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ORIGINAL ARTICLE

Ectomycorrhizal fungal diversity and community structure associated with cork oak in different landscapes

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Abstract

Cork oak (*Quercus suber* L.) forests play an important ecological and economic role. Ectomycorrhizal fungi (ECMF) are key components for the sustainability and functioning of these ecosystems. The community structure and composition of ECMF associated with *Q. suber* in different landscapes of distinct Mediterranean bioclimate regions have not previously been compared. In this work, soil samples from cork oak forests residing in different bioclimates (arid, semi-arid, sub-humid, and humid) were collected and surveyed for ectomycorrhizal (ECM) root tips. A global analysis performed on 3565 ECM root tips revealed that the ECMF community is highly enriched in *Russula*, *Tomentella*, and *Cenoccocum*, which correspond to the ECMF genera that mainly contribute to community differences. The ECMF communities from the rainiest and the driest cork oak forests were distinct, with soils from the rainiest climates being more heterogeneous than those from the driest climates. The analyses of several abiotic factors on the ECMF communities revealed that bioclimate, precipitation, soil texture, and forest management strongly influenced ECMF structure. Shifts in ECMF with different hyphal exploration types were also detected among forests, with precipitation, forest system, and soil texture being the main drivers controlling their composition. Understanding the effects of environmental factors on the structuring of ECM communities could be the first step for promoting the sustainability of this threatened ecosystem.

Keywords Cork oak · ECMF community · Environmental factors · Exploration types

Introduction

The microbial community present in the soil establishes a variety of associations with plants, which may be beneficial, neutral, or harmful and play an essential role in plant health and productivity (Rout 2014). Mycorrhizal symbiosis is a well-known, beneficial association. Indeed, through the establishment of ectomycorrhizae (ECM), ectomycorrhizal fungi (ECMF) are described as helpers in increasing plant survival and growth rate in forestry ecosystems (Selosse et al. 2000; Menkis et al. 2007), as well as in improving host tree health (Hyder et al. 2013). In the case of Mediterranean species, ECM are crucial for drought resistance improvement (Jany et al. 2002). Cork oak (*Quercus suber* L.) forests represent a very important Mediterranean ecosystem that is now facing increased threats from predicted climate change, cork exploitation, and oak decline. The combination of increasing mean temperatures and drought, which could also result in increasing wildfires, is one of the main concerns of cork oak forest producers (Acácio et al. 2009).

Cork oak is an evergreen oak tree species, typical in the Western Mediterranean region that has significant ecologic and economic importance, especially for the Iberian Peninsula. Cork oak forests with the highest value are in Portugal, which is the largest producer of cork (almost 50% of world production) and one of the largest importers of cork for processing industries (APCOR 2016). *Q. suber* grows in different forest systems, from forest types with densities of

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approximately 400 trees/ha (*sobreirais*) to low density stands (60–100 trees/ha; *montados*) with savannah-like landscapes. *Montados* are typical in the southern region of Portugal (Alentejo), where an extensive agro-silvo-pastoral exploitation is frequently found due to the scattered cork oak tree cover, whereas *sobreirais* are typically found in central and northern Portugal. Cork oak density and tree distribution are closely related to water availability (Joffre et al. 1999). Adaptations to local environmental conditions could drive cork oak population genetic divergence (Ramírez-Valiente et al. 2009a), but the moderate capacity of this species to cope with severe drought could lead to the disappearance/scarceness of actual populations (Ramírez-Valiente et al. 2009b). Since a reduction in water availability is expected in the near future (Giorgi and Lionello 2008), the long-term sustainability of these ecosystems may be further threatened, leading to a decrease in *Q. suber* growth and productivity (Moricca et al. 2014; Acácio et al. 2017). Regarding these environmental challenges, cork oak forest decline could have implications beyond economic losses, including microbial, plant, and animal diversity losses (Hector et al. 1999). Forest degradation affects the belowground microbial community (Zhou et al. 2018), which is known to play a key role in nutrient cycling and plant productivity (van der Heijden et al. 2016). The composition of ECMF communities is known to strongly vary with biotic factors, including forest use and management (Azul et al. 2010), forest vegetation composition and activity (Šťursová et al. 2016), and host genotype (Lamit et al. 2016). The main drivers of ECMF also include abiotic conditions, climatic factors (Mucha et al. 2018), and soil properties (Albornoz et al. 2016) or soil resources (Courty et al. 2016). The diversity and structure of *Quercus* ECMF communities have already been studied based on the ITS barcoding of ECM tips (Smith and Read 2008; Azul et al. 2010; Shi et al. 2011; Richard et al. 2011; Lancellotti and Franceschini 2013). The ECMF richness in cork oak *montados* was previously correlated with landscape and land use practices, but only cork oak forests situated on arid/semi-arid region climates were used (Azul et al. 2010). The present study aimed to (1) have a wide assessment of ECM root tips from *Q. suber* at different landscapes and determine the variation of ECM composition among sampling sites, (2) determine how the ECMF composition reflects the environmental features of each sampling site, and (3) determine a potential pattern of ECMF exploration types among distinct forests. For this purpose, soils from seven cork oak forests located in five different geographic regions of Portugal were sampled and surveyed for ECM root tips. Portugal displays diverse climate regions, ranging from humid to arid Mediterranean climate regions, as well as subhumid and semi-arid areas. The sampled forests corresponded to different climate regions and depicted distinctive characteristics (including forest type and uses, human disturbance, and soil features).

Material and methods

Selection of cork oak stands and sample collection

Sample collection occurred in five different geographic locations in Portugal (Fig. 1). Cork oak stands were selected based on information previously obtained on the stands (Varela and Eriksson 1995), as well as on the Mediterranean climate classification (Rego and Rocha 2014), determined by the climatic parameter of Emberger (*Q*; Emberger 1930). This parameter takes into account the annual precipitation (Pannual) and maximal (Tmax) and minimal (Tmin) temperatures of the hottest and coldest months, respectively, during the sampling year, where $Q = 100\ \mathrm{Pannual}\ (\mathrm{Tmax}^2 - \mathrm{Tmin}^2)$ (Tate and Gustard 2000). This information was obtained from the Portuguese Sea and Atmosphere Institute (IPMA) (Table 1). The annual precipitation means from the past 30 years (1986–2016) ranged between 1448.4 mm (National Park of Peneda-Gerês, PG) and 558 mm (Herdade da Contenda, HC), corresponding to the highest (186.6) and lowest (43.5) *Q* indexes, respectively. From each of the locations presenting extreme precipitation conditions, two independent forests were sampled [Ermida (PG-ER) and Rio Cabril (PG-RC) from the PG location; Contenda (HC-CT) and Monte Asparão (HC-MA) from the HC location]. Although they had similar annual precipitation means over the past 30 years, the other locations [Alcobaça (AL), Limãos (LI), and Grândola (GR)] had decreasing *Q* indexes from 102.7 to 77.5. Beyond distinct climatic parameters, the sampled cork oak forests also displayed distinctive features [*sobreiral* or *montado* forest systems; forest use for grazing domesticated livestock/wild animals (deer and wild boar) or wild forest; different human disturbances (mainly revealed by soil tillage); different vegetation covers (especially in regard to the presence of *Cistus* spp., which are able to form ECM (Comandini et al. 2006); and different soil features (pH and texture)].

During the autumn season (November and December 2013), all seven cork oak stands were sampled. In each stand, five apparently healthy trees were selected at least 30 m apart from each other to avoid direct interlacing/connection of their roots. The coil cores were collected from under the middle of the tree canopy. After removing the uppermost layer of soil that consisted of plant litter and other organic material, two independent soil cores (8 cm in diameter and 12 cm in depth) were collected in opposite directions from the cork oak trunk and kept at 4 °C until processing. In total, 70 soil cores (7 forests × 5 plots × 2 cores) were collected.

ECM root tip sorting

Each soil core was sieved twice. The particles retained by the 4-mm^2 sieve were discarded, and the residues retained by the

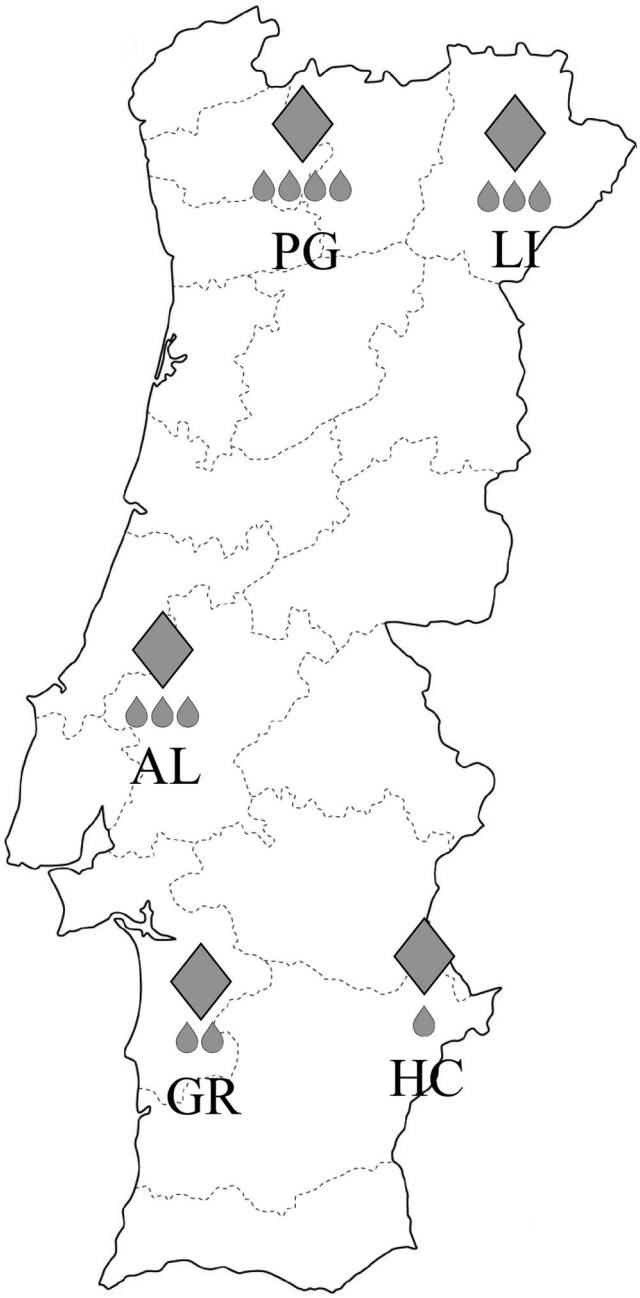

Fig. 1 Distribution of the studied Portuguese cork oak forests. Peneda-Gerês (PG), Limãos (LI), Alcobaça (AL), Grândola (GR), and Herdade da Contenda (HC) were selected based on the climatic parameter of Emberger and water availability conditions (water gradient is represented by the number of drops)

2-mm^2 sieve were thoroughly washed. The root tips were isolated and grouped according to their morphology, color, and characteristic features observed under a dissecting microscope. The subsamples of each morphotype in every soil core were selected based on the strategy described by Richard et al. (2005, 2011) with some adaptations as follows: (1) one ECM tip was sampled for each rare morphotype (i.e., represented by fewer than three ECM tips); (2) two ECM tips were sampled for all the morphotypes represented by more than three and less than ten mycorrhizae; and (3) three ECM tips were sampled for all the morphotypes represented by at least ten ECM tips. These ECM subsamples were stored at −20 °C until DNA extraction.

Molecular identification of ECMF

The molecular analysis was performed on each root tip. The total DNA was extracted using the Extract-N-Amp™ kit (Sigma-Aldrich, St. Louis, USA). Internal transcriptional spacer (ITS) PCR amplification was performed using 10 μl of the Extract-N-Amp™ PCR Reaction Mix (Sigma-Aldrich), 4 μl of stored DNA, 6 μl of distilled water (sterilized), and 1 μl of each primer at 10 mM. All the samples were amplified using ITS1F/ITS4 primers, and the negative amplifications were re-amplified using ITS1F/ITS2 primers (White et al. 1990; Gardes and Bruns 1993). In addition, the DNA samples resulting in two different fungal ITS products were further re-amplified with ITS1F/ITS4B primers (White et al. 1990; Gardes and Bruns 1993). The thermocycling program included an initial denaturation step at 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s; and a final elongation step at 72 °C for 10 min. The amplification products were separated by electrophoresis on a 1% (*v*/*v*) agarose gel, stained with Green Safe (NZYtech, Portugal) and visualized under UV light. The amplification products were sequenced by ITS1F primers at Macrogen (Amsterdam, The Netherlands). Fungal sequences were blasted against the UNITE (https://unite.ut.ee/) and NCBI (http://www.ncbi.nlm.nih.gov/) databases. The best BLAST hit was determined based on the *e*-value, higher similarity identity, and ecological considerations. The UNITE identification was preferred to the NCBI descriptions except when morphological characterization indicated the opposite. The sequences that were identified up to the genus level were further edited with the Seqman module of the programme DNASTAR (DNASTAR 8, Madison, USA). The alignments of the treated sequences were performed with the MegAlign module of the same programme, and the sequences with more than 97% identity were classified as the same species. After identification, the exploration type of each taxon was determined according to the literature, as evaluated by Agerer (2001, 2006), Hobbie and Agerer (2010), and Suz et al. (2014).

Statistical analyses

The ECMF community analysis was conducted based on the genera-abundance data. The ECMF community diversity was measured by computational indexes that combined both relative abundance and diversity (Magurran 2004). *Estimates S* - version 9 (RK Colwell, http://purl.oclc.org/estimates) was used to determine alpha [Simpson (*d*), Shannon (H′),

Table 1 Characterization of the cork oak sampling sites including geographic and environmental features. Averages of annual precipitation (Pannual) over the past 30 years (1986–2016), precipitation in the months with the lowest (Pmin) and highest (Pmax) precipitation levels, annual temperature (Tannual), and temperature of the coldest (Tmin) and hottest months (Tmax) were used to determine the indexes of Emberger (*Q*). Human disturbance (mainly from soil tillage), forest system (*sobreiral*/*montado*), and vegetation cover were assessed to understand the agricultural exploitation

Variables	National Park of Peneda-Gerês (PG)		Limãos (LI)	Alcobaça (AL)	Grândola (GR)	Herdade da Contenda (HC)	
	Ermida (PG-ER)	Rio Cabril (PG-RC)				Contenda (HC-CT)	Asparão (HC-MA)
GPS location	41° 42′ 39″ N	41° 45′ 43″ N	41° 31′ 51″ N	39° 27′ 41″ N	38° 11′ 32″N	38° 1′ 45″ N	38° 2′ 24″ N
	8° 6′ 14″ W	8° 1′ 39″ W	6° 49′ 56″ W	9° 2′ 42″ W	8° 37′ 11″ W	7° 0′ 27″ W	7° 1′ 55″ W
Altitude (m)	627	492	601	78	150	437	419
Pannual (mm)	1448.4		772.8	651.6	735.6	558	
Pmin (mm)	22 (July)		15.4 (July)	4.2 (July)	3.7 (July/Aug)	2.7 (July)	
Pmax (mm)	220.2 (December)		121.6 (December)	106.8 (November)	124.7 (December)	97.7 (December)	
Tannual (°C)	12.7		15.0	17.0	16.6	16.9	
Tmin (°C)	9 (January)		4.5 (January)	10.4 (January)	10.1 (January)	9.7 (January)	
Tmax (°C)	21.4 (July/August)		21.7 (July/Aug)	23.8 (August)	23.2 (August)	24.8 (August)	
Q	186.6 (humid)		88.9 (sub-humid)	102.7 (sub-humid)	77.5 (semi-arid)	43.5 (arid)	
Human disturbance	Non-tilled		Tilled	Non-tilled	Tilled	Non-tilled	
Forest system	*sobreiral*		*sobreiral*	*sobreiral*	*montado*	*sobreiral*	
Forest use	Wild forest		Pasture	Wild forest	Pasture/cork exploration	Pasture	
Vegetation cover	*Genista* sp., *Cistus* sp., *Ulex* sp., *Erica* sp.		*Cistus* sp.	*Pistacia* sp., *Ulex* sp., *Rubus* sp., *Rosa* sp.	*Cistus* sp.	*Cistus* sp., *Lavandula* sp.	
Soil pH	4.97 (strongly acid)	5.10 (strongly acid)	5.49 (strongly acid)	6.01 (slightly acid)	5.51 (slightly acid)	5.37 (strongly acid)	5.90 (slightly acid)
Soil texture	Sand		Sand	Sand	Loamy sand	Sandy loam	

Fisher's alpha] and beta diversity (Whittaker) indexes, as well as species richness estimators (first-order jackknife), whereas *Species Diversity and Richness version 5* (Pisces Conservation Ltd. Lymington, UK; 2014) was used for computing the rarefaction curves (Henderson and Seaby 2007). For evaluating the microbial community changes, an analysis of the similarity (ANOSIM; Clarke 1993) of fungal assemblages was performed by *Community Analysis Package version 5* software (Pisces Conservation Ltd. Lymington, UK; 2014). The same programme was used for determining the most important taxa among and between forests as evaluated by the similarity percentage analysis (SIMPER). A Mantel test (Mantel 1967) for correlating the community structure and geographic distances was conducted using the Microsoft Excel add-in programme *XLSTAT* (*version 2017*, Addinsoft, New York, USA). Other Excel tools were used for determining the Pearson correlations between climatic conditions and fungal structure. For testing the climate effect on ECM abundance and richness, a one-way ANOVA followed by Tukey's multiple comparison test was performed using the analysis tools of *GraphPad Prism 7.00* (GraphPad Software, La Jolla, CA, USA). Further analyses were performed with *R version 3.4.3* (R Foundation for Statistical Computing, 2017). A species indicator analysis (Dufrêne and Legendre 1997) was used to identify the indicator ECMF taxa (or exploration type) for cork oak forests and was performed as implemented by the *indval* function in the R package *labdsv* (Roberts 2010). The *IndVal* index varies from 0 to 1, measuring the association between the ECMF taxa and a site group. To take into account the rare species and undersampling that could disturb the following analyses, the fungal taxa with less than 20 observations in all samples and/or occurring in a single sample were excluded. The species composition across samples/forests was visualized using nonmetric multidimensional scaling (NMDS) based on the Bray-Curtis dissimilarity indexes of square-transformed results (Oksanen et al. 2012). The ordination plots were created using the *metaMDS* function of the *vegan* package. For identifying the main factors affecting ECMF community composition, the environmental variables were fitted to the ordination plots using the *envfit* function of the same package. The significance of the vectors was tested with 999 permutations. The evaluated continuous factors were the climatic parameters (temperature and precipitation values), bioclimatic regions where the cork oak forests are included (as evaluated by *Q* index), geographic parameters (altitude, latitude, longitude), and soil pH. The evaluated categorical factors were forest management (*sobreiral*/*montado*), human disturbance (mainly caused by tillage), forest use for grazing wild animals (deer and wild boar)/domesticated livestock (pasture), and vegetation cover (particularly *Cistus* presence). The effects of the categorical forest factors were further investigated using a permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) for determining

whether the variance promoted by the environmental factors differed significantly between *Q. suber* trees or the sampled soil cores nested within forests. PERMANOVA was implemented with the *adonis* function from the *vegan* package with the Bray-Curtis dissimilarity matrix using square-transformed results as a measure of community dissimilarity. The significance was tested with 999 permutations. Similar analyses were performed for the ECM exploration types.

Results

General description of the ECMF community

From the seven cork oak stands surveyed, 3565 ECM root tips were isolated and 796 tips were used for the molecular identification, 74% of which (587 sequences) had a successful identification. This analysis revealed 161 OTUs from 23 families and 32 different genera of ECMF (Table 2). Basidiomycota (seven orders) and Ascomycota (three orders) were the only phyla identified. From Basidiomycota, Agaricales was the richest order, comprising eight families and genera, followed by Thelephorales with two families and six genera. Among Ascomycota, the Pezizales order was the most diverse (three families and genera identified). The abundance analysis revealed Basidiomycota as the most abundant phylum (3229 root tips—82% of the collected root tips) followed by Ascomycota (651 root tips—18%). Within Basidiomycota, the most abundant families (from 18 identified) were Russulaceae (43% of basidiomycete root tips) and Thelephoraceae (26%). More precisely, *Russula* (76% of Russulaceae root tips) and *Tomentella* (99% of Thelephoraceae root tips) were the most dominant genera. Within the Ascomycota, the most abundant root tips belonged to the Mytilinidiales order (56% of Ascomycete root tips), which only comprised *Cenococcum*.

Variation in the ECMF communities among *Quercus suber* forests

The ECM fungal communities were compared between forests/samples by computation of the rarefaction curves (Fig. S1) and diversity indexes (Table 3; Table S1). The rarefaction curves suggested that the ECM root tips from all the forest sampling sites could provide information about the ECMF community, although they did not reach a clear plateau

Table 2 Number of ECM root tips with each genus in each sampled cork oak forest. Information about species identification and the corresponding exploration types is given in Table S5. Each forest is referred to by its code, as shown in Table 1

Phylum	Order	Family	Genus	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	Total
Ascomycota	Eurotiales	Elephomycetaceae	*Elaphomyces*	108	14	8	0	0	0	0	130
	Mytilinidiales	Gloneaceae	*Cenococcum*	18	178	0	46	29	74	20	365
	Pezizales	Helvellaceae	*Helvella*	0	0	0	0	0	7	0	7
		Pyronemataceae	*Humaria*	0	0	89	0	0	0	0	89
		Tuberaceae	*Tuber*	0	0	0	0	53	0	7	60
Basidiomycota	Agaricales	Amanitaceae	*Amanita*	0	13	3	3	4	0	0	23
		Cortinariaceae	*Cortinarius*	26	3	19	48	0	22	39	157
		Entolomataceae	*Entoloma*	0	0	133	0	0	0	2	135
		Hymenogastraceae	*Hebeloma*	0	26	0	0	0	0	0	26
		Hygrophoraceae	*Hygrocybe*	0	6	44	9	0	0	0	59
			Hygrophorus	0	0	0	11	0	0	0	11
		Inocybaceae	*Inocybe*	0	4	8	12	2	22	0	48
		Hydnangiaceae	*Laccaria*	6	1	0	0	0	0	17	24
		Tricholomataceae	*Tricholoma*	0	0	35	0	0	1	3	39
	Atheliales	Atheliaceae	*Piloderma*	0	0	0	5	0	0	0	5
	Boletales	Boletaceae	*Boletus*	31	4	23	3	0	0	0	61
			Leccinum	0	1	0	0	0	0	0	1
			Xerocomus	7	2	0	0	0	0	0	9
		Sclerodermataceae	*Pisolithus*	0	0	0	0	0	1	0	1
	Cantharellales	Cantharellacae	*Cantharellus*	127	0	0	0	0	0	0	127
		Clavulinaceae	*Clavulina*	16	9	38	0	0	0	0	63
		Hydnaceae	*Sistotrema*	0	1	0	0	0	0	0	1
	Russulales	Russulaceae	*Lactarius*	160	11	2	64	0	5	1	243
			Russula	127	0	601	35	15	133	111	1022
	Sebacinales	Sebacinaceae	*Sebacina*	0	65	6	30	0	0	0	101
	Thelephorales	Bankeraceae	*Hydnellum*	0	2	0	0	0	0	0	2
			Phellodon	1	0	0	0	0	0	0	1
			Sarcodon	1	0	0	0	0	0	0	1
		Thelephoraceae	*Pseudotomentella*	12	27	0	0	0	0	0	39
			Thelephora	0	11	0	0	0	0	0	11
			Tomentella	88	95	237	205	6	50	23	704
			Total	728	473	1246	471	109	315	223	3565

Table 3 Diversity parameters for the cork oak ECM fungal communities as determined by root tip barcoding. Total number of the ECM root tips (N), number of identified genera (S), alpha diversity indexes [Simpson's index (d), Shannon index (H′), Fisher's alpha], richness estimator [first-order jackknife], and the beta diversity Whittaker index are represented. The lowest estimates are highlighted in bold, and the highest estimates are italicized. Each forest is referred to by its code, as shown in Table 1. Letters indicate significant differences for each parameter ($p < 0.05$; one-way ANOVA, Tukey test) based on the values determined for each replicate (5 replicates/site). All the replicate values are described in Table S1

	N	S	d	H′	α Fisher	Jackknife index	Beta diversity (Whittaker)
PG-ER	728^{ab}	29^{ab}	3.67^{ab}	2.06^{ab}	5.35^{a}	50^{ab}	2.85
PG-RC	473^{bc}	36^{a}	3.43^{ab}	1.96^{a}	7.68^{a}	62.6^{a}	2.85
LI	$\mathit{1246}^{a}$	$\mathit{38}^{a}$	3.75^{ab}	2.19^{a}	6.57^{a}	$\mathit{67}^{a}$	*3.24*
AL	471^{bc}	36^{a}	$\mathit{3.83}^{a}$	$\mathit{2.32}^{a}$	$\mathit{7.69}^{a}$	61.8^{a}	2.94
GR	$\mathbf{109}^{c}$	$\mathbf{12}^{b}$	$\mathbf{3.06}^{b}$	$\mathbf{1.46}^{b}$	$\mathbf{3.03}^{a}$	$\mathbf{21}^{b}$	**2.10**
HC-CT	315^{bc}	22^{ab}	3.32^{ab}	1.73^{ab}	4.69^{a}	38.2^{ab}	2.38
HC-MA	223^{bc}	21^{ab}	3.68^{ab}	2.00^{ab}	4.86^{a}	35.6^{ab}	2.67

(Fig. S1). The northern forests presented a more diversified ECMF community, mainly LI and PG-RC but also PG-ER. However, the central AL forest exhibited the most diversified ECMF community. Accordingly, the AL forest and the most northeastern forest (LI) had the highest alpha diversity indexes, while the southern forest (GR) consistently represented the lowest values (Table 3). GR exhibited the least diversified community of all the sampled forests. The differences between the highest and lowest diversity indexes detected in these forests were always statistically significant ($p < 0.05$), except for Fisher's alpha (Table 3). A similar pattern was observed for beta diversity described by the Whittaker estimator, with the LI forest being the most diverse and GR being the least diverse communities. At the ecological level, both sets of biological diversity replicates (PG-ER/PG-RC and HC-CT/HC-MA) presented similar values for the beta diversity index, which validated our sampling strategy.

The northern and rainiest forests (PG-ER, PG-RC, and LI) had higher ECMF abundances than the southern and driest forests (HC-CT, HC-MA, and GR) (significant difference at $p < 0.001$), but no significant differences were revealed in the genera richness among the sampling sites (Fig. S2). A detailed analysis of the ten most abundant genera in each cork oak stand revealed that the *Tomentella* genus was the most common among all the cork oak forests, followed by *Russula*, which was only absent from the rainiest forest (PG-RC), and *Cenococcum*, which was only absent from the LI forest (Fig. 2). The southern forests had a more homogenous genera content and were always enriched in *Russula*, *Tomentella*, and *Cenococcum* (46–82% occurrence). The northern forests were more diverse and each included a genus that was somewhat specific unique to one of the forests, such as *Cantharellus* (PG-ER), *Sebacina* (PG-RC), and *Humaria* (LI). However, when performing an indicator species analysis, only *Cantharelus* was singled out as an indicator species of the PG-ER forests (*IndVal* = 0.40; $p < 0.01$). Due to their higher abundance, *Russula* was predicted as an indicator species of LI (*IndVal* = 0.71; $p < 0.001$), and *Cenococcum* was predicted as an indicator of PG-RC (*IndVal* = 0.49; $p < 0.001$).

The cork oak ECMF communities differed among the forests (PERMANOVA; $F_{6,34} = 3.028$, $R^2 = 0.394$, $p < 0.001$) but were not significantly different among the soil replicates

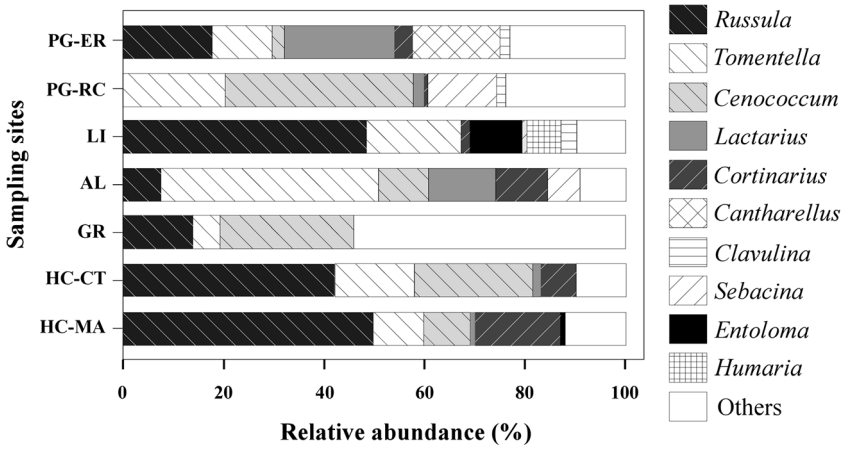

Fig. 2 Most abundant ECM fungal genera identified by the root tip analysis of all the sampled cork oak stands. Each forest is referred to by its code, as shown in Table 1

(PERMANOVA; $F_{29,69} = 0.961$, $R^2 = 0.353$, $p < 0.05$), revealing that the sampled forest could explain almost 40% of the ECMF community variance (Table S3). Distinct forests presented dissimilar ECMF communities (ANOSIM; $R = 0.450$, $p < 0.05$), but the dissimilarity among the northern forests (PG-ER, PG-RC, and LI) was greater (ANOSIM; $R = 0.256$, $p = 0.05$) than that among southern forests (HC-CT, HC-MA, and GR; ANOSIM; $R < 0$, $p > 0.05$). Even for those locations with extreme conditions (PG and HC), where two independent forests were sampled, the northern and wettest sites (PG-ER/PG-RC) had less similar communities (ANOSIM; $R = 0.460$, $p = 0.012$) than the southern and driest samples (HC-CT/HC-MA), which revealed no differences in the overall ECMF composition (ANOSIM; $R < 0$, $p > 0.05$) (Table S2A). The ECMF community of the northern LI cork oak forest was the least similar among the sampled geographic areas and could almost describe a singular ECMF community. As the most abundant genera, *Russula* (29% of the total root tips), *Tomentella* (20%), and *Cenococcum* (10%) were the genera that contributed the most to sample divergence (*Russula* contributed up to 33.4% of the total dissimilarity between samples, *Tomentella* up to 36.7%, and *Cenococcum* up to 33.4%, as detected by the SIMPER dissimilarity analysis between groups).

Environmental variables structuring *Quercus suber* ECMF communities

When considering geographical distance between the sampled forests, with the most distant forests (HC-MA and PG-RC) being 430 km apart, the ECMF community similarity significantly decreased as geographic distance increased (Mantel test for Jaccard's similarity index and geographic distance; $r = -0.188$, $p < 0.001$). This result was different at a regional scale. The most northern and rainiest forests (PG-ER, PG-RC, and LI), for which the greatest separation was 109 km, still presented a significant effect of geographic distance on ECMF community similarity (Mantel test; $r = -0.258$, $p = 0.008$). However, the ECMF communities from the most southern and driest forests (HC-CT, HC-MA, and GR), where the greatest separation was almost 150 km, did not reveal a significant effect of geographic distance on community similarity (Mantel test; $r = -0.034$, $p = 0.734$).

The community dissimilarities were visualized using nonmetric multidimensional scaling (NMDS) based on the Bray-Curtis dissimilarity index, which was also used for revealing the habitat effect on the ECMF community structure (Fig. 3(A)). Many environmental variables were significantly reflected in the ECMF composition, but the most significant drivers were the *Q* index and precipitation (annual and maximal), as well as the forest use for pasture and soil texture (all at $p < 0.001$; Table 4). Based on the Bray-Curtis distances, the effect of the categorical environmental factors was further investigated by a permutational multivariate analysis of

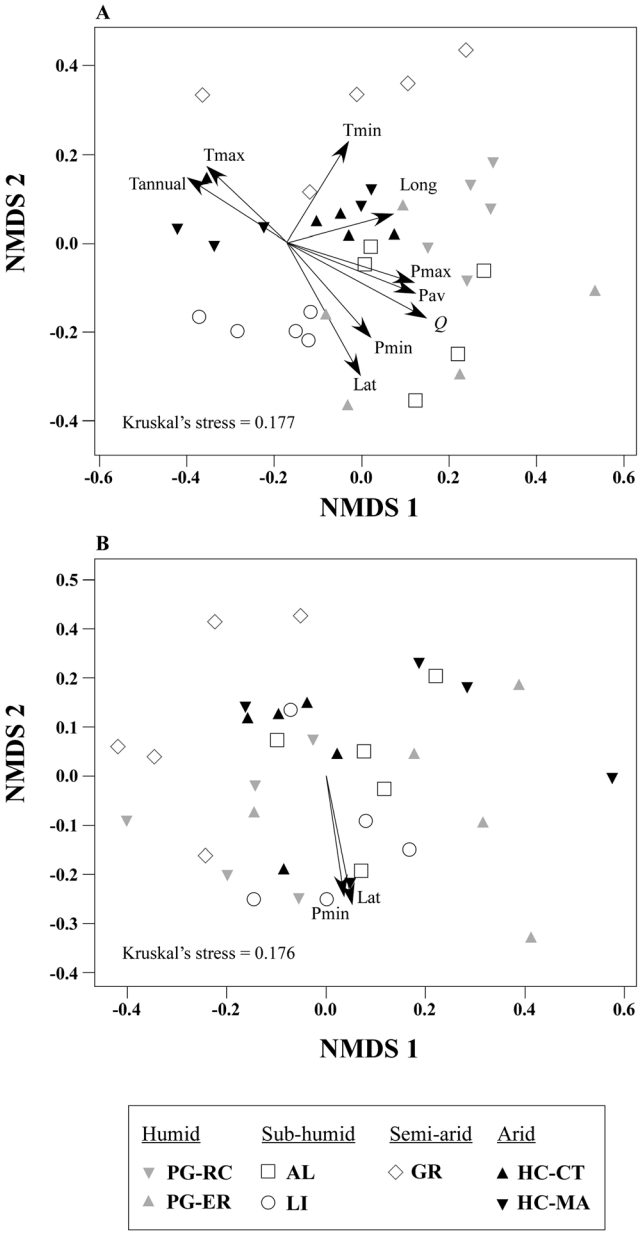

Fig. 3 Ordination (nonmetric multidimensional scaling, NMDS) of ECMF communities (**a**) and ECMF exploration types (**b**) found in different *Q. suber* forests. Each point represents a different sample (five samples from each forest). The environmental variables are fitted to the NMDS ordination, and arrows indicate correlations between the environmental factors and ECMF communities. The direction of arrows indicates positive correlations. Only those factors that were significantly correlated with NMDS ordination axes ($p < 0.05$) are shown. The significance of vectors is displayed in Table 4

variance (PERMANOVA; Table S3). The bioclimate, pasture, and soil texture explained most of the variation found in the ECMF communities among the forests (combined $R^2 = 0.54$), with the bioclimate alone being responsible for 24% of the variation. A positive correlation was also detected between the ECM occurrence and precipitation levels at the sampling sites, with fungal richness being more influenced than

Table 4 Effects of environmental variables on ECMF composition as described by the nonmetric multidimensional scaling scores fitted to the NMDS ordination and corresponding *p* values. The continuous variables and categorical environmental factors are displayed. Each forest is referred to by its code, as shown in Table 1. Statistical differences are denoted in italics for $p < 0.05$, in bold for $p < 0.01$, and in italicized-bold for $p < 0.001$

Variables	ECMF community		Exploration types	
	R^2	*p*	R^2	*p*
Continuous variables				
Tmax	*0.212*	*0.023*	0.146	0.074
Tmin	**0.268**	**0.009**	0.124	0.123
Tannual	**0.274**	**0.006**	0.137	0.096
Pmax	***0.329***	***< 0.001***	0.078	0.265
Pmin	**0.300**	**0.005**	*0.174*	*0.046*
Pav	***0.339***	***< 0.001***	0.089	0.224
Q	***0.456***	***< 0.001***	0.096	0.196
pH	0.102	0.166	0.069	0.305
Altitude	0.154	0.064	0.174	0.05
Latitude	**0.402**	**0.002**	*0.215*	*0.025*
Longitude	**0.282**	**0.004**	0.032	0.604
Categorical factors				
Forest	***0.620***	***< 0.001***	***0.427***	***< 0.001***
Location	***0.564***	***< 0.001***	*0.227*	*0.042*
Bioclimate	***0.470***	***< 0.001***	*0.204*	*0.026*
Forest system	**0.193**	**0.002**	**0.168**	**0.005**
Pasture	***0.272***	***< 0.001***	0.081	0.073
Human disturb.	0.052	0.052	0.081	0.055
Cistus cov.	0.076	0.093	0.013	0.629
Soil texture	***0.367***	***< 0.001***	**0.202**	**0.008**

abundance (Table S4). The temperatures registered at the sampling sites were negatively correlated with ECM root tip abundance and to a lesser extent with richness. The combination of these climatic variables in the Emberger index (which defines the bioclimate classification) revealed that ECMF richness was significantly increased in the forests with the wettest climates ($p < 0.01$), but ECMF abundance was not increased (Table S4).

Structure of exploration types in different habitats

The exploration types of ECMF were determined for 26 genera (81% of the identified genera). The most abundant was the short exploration type, which composed 40% of all the identified root tips (Tables S5 and S6). The distribution of exploration types was significantly different among *Q. suber* forests (PERMANOVA; $F_{6,34} = 2.385$, $R^2 = 0.338$, $p < 0.01$) but not among soil replicates (PERMANOVA; $F_{29,69} = 1.125$, $R^2 = 0.399$, $p > 0.05$). Forests can thus explain almost 34% of the exploration-type variance (Table S3). According to the ANOSIM, the rainiest forests (LI and PG forests) and the driest forests (GR and HC forests) had significantly dissimilar exploration-type compositions ($R = 0.18$, $p = 0.005$), but their intra-diversity was highly similar (Table S2B). For the exploration types, a weak clustering of samples from the same forest was revealed by NMDS ordination based on the Bray-Curtis dissimilarity index (Fig. 3(B)). Precipitation in the driest months (July/August) and altitude revealed to be significantly correlated with NMDS vectors based on the environmental fitting test ($p < 0.05$). However, the most significant drivers were the forest system (*sobreiral*/*montado*) and soil texture (both at $p < 0.01$; Table 4). As evaluated by PERMANOVA, the combination of both environmental categorical factors only explained 23% of the variance found in communities distinguished by their ECMF exploration type (Table S3). The short and contact exploration types were revealed as indicator exploration types for LI forests (*IndVal* = 0.41 and *IndVal* = 0.40, respectively; both at $p < 0.05$), while forests used for grazing animals displayed long (*IndVal* = 0.35; $p < 0.05$) and contact (*IndVal* = 0.43; $p < 0.05$) exploration types as indicators.

Discussion

ECMF community associated with cork oak in different landscapes

To the best of our knowledge, this is the first report that compares ECMF structure in different landscapes and distinct Mediterranean bioclimates. The ECM fungal community associated with cork oaks was mainly represented by *Russula* (29%), *Tomentella* (20%), and *Cenoccoccum* (10%), which is in accordance with other reports on *Q. suber* forests that also describe a highly enriched ECMF community with *C. geophilum*, Russulaceae, and Thelephoraceae (Yakhlef et al. 2009; Azul et al. 2010; Lancellotti and Franceschini 2013; Maghnia et al. 2017). This trend is commonly seen in other Fagaceae forests (reviewed by Reis et al. (2017)). While *C. geophilum* was the main ECM root tip identified in the *Q. ilex* forests (Richard et al. 2011), *Russula* was the most abundant genus in *Q. suber* landscapes (Azul et al. 2010). In this work, due to their abundance, both genera were found to be indicator genera of specific forests (*Cenococcum* for PG-RC and *Russula* for LI forests) but were absent in other forests (*Cenococcum* from LI and *Russula* from PG-RC). The heterogeneity of forest microbial diversity has been well described and results from dynamic ecosystem processes occurring at spatial and temporal scales (reviewed by Baldrian (2017)). Variations in bioclimate, soil texture, and forest management may account for the observed differences in *Russula* and *Cenococcum* abundance between the sampled forests. Accordingly, previous studies have reported shifts in ECMF abundance in response to management practices. For

example, an increase in nitrogen supply was observed to stimulate *Russula* to produce more sporocarps and colonize relatively more oak seedling roots (Avis et al. 2003). Similarly, tillage was reported to decrease the presence of *Cenococcum* and other ectomycorrhizae with a long-distance exploration type, as rhizomorphs are broken in the process (Maghnia et al. 2017). Accordingly, the absence of *Russula* or *Cenococcum* in Fagaceae forests has been reported, such as in *Q. ilex* (De Román and De Miguel 2005) and *Q. petrea* (Voříšková et al. 2014) forests. Also, an analysis of the ECMF community associated with different cork oak forests (only *montados*) revealed pronounced differences in the community structure and reported an absence of *Russula* in specific cork oak forests (Azul et al. 2010).

Cork oak ECMF community dependence on environmental factors

Due to their high abundance, the occurrence of ECM root tips from *Russula*, *Tomentella*, and *Cenococcum* well discriminated all the samples, but a clear pattern of taxa distribution among forests is missing, even between geographic replicates (PG-ER/PG-RC, and HC-CT/HC-MA). When comparing the northern and rainiest forests (PG-ER/PG-RC and LI) with the southern and driest forests (HC-CT/HC-MA and GR), the ECMF community differences were evident. This result is associated with a more heterogeneous picture of the ECMF community found in northern forests, which contain a diversified and abundant ECMF community. In contrast, the southern samples were more similar to each other and presented a lower number of ECM root tips. Even among forest replicates, a greater similarity was always found among soil samples from the southern forests than from the northern forests. Another indication of northern ECMF heterogeneity is given by the Mantel test statistic that described a significant correlation between ECMF similarity and geographic distance for the closer northern forests (109 km apart) but a non-significant correlation between the more separated southern forests (150 km apart). These results suggest that distance is not the main driver for ECMF occurrence, especially for the driest regions, and agree with Miyamoto et al. (2015) who described geographic distance as a minor driver of ECMF community structure at a regional scale.

The composition of ECMF communities is strongly influenced by biotic and abiotic conditions. In an attempt to study the most diverse cork oak landscapes, the sampling sites were chosen mainly based on bioclimate regions. In addition to having the most extensive *Q. suber* forest area, Portugal also has a wide range of Mediterranean bioclimates (from humid to arid), allowing a wide view of ECMF associations with *Q. suber* in natural habitats. The sampled forests also have other features (forest system and management, forest use, and human disturbance) that make the sampled forests distinct from each other. Many environmental variables were indeed significantly reflected on the ECMF structure, indicating that cork oak-associated ECMF are susceptible to many environmental components. This finding is not new, as many reports describe the effect of several environmental drivers of ECMF communities (e.g., Suz et al. 2014). In the present work, bioclimate and precipitation, as well as forest use for grazing and soil properties, were the drivers that presented a major significant effect on ECMF community structure. Land use practices (including permanent grazing or tillage) have already been described to affect the structure of ECMF in a particular region of southern Portugal where cork oaks are found in managed *montados* (Azul et al. 2010). However, in this study, neither different bioclimate regions nor climate variables were considered. Although several environmental conditions known to govern ECMF composition [e.g., soil features, soil properties (Albornoz et al. 2016) and resources (Courty et al. 2016)] were not evaluated in the present work, the influence of bioclimate and its components (precipitation and temperature) for shaping ECMF structure was revealed. Castro et al. (2010) and Jarvis et al. (2013) also described precipitation as a main driver of ECMF community composition. Interestingly, we found a stronger correlation between the rainiest climates and ECMF richness, while ECMF abundance seemed to be more influenced by temperature than precipitation levels. As no alterations in ECMF richness were observed in response to simulated warming in boreal or temperate tree species, Mucha et al. (2018) suggested that warming would have a smaller impact on root symbiotic fungi when precipitation was held constant. We suggest that limitations on the precipitation levels occurring in drier regions could lead to a decrease in ECMF richness, making the driest and warmer sites (southern cork oak forests) more susceptible to environmental filtering than the rainiest and colder sites. This suggestion is supported by the more homogeneous picture of southern forests.

The different abundances of certain fungal lineages could be partly explained by differences in ECMF tolerance/susceptibility to environmental conditions. *Elaphomyces* and *Lactarius* were more frequently found in humid climates than in arid climates, as well as all identified genera from Thelephoraceae and Sebacinaceae and from the Cantharellales order. Accordingly, the temperature assays revealed that the Thelephoraceae and Sebacinaceae species are susceptible to temperature increases, while *Cenococcum geophilum* and *Rhizopogon* sp. are more tolerant (Kipfer et al. 2010; Fernandez and Koide 2013). In a rainfall exclusion experiment in *Q. ilex* forests, the global richness of the community was not affected, but significant shifts in the community composition were reported (Richard et al. 2011). Sebacinaceae were also less represented in a drought scenario, while members of the Cortinariaceae species were significantly more abundant.

Are ECMF exploration types distinct among different landscapes?

ECMF can be classified according to their ability to form specific features, such as extramatricial mycelia and rhizomorphs (Agerer 2001). Several types of ECMF have been distinguished, ranging from the contact (with a smooth mantle and few emanating hyphae) to the long-distance (able to form rhizomorphs) exploration types. A distinct distribution of exploration types was found between the rainiest and the driest cork oak forests, but not within them. Additionally, precipitation (occurring during the driest season) was the environmental condition that was most correlated with exploration-type distribution, together with the forest system (tree density in *sobreirais* or *montados*). However, we cannot exclude other environmental factors that may govern the distribution of exploration types, as many features were not considered (mainly soil nutrients). Indeed, most studies on ECMF exploration types have focused on soil resources and their relation to the relative abundance of exploration types (e.g., Hobbie and Agerer 2010; Suz et al. 2014). However, the influence of temperature and root density were reported to affect their distribution (Peay et al. 2011; Jarvis et al. 2013). Accordingly, manipulated warming was reported to decrease medium-smooth and contact exploration types (Mucha et al. 2018). To the best of our knowledge, the effect of precipitation has never been related to exploration-type distribution. Two findings from our work point to the importance of precipitation levels as follows: (1) the distribution pattern was different among the rainiest and driest forests and (2) vector fitting revealed a strong influence of precipitation in the driest season on exploration-type distributions. According to Peay et al. (2011), the spatial and temporal patterns of ECMF assemblages are also related to root density, with short-range exploration ECMF being more effective in colonizing denser roots, while longer distance types are more efficient in finding and colonizing new roots. This hypothesis could partly explain the strong effect of forest system (tree density) on exploration-type distributions.

Conclusions

This study evaluated the ECM fungal community of cork oak forests in different locations and in distinct Mediterranean climates ranging from humid to arid. To the best of our knowledge, the present work comprises the most complete assessment of ECMF communities associated with cork oak in different landscapes. This work revealed that the occurrence of ECM root tips from *Russula*, *Tomentella*, and *Cenococcum* well discriminated cork oak forests. The southern and most arid forests were revealed to be less diverse and more homogenous than the northern and humid forests. From the 18 environmental parameters evaluated, the bioclimate and its components (precipitation and temperature) had the strongest effect on ECMF community structure. The precipitation occurring during the driest season also significantly affected the distribution of ECMF exploration types. When relating the ECMF community structure with each climatic parameter individually, it was found that temperature is negatively correlated with ECMF abundance, whereas richness is more correlated with precipitation levels. Although further experimental support is needed for conclusions about climate implications on the occurrence of ECMF root tips, the results suggest possible effects on the structuring of ECMF communities and/or the promotion of mycorrhization. As current climate models predict a general temperature increase and precipitation decline in the near future, possible resulting alterations in ECMF community could compromise cork oak forest sustainability.

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