, *Halimium ocymoides*; HL, *H. lasianthum* subsp. *alyssoides*; AU, *Arbutus unedo*; CL, *Cistus ladanifer*; CS, *C. laurifolius.*

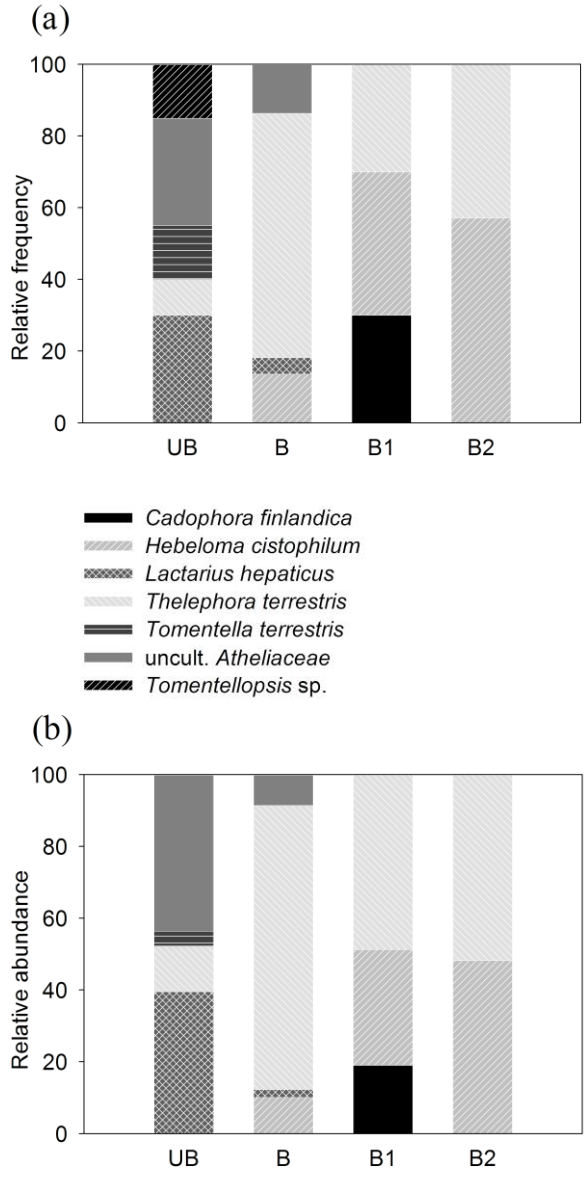

Figure 2 Relative frequency (a) and abundance (b) of different ECM taxa found in four stands affected by differing fire frequency. UB, unburnt stand; B, long fire return interval stand; B1, short fire return interval stand dominated by *Cistus ladanifer*; B2, short fire return interval stand dominated by ericaceous shrubs, *Halimium* spp. and *Pterospartum tridentatum*.

The most frequent and abundant taxa across the whole area were *Thelephora terrestris* Ehrh., *Cenococcum* sp., *Hebeloma cistophilum* Maire, *Lactarius hepaticus* Plowr. and an uncultured *Atheliaceae* (Fig 2; Table 2). The frequency and abundance of *T. terrestris* were significantly higher in stand B when compared with all the other stands except for the abundance in stand B2 that was not significantly different from that in B (Fig 2; Table 3).

Hebeloma cistophilum colonized all shrubs exclusively in the burnt stands and its frequency and abundance were significantly higher in both short fire return interval stands if compared with the unburnt stand (UB). *Lactarius hepaticus* and the uncultured *Atheliaceae* were the most frequent and abundant species found in the unburnt stand (UB). The frequency and abundance of *L. hepaticus* were significantly higher in stand UB if compared with all the burnt stands. The uncultured *Atheliaceae* was significantly more frequent in UB than in the short fire return interval stands and more abundant in UB than in all the burnt stands.

The detrended correspondence analyses (DCA) separated most UB samples from those of all burnt stands (Fig 3). Samples from UB and B1 were placed at opposite sides of axis 1, both in terms of frequency (data not shown) and abundance (Fig. 3).

Table 2 Database matches of ITS sequences obtained from ECM fungi colonizing four shrubs and maritime pine grown in stands with different fire regimes and relative frequences. Blast expected value was equal to 0.0 for all the alignments. 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 ericaceous shrubs, *Halimium* spp. and *Pterospartum tridentatum*. PP, *Pinus pinaster*; HO, *Halimium ocymoides*; HL, *H. lasianthum* subsp. *alyssoides*; AU, *Arbutus unedo*; CL, *Cistus ladanifer* and CS, *C. laurifolius*.

Morphotype	Accession number	Best blast hit	% Similarity/ Overlap (bp)	UB		B			B1			B2	
				PP	HO	PP	HO	HL	CL	CS	AU	HO	HL
Amanita sp.	HQ625456	*Amanita* sp. FJ013072	97/540		1								
Cadophora sp.	HQ625450	*Cadophora finlandica* AF486119	98/868						2	1	3		
Cenococcum sp.	HQ625444	*Cenococcum* sp. FJ152539	98/916		3		2	2	4	2	5	2	2
Cortinarius sp	HQ625460	*Cortinarius belleri* AY669685	96/587						3				
Cortinarius sp.	HQ625476	*Cortinarius* sp. AY634134	98/590			1							
Hebeloma sp.	HQ625447	*Hebeloma cistophilum* EU570177	99/674				1	2	5	3		3	
Inocybe sp.	HQ625486	*Inocybe jacobi* FN550883	99/573				1						
Lactarius sp.	HQ625465	*Lactarius hepaticus* UDB000861	99/686	4	2			1					
Rhizopogon sp1	HQ625448	*Rhizopogon luteolus* GQ267481	99/828	1		2						1	
Rhizopogon sp2	HQ625451	*Rhizopogon roseolus* HM036649*	99/698		1	5			1				
Rhodocollybia sp.	HQ625469	*Rhodocollybia butyracea* AF505750	93/545	1									
Russula sp1	HQ625470	*Russula densifolia* UDB000336	99/659					1					
Russula sp2	HQ625452	*Russula drimeia* EU557320	99/663	2			1		1				
Russula sp3	HQ625471	*Russula gracilis* FJ845431	99/667						1		1		
Terfezia sp1	HQ625472	*Terfezia leptoderma* AF396863	91/565			2						1	
Terfezia sp2	HQ625473	*Terfezia leptoderma* AF396863	96/587					1					
Thelephoraceae 1	HQ625443	*Thelephora terrestris* UDB003345	99/617	1	1	8	5	3	2	2	2	2	1
Thelephoraceae 2	HQ625474	*Tomentella terrestris* UDB003315	99/641	2	1								
Thelephoraceae 3	HQ625483	*Tomentellopsis* sp. FJ188365	99/767	2	1								
Ascomycotina 1	HQ625454	uncultured Ascomycota EU046087	99/469	2									
Atheliaceae	HQ625445	uncultured *Atheliaceae* EU557321	98/581	5	1	2		1					
Basidiomycotina	HQ625449	uncultured Basidiomycota FM866342	99/512				4		1		1	1	
Cantharellaceae 1	HQ625475	uncultured Cantharellales AY880947	99/589									1	
Cantharellaceae 2	HQ625485	uncultured Cantharellales DQ822796	95/627	2									
uncultured ECM 1	HQ625477	uncultured ectomycorrhizal fungus FJ013076	99/605				1	2	1			1	
uncultured ECM 2	HQ625455	uncultured ectomycorrhizal fungus FJ013067	90/624						1				
Sebacinaceae	HQ625482	uncultured *Sebacinaceae* FM992969	99/579								1		
Wilcoxina sp.	HQ625484	*Wilcoxina* sp. FM992988	96/562						1				
Total species richness				10	8	6	7	8	12	4	6	8	2
Total n. of samples				9	6	9	7	6	9	7	7	7	3

*according to Martín & García (2009) this sequence corresponds to R. rubescens (Tu. & C. Tul.) Tul. & C. Tul.

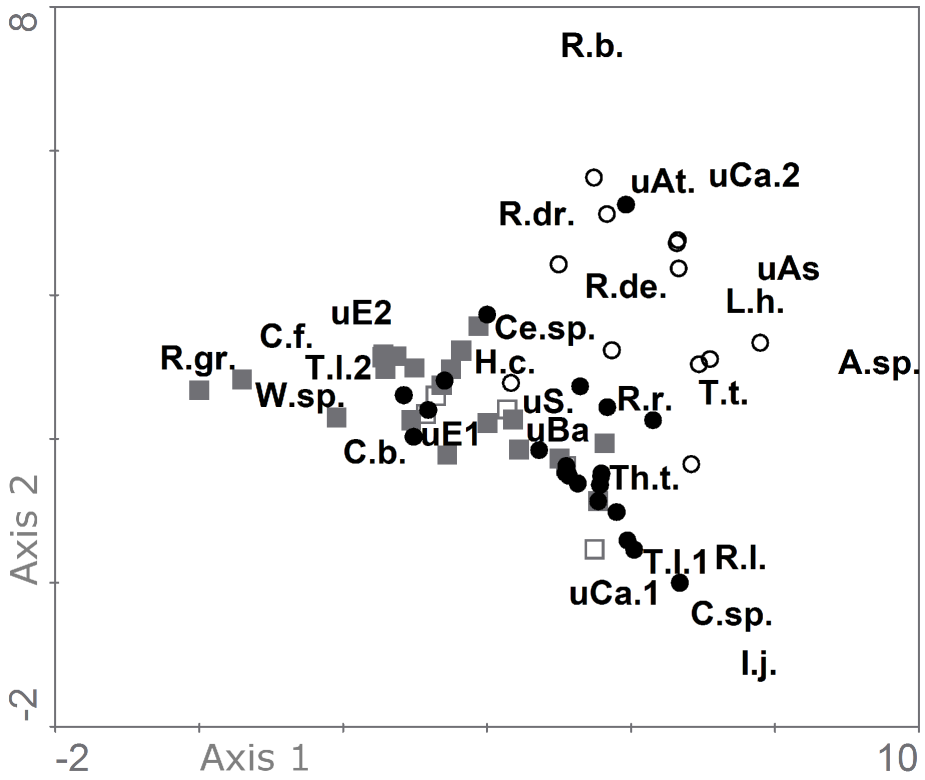

Figure 3 Results of DCA performed on abundances data of ECM species in four stands affected by differing fire frequency: ○ unburnt stand (UB); ● long fire return interval stand (B); ■ short fire return interval stand dominated by *Cistus ladanifer* (B1); □ short fire return interval stand dominated by ericaceous shrubs, *Halimium* spp. and *Pterospartum tridentatum* (B2). Each point represents a soil sample. Eigenvalues of axis 1 and 2 are 0.863 and 0.731 respectively and explain 14.1% of the variance of the species data. Specie abbreviations are: A.sp. (*Amanita* sp.), C.f. (*Cadophora finlandica*), C.b. (*Cortinarius belleri*), C.sp. (*Cortinarius* sp.), H.c. (*Hebeloma cistophilum*), I.j. (*Inocybe jacobi*), L.h. (*Lactarius hepaticus*), R.l. (*Rhizopogon luteolus*), R.r. (*Rhizopogon roseolus*), R.b. (*Rhodocollybia butyracea*), R.de. (*Russula densifolia*), R.dr. (*Russula drimeia*), R.gr. (*Russula gracilis*), T.l.1 (*Terfezia leptoderma* 1), T.l.2 (*Terfezia leptoderma* 2), Th.t. (*Thelephora terrestris*), T.t. (*Tomentella terrestris*), To.sp. (*Tomentellopsis* sp.), uAs (uncultured Ascomycota), uAt. (uncultured *Atheliaceae*), uBa (uncultured Basidiomycota), uCa.1 (uncultured *Cantharellales* 1), uCa.2 (uncultured *Cantharellales* 2), Ce.sp. (*Cenococcum* sp.), uE1 (uncultured ECM fungus 1), uE2 (uncultured ECM fungus 2), uS. (uncultured *Sebacinaceae*), Wi.sp. (*Wilcoxina* sp.).

Host specificity

Overall, 32.1% of the identified ECM species were common to pine and shrubs. All common fungal species (*Lactarius hepaticus, Rhizopogon luteolus, R. roseolus, Russula drimeia* Cooke, *Terfezia* sp. 1, *Thelephora terrestris, Tomentella terrestris, Tomentellopsis* sp. and the uncultured *Atheliaceae*) were present on the roots of *Halimium ocymoides* and a lower percentage occurred on the other shrubs species (Fig. 4a). *Thelephora terrestris* occurred on all host plants and was the only species in common between *Pinus pinaster* and *Arbutus unedo* while *Cistus* spp. and *Halimium lasianthum* subsp. *alyssoides* had three fungal species in common with *P. pinaster*. The highest number of ECM species occurred on *Halimium* spp. (17 species), followed by *P. pinaster* (14), *Cistus* spp. (12), and *A. unedo* (6). All together, shrub species shared three ECM fungi: *T. terrestris*, the uncultured Basidiomycota and *Cenococcum* sp. (Fig. 4b).

The proportion of ECM symbionts potentially common between pine and shrubs decreased from the unburnt stand (62%), to stands B (53%), B1 (38%) and B2 (23%). ECM species common to pine and shrubs represented 78% of the total fungal abundance in UB, 64% in B and 34% and 25% in B1 and B2 respectively.

Table 3 Results of a non-parametric Kruskal-Wallis test that compares the frequencies and abundances of ECM taxa colonizing maritime pine and understorey shrub species in four stands subjected to different fire return intervals.

	Frequency		Abundance	
	χ^2	*P-value*	χ^2	*P-value*
Cadophora finlandica	13.11	0.003	13.09	0.003
Hebeloma cistophilum	7.61	0.050	7.50	0.050
Lactarius hepaticus	21.23	0.000	21.61	0.000
Thelephora terrestris	14.41	0.002	10.90	0.009
Tomentella terrestris	12.53	0.011	12.53	0.013
Tomentellopsis sp.	12.53	0.011	16.64	0.001
uncultured *Atheliaceae*	16.31	0.001	ns	ns

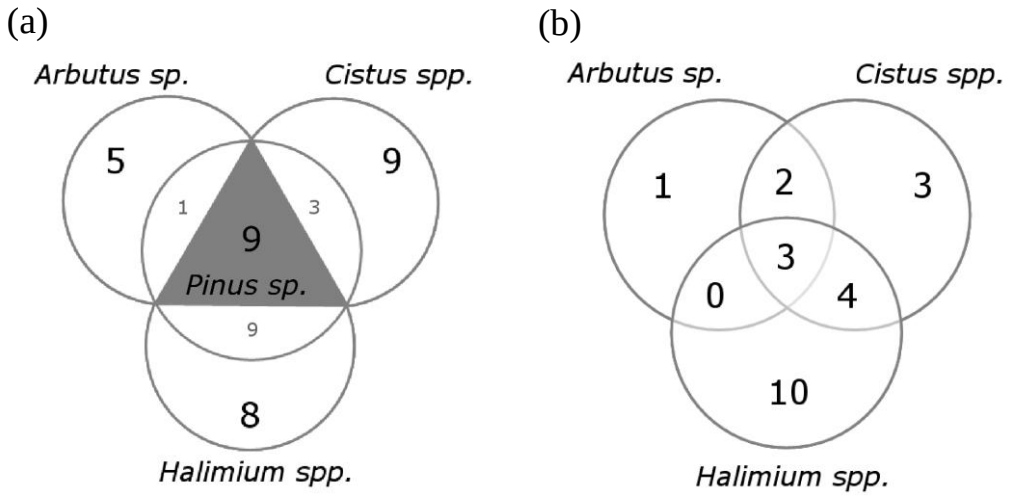

Figure 4 Distribution of ECM fungal species common to *Pinus pinaster* and understorey shrub species (a) and to different shrubs genera (b). Values represent the number of ECM species for each plant host. Numbers at intersections are ECM species common between the intersecting species. The value in the triangle represents the total number of species in common between pine and shrubs and small values in grey represent the number of species in common between pine and each of the three shrubs genera: *Arbutus unedo* (*Arbutus* sp.), *Cistus ladanifer* and *C. laurifolius* (*Cistus* spp.), and *Halimium ocymoides* and *H. lasianthum* subsp. *alyssoides* (*Halimium* spp.).

Significant differences in the frequency and abundance of common ECM species were found between the short fire return interval stands (B1 and B2) and the other two stands – the unburnt stand (UB) and the long fire return interval (B) (Table 4). The non-mycorrhizal root inhabitants fungal species identified from the analysed root tips occurred with very low frequency (Table S1). The two most

frequent and abundant species were an unidentified ericoid fungi and *Phialocephala fortini* C.J.K. Wang & H.E. Wilcox. The unidentified ericoid mycorrhizal fungi was the most common species found in the short fire return interval stands, while *P. fortini* was the most frequent species in the unburnt stand.

Discussion

With this study the ECM fungi on the root system of five typical Mediterranean understorey shrub species and *Pinus pinaster* in four areas affected by different fire return interval length were identified, multiple-host symbionts were detected, and the influence of fire frequency on the potential for common ECM networks between shrub species and pine was verified.

While some ECM fungal species exhibit host specificity, most of them are generalist (Bruns *et al.* 2002). Thus, plants benefit from the association with a large variety of ECM fungal species that may allow them the access to additional nutrient pools and increase the probabilities that seedlings will not be limited after dispersal in new settings (Bruns *et al.* 2002). Non-specific ECM fungi might help to stabilize the forest community through disturbance events by associating with both pre- and post-disturbance hosts (Perry *et al*. 1989).

Table 4 Results of Mann-Whitney U-tests used to test for differences in frequencies and abundances of ECM taxa potentially common to maritime pine and understorey shrub species in four stands subjected to different fire 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 ericaceous shrubs, *Halimium* spp. and *Pterospartum tridentatum*.

	UB-B	UB-B1	UB-B2	B-B1	B-B2	B1-B2
Frequency						
U	ns	32	22.5	139	78.5	ns
P-value		0.000	0.000	0.000	0.012	
Abundance						
U	ns	37.5	17.5	141	73	ns
P-value		0.007	0.010	0.000	0.007	

The presence of ECM fungi on early colonists is therefore essential for facilitating the establishment of neighboring seedlings of successional woody plant species (Nara & Hogetsu 2004), providing compatible fungal inoculum even to phylogenetically distant plant individuals as occurs in primary succession (Nara 2006). Our study revealed that approximately one third of the ECM species occurred on both pine and shrubs. The same value was found in a mixed evergreen forest between *Pseudotsuga menziesii* (Mirb.) Franco and *Lithocarpus densiflora* (Hook & Arn.) Rehd. Liebm. Tanoak (Kennedy *et al*. 2003) but is higher than the 13% found in common between *A. unedo* and *Quercus ilex* L. in a Mediterranean forest (Richard *et al.* 2005).

Disturbances such as fire generally induce a shift in the ECM community from narrow host range fungi to wide host range fungi (Jones *et al*. 1997; Simard *et al.* 1997a; Horton & Bruns 1998). By contrast, in the present study, the proportion of potential ECM symbionts in common between pine and shrubs did not change significantly between the unburnt stand (62%) and the low fire return interval stand B (53%). Considering that the composition of post fire ECM communities has a strong resemblance to the composition of the pre-fire resistant propagules community (RPC) (Baar *et al.* 1999; Taylor & Bruns 1999) a rough estimation of the composition of the ECM fungal community able to colonize regenerating aboveground vegetation immediately after a fire event could be done by looking at the composition of the RPC in the study area. Based on results from a previous study of the RPC in the same stand (Buscardo *et al.* 2010), the proportion of potential non-specific symbionts was lower in the unburnt stand (67%) if compared with all burnt stands (B, 80%; B1 and B2, 75%) supporting the dominance of multiple host species immediately following disturbance. Nevertheless, fire-induced shift to wide host range fungi might not persist longer than 4-6 years (Twieg *et al.* 2007), which could explain the lack of current significant differences between stands UB and B.

Our hypothesis that recurrent wildfires reduce the number of potential shared symbionts between understorey shrub species and maritime pine was supported by the results of this study. While differences in the percentage and abundance of common species were not significant between the unburnt stand and the long fire return interval stands, they were pronounced between UB and B on one side and both short fire return interval stands on the other side. Although it has to be considered that ECM tips were found in a reduced number of samples in stand B2 (n=10) if compared with the other stands and that this could have affected diversity and community composition measures, the number of species in common between pine and shrubs did not substantially increase with sample size. Therefore, the observed changes have to be attributed to a series of direct and indirect effects of wildfire aboveground and belowground. Soil nutrient composition of the burnt stands revealed reduced organic C, Ca^{2+} and Mg^{2+} compared to the unburnt stand, and lower N concentrations in the short fire return interval stands than in UB and B (Buscardo *et al*. unpublished). Furthermore, natural pine regeneration was prevented by the short fire return intervals with consequent changes of the aboveground vegetation structure and obvious alterations of the total soil fungal communities' structure and composition (Buscardo *et al*. unpublished). Some ECM species appeared with higher frequencies and colonized exclusively shrubs growing in the burnt areas. For example, *Hebeloma cistophilum,* considered to be a *Cistus*-specific symbiont (Comandini *et al.* 2006), colonized both *Cistus* spp. and the two species belonging to the closely related genus *Halimium*, which has recently been included into *Cistus* (Demoly 2006). Wind-born spores were probably the primary inoculum source for *Hebeloma* sp. (Baar *et al.* 1999; Buscardo *et al.* 2010) and its absence from the unburnt pine forest could be attributed to its poor competitive nature in a mature forest where other competing taxa associated with the canopy dominant tree (Gardes & Bruns 1996; Baar *et al.* 1999; Smith *et al*. 2004; Martín-Pinto *et al.* 2006). The reduced number of species that can associate with both shrubs and pine

in the short fire return interval stands might also be attributable to the absence of pine in these stands because ECM fungi have the tendency to switch between hosts that share the same environment (Comandini *et al.* 2006).

Changes of vegetation structure and composition caused by repeated burnings may, in part, be responsible for changes in belowground components (Hart *et al.* 2005; Anderson *et al*. 2003). Plant host identity strongly influences ECM communities especially when there is an increasing phylogenetic distance between hosts (Ishida *et al.* 2007). Single-host ECM taxa across the four stands corresponded to 46% of total species richness and approximately three fourth of them were found only once, so an underestimation of the actual number of fungi that would have probably been detected with a more extensive sampling including other host species cannot be excluded. For example, typical components of the macromycetus fungal flora in *Cistus*-dominated plant communities, known to associate with maritime pine, such as *Amanita muscaria* (L.) Lam. and *Laccaria laccata* (Scop.) Cooke (Comandini *et al.* 2006) were not detected in plant roots but found as sporocarps in stand B1.

Other host specific species like *Cortinarius belleri* M.M. Moser (Comandini *et al.* 2006) also occurred exclusively in the *Cistus* dominated stand (B1). However, more than 60% of the ECM species of B2 occurred in B1 and these two stands also had a similar pattern of ECM abundance, which suggests that similar fire history is playing a larger role in ECM fungal composition than vegetation differences.

The root system of many plant hosts is often colonized by facultative root-associating fungi such as endophytes (Vrålstad *et al.* 2002) that, together with mycorrhizosphere microbes, enhance the functioning of plant-soil interactions (Tedersoo *et al.* 2009). In this study, 17 ectomycorrhizal associated fungi mostly closely related to root endophytes and ericoid mycorrhizal fungi that are common root inhabitants of non-ericaceous plants (Bergero *et al.* 2000; Chambers *et al.* 2008) were detected. The distribution of these microfungi appears to be influenced by host specificity and by changes in the vegetation structure (Anderson *et al*. 2003; Tedersoo *et al.* 2009). Although most of the root inhabitant fungi occurred with very low frequency, differences could still be detected between the unburnt stand and the short fire return interval stands. An abundant ericoid mycorrhizal fungus colonized preferentially the host in both short fire return interval stands, while *Phialocephala fortinii* colonized preferentially plants in the unburnt stand. In a previous study on the total soil fungal community higher abundances of Helotiales species belonging to the *Pezoloma ericae* (D.J. Read) Baral aggregate (synonymous: *Hymenoscyphus ericae* (D.J. Read) Korf & Kernan) were found in stand B2 than in all the other stands (Buscardo *et al*. unpublished). Ericoid mycorrhizal fungi are characterized by high enzymatic competence, slow growth, and apparent absence of reproductive structures and are well suited to persist under the stressful conditions of Mediterranean summers, even in the absence of the host plant (Bergero *et al.* 2003). These functional traits might also provide a competitive advantage in a highly disturbed environment of high fire return interval stands where qualitative and quantitative fluctuations of N supply occur at both temporal and spatial scales (Grelet *et al.* 2009).

The presence of compatible pine symbionts on understorey shrubs after a fire event may allow pine seedlings to tap into already functioning ECM networks, potentially lowering the costs of establishing mycorrhizal infection and offering the access to larger nutrient pools (Newman 1988; Dickie *et al.* 2004). This study shows how fire frequency affects the availability of compatible pine symbionts and the potential facilitation offered by the ECM networks for pine regeneration. To demonstrate the mycorrhizal connection between understorey shrubs and pine trees future research should address the identification of fungal genotypes with taxon specific primers or fingerprinting methods (Egger 1995; Grelet *et al.* 2010).

The main conclusion obtained with this work is that while the stand affected by low fire interval return seemed to maintain the potential for common ECM networks between pine and understorey shrub species, both short fire return interval stands showed a drastic reduction of common potential symbionts between shrubs and pine. Recurrent fires could therefore limit the bootstrapping effect of shrubs and delay the colonization by pine seedlings of frequently burnt forest areas of the Mediterranean region.

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Supplementary information

Table S1 Database matches of ITS sequences obtained from ectomycorrhizal associated fungi of four shrubs and maritime pine grown in stands with different fire regimes. Blast expected value was equal to 0.0 for all the alignments. 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 ericaceous shrubs, *Halimium* spp. and *Pterospartum tridentatum*. PP, *Pinus pinaster*; HO, *Halimium ocymoides*; HL, *H. lasianthum* subsp. *alyssoides*; AU, *Arbutus unedo*; CL, *Cistus ladanifer* and CS, *C. laurifolius*.

Accession number	UB		B			B1			B2		Best blast hit	% Similarity/ Overlap(bp)
	PP	HO	PP	HO	HL	CL	CS	AU	HO	HL		
HQ625481						1					AB512345 *Mycena* sp.	96/705
HQ625466			1		1						AF062789 *Oidiodendron chlamydosporicum*	94/527
HQ625467									1		AF062800 *Oidiodendron maius*	99/527
HQ625442		2			1	5	4	3	4	1	AF072303 ericoid mycorrhizal sp.	99/524
HQ625468				1					1	1	AM887700 *Rhizoscyphus ericae*	98/540
HQ625462							1				AY627824 *Epacris pulchella* root associated fungus	99/536
HQ625446	4	4					1				EF093161 *Phialocephala fortinii*	99/542
HQ625463				1							EF093184 *Gyoerffyella* sp.	99/526
HQ625461	1										EF619850 uncultured Ascomycota	94/512
HQ625459										2	EU082785 *Coniochaetaceae* sp.	93/554
HQ625457						1					EU639688 *Helotiales* sp.	97/525
HQ625480							1				FJ152529 uncultured Leotiomycetes	95/509
HQ625479						1					FJ475762 uncultured *Helotiales*	99/533
HQ625458						1					GQ411520 *Colpoma* sp.	93/523
HQ625453									1	2	GU092954 *Hamigera avellanea*	94/438
HQ625478	1										GU289426 uncultured fungus	99/632
HQ625464					1						HM036626 fungal sp.	98/477

Factors involved in post-fire pine regeneration

Maritime pine regeneration and subsequent development of seedlings depends not exclusively on post-fire environmental factors but also on seedlings association with symbiotic ectomycorrhizal (ECM) fungi. This research revealed that different environmental factors explained post-fire regeneration variability at the Ligurian study area. Sapling performance was enhanced at higher elevations, moderate slopes, shallower soils, and a reduced bare ground cover. These conditions were found in the proximity of post-fire residual pine trees and reduced cover of ericaceous shrubs.

Common environmental factors were responsible for shaping the ECM fungal distribution and for describing most of the explained regeneration variability. ECM fungal diversity was promoted by the proximity to residual pine trees and by high pine pre-fire basal area and was negatively affected by shrub height and high degrees of erosion. The presence of typical ECM fungal k-selected species in the proximity of unburnt pine trees and the fact that more than 30% of the ECM fungal species associated with pine saplings are also known to colonize different species of the genus *Cistus*, highlighted the potential ecological role played by residual pine trees and ECM shrubs in promoting pine regeneration. While high covers of ericoid mycorrhizal shrubs reduced pine saplings performance, pine seedlings also benefitted from an existing ECM fungal network in the proximity of mature pine trees and ECM shrubs that possibly improved their establishment and survival.

Digging deeper: wildfire regime and soil fungal communities

A more detailed study of the response of belowground fungal communities to wildfires and of the interactions between ECM fungi and pine regeneration was performed using forests from Portugal. With this study the long-term effects of wildfire and different wildfire frequency (measured as fire interval length) on the structure and composition of distinct components of the soil fungal communities associated to a maritime pine forests in central Portugal were evaluated. Wildfire shaped structure and composition of both the total soil fungal communities and the ECM fungal resistant propagules associated to *Pinus pinaster* and *Quercus suber*. While a gradual recovering towards pre-fire fungal belowground species composition was perceptible in areas characterised by long fire return interval length, the effects of repeated wildfires several years after the last disturbance event were still evident on the structure and composition of these components of the fungal community and the associated aboveground vegetation. Wildfire induced a general shift of the soil fungal communities towards the Ascomycota in stands characterized by the same shrub cover and favoured the abundance of fungal species that preferentially inhabit mineral soils and pioneer species in all the burnt stands. The effect of wildfire frequency on the ECM fungal resistant propagules community (RPC) varied greatly depending both on host and fungal species. While short fire return interval reduced drastically the number of ECM symbionts associated with cork oak, effects on ECM fungal species richness

associated with maritime pine were absent. In general infrequent species found in the unburnt soil were completely eliminated in the short fire return interval sites, some species were favoured by long fire return interval but suffered with short fire return interval and others positively responded to both fire regimes benefitting from the release of competition induced by fire.

Underground networks

As shown in this research, the regeneration of forests after disturbances like wildfires can be facilitated by the presence of other plant species that are a source of compatible ECM fungi. Therefore, the influence of fire frequency on the potential for common ECM networks between understorey shrub species and *Pinus pinaster* was investigated. Fire frequency affected the potential for common ECM networks between understorey shrub species and *Pinus pinaster*. The main conclusion obtained with this work is that the potential for common ECM networks between pine and understorey shrub species seems to be maintained in the low fire return interval stands, but recurrent fires strongly reduced the occurrence and abundances of common symbionts species. Recurrent fires could therefore limit the bootstrapping effect of shrubs and delay the colonization by pine seedlings of frequently burnt forest areas of the Mediterranean region.

Wildfires, vegetation, soil chemistry and fungal communities

The changes observed on belowground fungal communities were the result of direct effects on soil and indirect effects on the above-ground plant community. Short fire return interval changed radically the vegetation cover from a maritime pine young forest to a shrubland dominated by either *Cistus* spp. or *Pterospartum tridentatum, Erica* spp. and *Halimium* spp.. The aboveground vegetation determined significant differences in the abundance of specific ECM fungal species detected in the total soil fungal community but played a minor role on both the proportion of ECM species common to pine and shrubs and on the structure and composition of ECM fungal RPC. Highly recurrent fires led to changes in soil properties characterized by significantly reduced values of organic C, N and inorganic ions if compared to the unburnt stand. These changes could explain part of the variability of some of the belowground fungal components.

Closing up…

The characterization of multiple components of the fungal community provided an overall picture of the effects of wildfire on belowground fungi. Some of the effects of wildfire frequency were corroborated by the different methodologies used in this work, like the differential effect of fire return interval length in different fungal species. For example, both semi quantitative (454 pyrosequencing/denaturing gradient gel electrophoresis) and quantitative (ECM root tips) measurements of ECM abundance revealed that species belonging to the *Atheliaceae* and *Russulaceae* dominated both the total soil fungal and the ECM communities in the control stand unaffected by wildfire in the last 40 years. On the other hand, the studies on the total soil fungal community, RPC

and common mycorrhizal fungi revealed that some species of the genera *Thelephora* and *Hebeloma* were dominant in all the burnt stands, probably benefitting from the release of competition induced by fire.

….and take-home message

The changes induced by repeated stand replacing wildfires on the belowground fungal community are less visible than those on the aboveground vegetation but they could lead to profound alterations in the dynamics of Mediterranean pine ecosystems. Frequent wildfires prevented pine regeneration in the short fire return interval stands and altered their vegetation structure. They also modify soil nutrient composition and belowground soil fungal community, reducing the number of pine compatible symbionts. Since pines are ecological obligate mycorrhizal species, changes induced by repeated fires on the ECM fungal community could strongly delay succession and post-fire recovery of forests in the Mediterranean.

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