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Research Article

RESEARCH ARTICLE

Encroachment of shrubs into subalpine grasslands in the Pyrenees modifies the structure of soil fungal communities and soil properties

Oriol Grau[1,2,*,†], Karita Saravesi[3], Josep M. Ninot[4], József Geml[5,6], Annamari Markkola[3], Saija H.K. Ahonen[3] and Josep Peñuelas[1,2]

[1]CSIC, Global Ecology Unit, CREAF-CSIC-UAB, 08193, Cerdanyola del Vallès, Catalonia, Spain, [2]CREAF, 08193, Cerdanyola del Vallès, Catalonia, Spain, [3]Department of Ecology and Genetics, P.O. Box 3000, FI-90014, University of Oulu, Oulu, Finland, [4]Institute for Research in Biodiversity (IRBio) and Department of Evolutionary Biology, Ecology and Environmental Sciences, University of Barcelona, Av. Diagonal 643, 08028, Barcelona, Catalonia, Spain, [5]Naturalis Biodiversity Center, Vondellaan 55, P.O. Box 9517, Leiden, The Netherlands and [6]Faculty of Science, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

*Corresponding author: Oriol Grau, Global Ecology Unit, CREAF-CSIC-UAB, 08193 Cerdanyola del Vallès, Catalonia. Tel: +34 935813251; E-mail: grau.oriol@gmail.com

One sentence summary: The expansion of shrubs into subalpine grasslands in the Pyrenees modifies the distribution and role of soil fungal communities and soil quality.

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†Oriol Grau, http://orcid.org/0000-0002-3816-9499

ABSTRACT

The encroachment of shrubs into grasslands is common in terrestrial ecosystems dominated by grass. Land abandonment and favourable climatic trends in recent decades have favoured the expansion of shrubs into subalpine grasslands in many mountainous regions across Europe. The advance of the succession from grassland to shrubland is expected to have a major impact on ecosystem functioning. We used DNA metabarcoding to assess whether the structure of soil fungal communities varied along the succession from subalpine grassland to shrubland in the Pyrenees, and investigated whether shrub encroachment was associated with changes in soil properties. The expansion of shrubs increased the soil C:N ratio and/or reduced the N, P or K contents. Plant-driven changes in soil properties were strongly associated with the compositional turnover of fungi, including arbuscular mycorrhizal, ectomycorrhizal, ericoid, root endophytic, saprotrophic, lichenised and pathogenic fungi. Total richness and the richness of most functional groups were correlated with soil P, N and the C:N or N:P ratios. We show that the interplay between abiotic factors (changes in soil properties) and biotic factors (occurrence and identity of shrubs) played a key role in the structure and uniqueness of soil fungal communities along the succession.

Keywords: compositional turnover; functional group; land abandonment; fungi; secondary succession; shrub expansion

INTRODUCTION

The ecological succession from grassland to shrubland is common in many terrestrial ecosystems dominated by grass in contrasting biomes around the globe (Knapp *et al.* 2008). This succession, widely referred to as 'shrub encroachment', has been observed in arctic and subarctic tundra (Sturm, Racine and Tape 2001; Tape, Sturm and Racine 2006; Naito and Cairns

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2011; Myers-Smith *et al.* 2015), alpine and subalpine grasslands (Dullinger, Dirnböck and Grabherr 2003; Anthelme, Villaret and Brun 2007; Montané, Rovira and Casals 2007; Brandt *et al.* 2013), mesic grasslands (Briggs, Blair and Mccarron 2005), Mediterranean steppes (Maestre *et al.* 2009), and semi-arid and arid grasslands or savannas (Eldridge *et al.* 2011). The expansion of shrubs into alpine and subalpine grasslands has increased in many mountainous regions across Europe in recent decades. This trend has been observed in most mountain ranges and massifs in Europe, such as the Pyrenees, the Cantabrian range, the Alps, the Apennines, the Carpathians, the Balkans and the Scandes (MacDonald *et al.* 2000; Dullinger, Dirnböck and Grabherr 2003; Roura-Pascual *et al.* 2005; Montané, Rovira and Casals 2007; Hallinger, Manthey and Wilmking 2010; Barrio *et al.* 2013; Wiezik *et al.* 2013; Pedashenko, Apostolova and Oldeland 2015). Shrub encroachment into subalpine and alpine grasslands generally responds to the abandonment of traditional practices, such as extensive livestock grazing, and/or to a decrease in the frequency of intentional fires to create summer pastures (MacDonald *et al.* 2000). Ongoing global warming is also expected to favour the growth and expansion of shrubs in these high-altitude, temperature-limited ecosystems across Europe (Sanz-Elorza *et al.* 2003; Körner and Paulsen 2004; Wookey *et al.* 2009).

Shrub encroachment modifies the functioning of grass-dominated ecosystems by changes in primary production, allocation of plant biomass, litter quality, rooting depth, and the composition and diversity of soil faunal communities (Van Auken 2009; Eldridge *et al.* 2011; Wiezik *et al.* 2013) and is expected to affect nutrient cycling and C flow in these ecosystems (Jackson *et al.* 2002). Soil microbial communities are also sensitive to these plant-driven changes during secondary succession (Cline and Zak 2015). The composition of fungal communities varies markedly along the succession from grassland to shrubland across North America (Knapp *et al.* 2008; Hollister *et al.* 2010; Yannarell, Menning and Beck 2014; Veach, Dodds and Jumpponen 2015; Creamer *et al.* 2016) and in Inner Mongolia (Li *et al.* 2017).

Fungi are essential for the functioning of terrestrial ecosystems, because they play fundamental ecological roles as decomposers, mutualists or pathogens of plants and animals and contribute highly to nutrient cycling, C flow and vegetation dynamics (Öpik *et al.* 2006; Talbot *et al.* 2014; Tedersoo *et al.* 2014; Saravesi *et al.* 2015). The role of soil fungi is therefore important for understanding the interplay between below- and aboveground processes and ecosystem functioning in grasslands where shrub cover is expanding. The impact of shrub encroachment on soil fungal communities, though, has been neglected in most grass-dominated ecosystems, such as subalpine grasslands. As far as we know, this topic has only been partly investigated in the White Mountains of California (Collins *et al.* 2016) and in the French Alps (Schwob *et al.* 2017), but no studies have assessed the richness and compositional patterns of contrasting functional groups of fungi in subalpine grasslands undergoing shrub encroachment in other regions, such as the Pyrenees.

The encroachment of shrubs is expected to cause important changes in soil and litter properties (Zhou *et al.* 2017) and in the structure and function of fungal communities (Wardle 2004; Lauber *et al.* 2008; Tedersoo *et al.* 2014). Previous studies conducted in the Pyrenees found that the encroachment of some shrub species into subalpine grasslands altered soil properties; for example, Montané, Rovira and Casals (2007) and Montané *et al.* (2010) found that *Juniperus communis* and *Cytisus balansae* ssp. *europaeus* increased soil C and N stocks in the top soil layer. Whether these impacts on soil properties vary between sites that differ in shrub species composition, and whether the encroachment of shrubs into subalpine grasslands and the changes in soil properties are associated with structural changes in soil fungal communities, however, remain unknown. Regional differences in climatic and edaphic conditions across the subalpine belt in the Pyrenees favour the occurrence of a large number of shrub species with contrasting functional characteristics (Grau *et al.* 2012; Illa *et al.* 2017). The impacts of shrub encroachment on soil properties and soil fungal communities may therefore differ greatly between sites with contrasting abiotic conditions and shrub composition and between successional stages of shrub expansion.

The aim of our study was to determine whether shrub encroachment into subalpine grasslands altered the structure (e.g. the richness and the composition) of soil fungal communities. We also determined whether these structural changes were consistent amongst contrasting functional groups of soil fungi and if they were associated with plant-driven changes in soil properties (Cline and Zak 2015). We chose at two contrasting sites where shrub encroachment takes place: one north-facing site (hereafter North face) and one south-facing site (hereafter South face) in the Pyrenees. Each study site represents an independent case study because the shrub species are not shared between the two sites and aspect and climate differ. At each site we assessed the structure of the fungal communities along the succession from pure grassland to shrubland, and amongst vegetation types (i.e. grasses or shrubs, along the succession) to analyse the interplay of biotic (i.e. shrub occurrence and identity) and abiotic (i.e. soil properties) filters (Talbot *et al.* 2014). The succession from grassland to shrubland was divided into three main successional stages within each site: (i) pure grassland, where shrubs were absent, (ii) young, mixed shrubland, where grasses and various shrub species co-occurred and (iii) mature shrubland, dominated by a shrub species. The shrub species considered in this study differed in their functional and ecological characteristics. At North face, we analysed three ericoid mycorrhizal shrub species that typically promote the encroachment of subalpine grasslands on the north-facing slopes of the Pyrenees: *Calluna vulgaris*, *Rhododendron ferrugineum* and *Vaccinium myrtillus*. At South face, we analysed three shrub species that typically promote the encroachment on south-facing slopes: the arbuscular mycorrhizal (AM) shrubs *J. communis* and *Juniperus sabina* and the ectomycorrhizal (ECM) and/or ericoid mycorrhizal shrub *Arctostaphylos uva-ursi* (note that *Arctostaphylos* forms an 'arbutoid' type mychorrhiza with normally ECM fungi; the resulting symbiosis, specific to *Arbutoidae,* is functionally similar to ECM, the only difference being that some fungal hyphae do penetrate the root cells (Smith and Read 2008)).

We expected that the advance of the succession would modify soil properties, that the richness and composition of the soil fungal community would differ along the succession at both sites and that the structure of mycorrhizal communities would be more sensitive to changes in vegetation structure than non-mycorrhizal groups. On this point, we hypothesised that the occurrence of AM, ericoid or ECM mycorrhizal shrubs along the succession would markedly shape the richness and composition of AM, ericoid or ECM fungal communities, respectively. For example, we hypothesised that the richness of AM fungi at South face would be higher in AM mycorrhizal shrubs like *J. communis* or *J. sabina* than in *A. uva-ursi*, which does not establish symbioses with AM fungi; or that the richness of ericoid fungi would be primarily driven by the occurrence of shrubs that

Table 1. Description of the study sites.

	Bargadèra (North face)	Fogueruix (South face)
Orientation and aspect	North facing, smooth slope	South facing, steep slope
Altitude (m a.s.l.)	1800–1900	2000–2100
Regional climate	Atlantic	Continental, with Atlantic influence
Annual precipitation (mm)	800–900*	600–800**
Mean annual temperature (°C)	8–9*	8–10**
Maximum annual temperature range (°C)	−14 to 30*	−15 to 30**
Dominant shrub species	*C. vulgaris*, *R. ferrugineum*, *Vaccinium myrtillus*	*A. uva-ursi*, *J. communis*, *J. sabina*
Grassland type and dominant species	Mesophilic, dense subalpine grassland dominated by *Festuca eskia*, *Nardus stricta*, *Trifolium alpinum*, *Festuca nigrescens*, *Alchemilla* sp. and *Cerastium arvense*	Xerophilous, open subalpine grassland dominated by *Festuca ovina*, *Festuca gautieri*, *Hieracium pilosella*, *Achillea millefolium* and *Potentilla neumanniana*

*Data from the nearest meteorological station at North face, at Bagergue (1400 m a.s.l., available since 2015); **data from the nearest meteorological station at South face, at Planes de Son (1540 m a.s.l, available since 2015). Both meteorological stations are at a lower altitude (ca. 400 m) than the study sites, so precipitation is expected to be slightly higher and temperature is expected to be slightly lower, than the reported values at both sites. The two sites are only 22 km apart horizontally but are in contrasting climatic regions. The mean annual number of days with precipitation or fog is higher at North face, which has a marked Atlantic climate (Servei Meteorològic de Catalunya, http://meteo.cat/wpweb/climatologia, accessed on May 2018), although these two climatic variables have not been recorded by these nearby meteorological stations.

establish ericoid mycorrhizae (*A. uva-ursi*, *C. vulgaris*, *R. ferrugineum*, *V. myrtillus*). We also hypothesised that the composition of soil fungal communities would be more similar amongst shrubs that share mycorrhizal type than amongst shrubs that do not. And finally, that the structure of soil fungal communities that actively contribute to nutrient cycling, such as mycorrhizal or saprotrophic fungi, would be particularly sensitive to changes in soil properties.

MATERIAL AND METHODS

Study area and data collection

The fieldwork was conducted in October 2013 at two sites (approximately 22 km apart horizontally) in the subalpine belt of the Central Pyrenees (Table 1)—one site on the south face (Fogueruix, Pallars Sobirà, Catalonia; 42°35′32.9″N, 01°04′28.8″E) on a Devonian slaty lime substrate and one site on the North face (Bargadèra, Val d'Aran, Occitania; 42°39′53.9″N, 00°50′07.8″E) on Cambro–Ordovician schists, sandstones and quartzites, both on the periphery of the Aigüestortes i Estany de Sant Maurici National Park. Although mean annual precipitation does not vary much between the two study sites, the mean annual number of days with precipitation or fog and the relative air humidity are much higher at North than South face (Servei Meteorològic de Catalunya, http://meteo.cat/wpweb/climatologia, accessed on November 2018). We identified three successional stages at each of these two sites: (a) pure grassland consisting of a homogenous mixture of grasses and forbs, with no shrubs, (b) young shrubland that comprised a mosaic of grassland and small patches (ca. 1–3 m^2) of co-occurring shrubs (*C. vulgaris*, *R. ferrugineum* and *V. myrtillus* at North face, and *A. uva-ursi*, *J. communis* and *J. sabina* at South face) and (c) mature shrubland, with large, monodominant patches (>50 m^2) of each shrub species. No adult or young trees were present inside the sampled plots along the succession, but there were forest stands of *Pinus uncinata* and *P. sylvestris* near the study sites (>120 m at North face and >30 m at South face). At South face, though, there were individuals of adult pines scattered across the study area which formed small, open stands around the plots. The pure grasslands are still grazed in summer, despite the general decrease in livestock pressure in recent decades in the subalpine pastures in the Pyrenees, but not (or very seldom) in areas already covered with shrubs (Oriol Grau, pers. obs.).

The replication of the successional stages was done in each site separately; the two sites were not treated as replicates in the analyses but as two independent study cases. Sampling plots were established to reproduce the extant structure of the vegetation in each successional stage. We established four replicate 5 × 5 m plots (separated by a minimum of 10 m) in the pure grassland, and in each of three mature shrublands at each site (Fig. S1, Supporting Information). In the mixed shrubland, though, the plots for each vegetation type (grass, shrub 1, shrub 2, shrub 3) were grouped because all vegetation types co-occurred; in this intermediate stage, we established four groups of four plots. In total, we sampled 32 plots per site along the succession from pure grassland to mature shrubland. The distance between successional stages or between the three mature shrublands at each site was at least 100 m. We collected five soil samples from each of the 32 plots in each site to a depth of 5 cm using a 5-cm corer, which were pooled in a plastic bag as a composite sample (replicate); the coarse litter layer was removed before the samples were collected. One composite sample was collected in each plot in the pure grassland and the three mature shrublands and in each of the grouped plots in the mixed shrubland. Each sample was divided into two subsamples: one was used for the extraction of DNA, and the other was used for the chemical analyses of the soil. The samples were kept cold during transport from the field to the laboratory, where they were frozen (-20°C) until processed.

Molecular, bioinformatic and soil chemical analyses

The soil samples were freeze-dried at -40°C for 48 h before DNA extraction. Genomic DNA was extracted from ca. 0.25 g of homogenised soil with a MoBio Power Soil® DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, Canada) following the manufacturer's protocol. Fungal DNA was amplified from the internal transcribed spacer (ITS2) region of ribosomal DNA using the primer pair ITS4 and FITS7 (White *et al.* 1990; Ihrmark *et al.* 2012). This primer pair is commonly used but it is known to underrepresent Glomeromycotina (Kohout *et al.* 2014). As a result, a limitation of this study is that fungi that form arbuscular mycorrhizae may be artificially decreased. The ITS4 primer was

labelled with a sample-specific, 10-base barcode. PCR was performed in triplicate 20-μl reactions containing 10 ng of DNA template, 2 μM of each primer, 0.2 mM dNTPs, 1U Phusion® High-Fidelity polymerase and 1 × Phusion HF buffer (ThermoFisher Scientific, Waltham, USA). The following conditions were used to conduct the PCR: initial denaturation at 98°C for 1 min followed by 27 cycles of 98°C for 10 sec, 57°C for 20 sec and 72°C for 30 sec, with a final extension at 72°C for 7 min. Triplicate amplicons were pooled and checked by MultiNA microchip electrophoresis (Shimadzu, Kyoto, Japan). The amplicons were cleaned with AMPure XP (Beckman Coulter, Brea, USA) and quantified with PicoGreen (Invitrogen, Carlsbad, USA) following the manufacturer's protocols. The amplicons were sequenced at the Biocenter Oulu Sequencing Center (Finland) using an Ion Torrent PGM platform (Ion 316 Chip v2; ThermoFisher Scientific). Four samples were lost during this process, so 60 of the 64 composite soil samples were sequenced (Data available at the Dryad Digital Repository, https://doi.org/10.5061/dryad.j02mk45).

We analysed the data using Qiime (Caporaso *et al.* 2010). The raw sequences were assigned to samples based on their sample-specific barcodes and were quality filtered with the following criteria: Phred quality score >20, minimum sequence length of 150 bp and maximum of two mismatches in either primer. Only full-length ITS2 sequences with both reverse and forward primers attached were analysed. The primer sequences were removed, the ITS2 sequences were clustered at 97% similarity using the USEARCH algorithm, and chimeric sequences were discarded using UCHIME implemented in USEARCH (Edgar 2010; Edgar *et al.* 2011). Combining different clustering methods may decrease the number of operational taxonomic units (OTUs) (Nguyen *et al.* 2015), so OTU selection was repeated using the UCLUST algorithm (Edgar 2010). Representative sequences were chosen for each OTU and queried against the UNITE fungal database (using Qiime release 7.0 with Species Hypothesis clustered at a threshold level of 97%) (Kõljalg *et al.* 2013) using BLAST (Altschul *et al.* 1990). These representative sequences were the centroid for each OTU cluster. Identified OTUs were classified into functional groups based on published ecological information of the taxa in question (Tedersoo and Smith 2013; Nguyen *et al.* 2016). The data set containing only fungal OTUs comprised a total of 1393 254 high-quality sequences with an average of 23 220 ± 8395 reads per sample. Next-generation sequencing libraries are generally normalised by random subsampling to the size of the smallest library, but this practice often preserves only a small fraction of the useable data, which can increase type I (decreased specificity) and type II (decreased sensitivity) errors and has been strongly discouraged by McMurdie and Holmes (2014). We avoided this problem by including all high-quality fungal sequences in the final analyses and using only rarefied data for exploratory purposes and checking the robustness of our data by rarefying the sequence counts of all samples to the smallest library (3832 sequences). We also calculated rarefied OTU accumulation curves, which indicated that the sequencing depth of our samples and the completeness of our sampling were very good (Figs S2 and S3, Supporting Information).

The frozen soil samples were thawed at room temperature and sieved to remove fine roots. Twenty millilitres of soil were added to 50 ml of distilled water for measuring pH and electrical conductivity using standard protocols. The contents of soluble Ca, Mg and K were determined by weighing and adding 22 ml of homogenised soil to 100 ml of 1 mol/l acid ammonium acetate, shaken for 1 h, vacuum-filtered and analysed using a SpectrAA 220FS atomic absorption spectrophotometer (Varian, Veriton, Australia). Total P content was determined colorimetrically by diluting 0.25 g of soil in 25 ml of a nitric-hydrochloric acid solution. The remainder of the sample was further dried and pulverised, and its total C and N contents were determined using an automatic CHN analyser (Fisons Instruments, Milan, Italy). The amount of organic matter was determined by ignition loss after heating the samples at 500°C for 3.5 h.

Data analysis

We retained the OTUs with five or more sequences (2836 of 3115 OTUs) to minimise false positives and contaminants with low abundances, as suggested by (Lindahl *et al.* 2013). We transformed the read-abundance matrix into a presence–absence matrix to avoid the uncertainty about using read abundance as a reliable indicator of OTU abundance in the samples (Amend, Seifert and Bruns 2010). We ran non-metric multidimensional scaling (NMDS) ordinations with the presence–absence matrix using the Jaccard dissimilarity index ('metaMDS' R function, 'BiodiversityR' package; Kindt and Coe 2005) to determine the similarity of the fungal communities amongst the grassland, young shrubland and mature shrubland. For exploratory purposes and to check the robustness of the data, we also performed ordinations using (i) raw read-abundance data, (ii) normalised read-abundance data (by dividing the number of sequences of each OTU by the total number of sequences in the sample) or (iii) rarefied read-abundance data (by randomly subsampling each sample to the lowest number of sequences in a soil sample, including the complete data set) using the Bray–Curtis index. The stress value of the NMDS was higher when read-abundance data were used, the NMDS gave very similar results, and the abundance data were inherently uncertain, as mentioned above, so we focused on the presence–absence data. We also assessed the dispersion of the community composition for each successional stage and for each site using the 'betadisper' R function ('vegan' package, Oksanen *et al.* 2015) and tested whether the distances to the centroids differed between successional stages using a permutational test ('permutest' R function). We calculated the absolute richness of various functional groups and their proportional richness (number of OTUs of a given functional group/total number of identified OTUs on a per-sample basis) to estimate and compare the prevalence of each functional group amongst sites and stages.

A total of 1673 of 2836 OTUs (59%) were assigned to functional groups (Table S1, Supporting Information). This percentage—59%—is similar or higher than those found in studies on the functional ecology of fungi (e.g. Tedersoo *et al.* 2014; Semenova *et al.* 2015; Grau *et al.* 2017; Geml 2019), and we deemed our conservative assignment appropriate for describing the general ecological patterns of the most common functional groups (AM, ECM, ericoid, root endophytic, saprotrophic, lichenised and plant pathogenic fungi), that are represented by a minimum of 50 OTUs or 15 000 sequences per group. We must still bear in mind that an important proportion (41%) of OTUs could not be assigned to functional groups. Although we believe it is very unlikely that any soil inhabiting functional group would be represented by a disproportionately large fraction of unknown species, the fraction that is still not assigned to functional groups could, in theory, alter the functional patterns observed. Therefore, in this study we will present the most obvious and clear patterns of the functional groups analysed. We used the

'envfit' R function to fit the soil variables and OTU richness of each functional group onto the NMDS ordination and used a PERMANOVA ('adonis' R function) to test the compositional differences of the fungal communities between sites, successional stages or vegetation types.

We also analysed the changes in soil variables, total richness of soil fungi and richness of each functional group between sites, successional stages, and vegetation types at each site (grass and shrub 1, shrub 2 and shrub 3). Although in our sampling design we maximised the independence of samples as much as possible given the extant structure of vegetation along the succession, it was not possible to find a completely independent, random distribution of combinations of successional stage × vegetation type in the field sites. We, therefore, firstly checked if there were any underlying patterns of spatial aggregation in our data with autocorrelation semivariograms ('nlme' package; Pinheiro *et al.* 2015), that are used for measuring the degree of spatial dependence between observations as a function of distance. The visual interpretation of the semivariograms indicated that in most cases there were no autocorrelated patterns in the data despite the plots were not completely randomised. Yet, we preferred to statistically check if autocorrelation should be accounted for in the models. We therefore built two types of models: (i) generalised least square (GLS) models that did not account for spatial autocorrelation, (ii) generalised least square models that included the 'corSpatial' R function to account for potential spatial autocorrelation. For this second type of generalised least square models, the coordinates (longitude and latitude) of each plot were specified and we fitted five different models using a different autocorrelation structure each time (e.g. exponential, linear, gaussian, rational quadratic or spherical). After running all the models (one without and five with autocorrelation structure) for each soil or richness variable, we compared and selected the best model using scores of the Akaike information criterion (AIC). We used maximised log-likelihood in all cases and log-transformed the soil variables in a few cases to obtain normally distributed data to run the models. The pure grassland was the initial stage of the succession from grassland to shrubland, so we considered it as the reference level in all models.

We conducted Pearson's product-moment correlations between the soil variables and the total richness and the richness of each functional group of fungi within each site. We also performed Mantel tests to determine the correlation between the changes in soil properties and the compositional changes of the fungal communities along the succession. Dissimilarity matrices were calculated with the 'vegdist' function ('vegan' package; Oksanen *et al.* 2015) using the Euclidean index for the soil data and the Sorensen index for the compositional data; the soil data were scaled before calculating the dissimilarity matrix. All analyses were performed in R v. 3.2.2 (R Core Team 2017).

RESULTS

A total of 2836 OTUs with five or more sequences were identified at 97% sequence similarity. Ascomycota represented 61% of the OTUs, followed by Basidiomycota (26%), Zygomycota (3%) and Glomeromycota (2%). Agaricales and Helotiales were the most abundant orders (11% of the OTUs each), followed by Chaetothyriales (7%), Pleosporales (5%), Sebacinales (2%), Verrucariales (2%), Morteriellales (2%) and Archaeorhizomycetales (2%). Incertae sedis represented 6%.

Richness patterns and uniqueness of soil fungal communities

Total fungal richness was higher at South face than North face (Fig. 1a; Table S2, Supporting Information) but varied little along the succession from the pure grassland to the mature shrubland within each site. Fungal richness was higher only in the young shrubland of *A. uva-ursi* compared to the initial stage of the succession. The richness of the AM, ECM, root endophytic, saprotrophic and plant pathogenic fungi was generally higher at South face (Fig. 1b–h). The richness of ericoid and lichenised fungi did not differ significantly between sites.

The richness of the functional groups differed to some extent between successional stages. At North face, the richness of AM fungi was lower in several vegetation types in the young and mature shrublands (but not in *R. ferrugineum*), whereas the richness of lichenised fungi tended to be higher in the young and mature shrublands. At South face, the richness of ECM, ericoid and root endophytic fungi was higher in the young and mature shrublands of *A. uva-ursi* compared to the pure grassland. Most of these trends were also apparent when the proportional richness of each group was used instead of the absolute richness (Fig. S4, Supporting Information).

The number of exclusive OTUs was twice as high at South face than North face (1365 vs 638 unique OTUs). About one third of the total number of OTUs (2836) occurred at both the study sites (834 OTUs), and approximately 22% of the OTUs were shared by all vegetation types within each site (Fig. 2). About 30% of the OTUs were shared uniquely between the young and the mature shrubland, but very few OTUs (2%) were shared uniquely between the pure grassland and the mature shrubland within each site (Fig. 2a and b). The increase in the number of unique OTUs was highest between the pure grassland and the young shrubland (about two-fold at North face and seven-fold at South face). The number of unique OTUs in the young shrubland was highest for the grass patches and *V. myrtillus* at North face and the grass patches and *A. uva-ursi* at South face (Fig. 2c and d). The number of OTUs shared by all shrubs increased from the young to the mature shrubland at North face (46 vs 122 OTUs), whereas it decreased at South face (72 vs 47). The number of exclusive OTUs in the mature shrubland was highest for *R. ferrugineum* and *C. vulgaris* at North face and for *A. uva-ursi* and *J. communis* at South face. The number of exclusive OTUs for all shrubs increased from the young to the mature shrubland, except for *V. myrtillus*, which did not vary.

Compositional patterns of soil fungal communities

A PERMANOVA ($P < 001$) found that the fungal-community composition of OTUs based on the presence–absence data varied significantly between North face and South face (obviously separated along the first axis in Fig. 3). This pattern was also obtained whether we used raw, normalised or rarefied read-abundance data (Fig. S5, Supporting Information). Compositional turnover was also clear along the second axis in Fig. 3, which encompassed the compositional change along the succession from grassland to shrubland. This compositional turnover between the pure grassland and the shrublands was also consistent within each site (Fig. 4a), regardless of the species of shrubs along the succession (Fig. S6, Supporting Information). The composition of the fungal community differed significantly amongst shrub species in the mature stage at South face and in the young and mature stages at North face (Fig. 4a). Compositional turnover was also significant

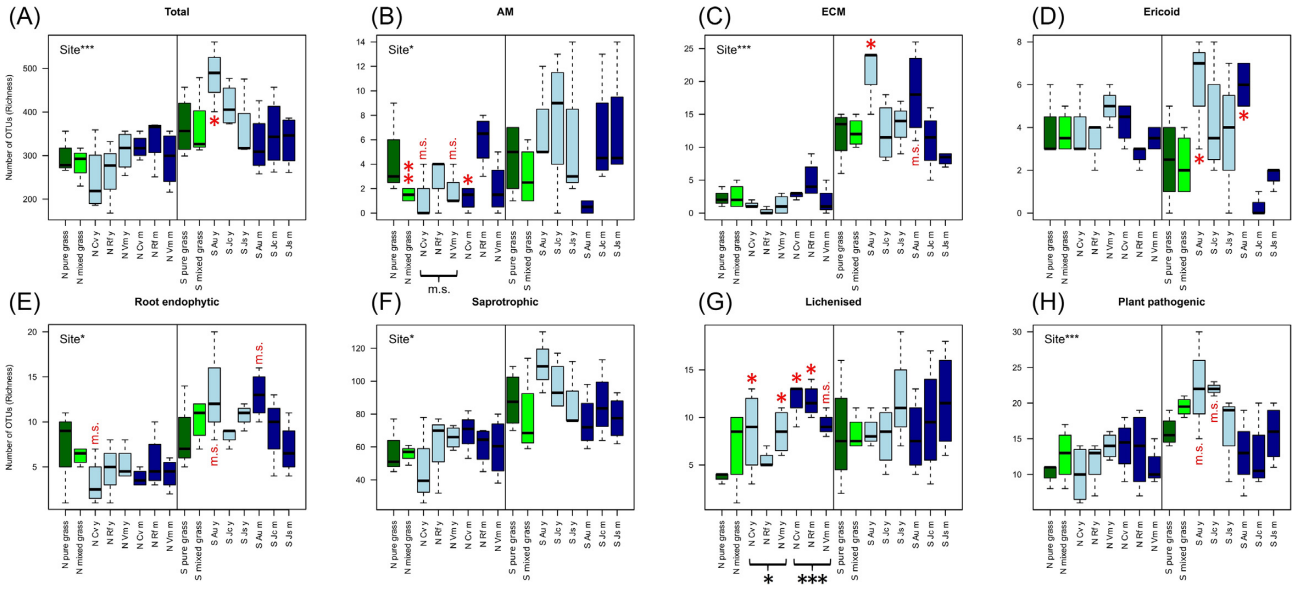

Figure 1. OTUs representing total richness and the richness of AM, ECM, ericoid, root endophytic, saprophytic, lichenised and plant pathogenic fungi. N, North face (left half of each panel); S, South face (right half of each panel); pure grass, pure grassland (dark green); mixed grass, grass patches in the mixed, young shrubland (light green); y, shrub in the mixed, young shrubland (light blue); m, shrub in the monodominant, mature shrubland (dark blue); Cv, *C. vulgaris*; Rf, *R. ferrugineum*; Vm, *V. myrtillus*; Au, *A. uva-ursi*; Jc, *J. communis*; Js, *J. sabina*. The black asterisks in the panels indicate significant differences between the two sites. The significant differences between the pure grassland (determined as the reference level in the models) and the other combinations of vegetation type × stage are marked in red; the significances are based on the Akaike information criterion selection of the best of the three models tested (see Methods). The curly brackets below the panels indicate the significance between successional stages (between the pure grassland (dark green) and the shrubs in the young shrubland (light blue), or between the pure grassland and the shrubs in the mature shrubland (dark blue)). ***, $P < 0.0001$; * or ∗, $P < 0.01$; m.s., marginally significant. See Table S2 (Supporting Information) for the mean ± standard deviation of the absolute richness for each vegetation type and successional stage.

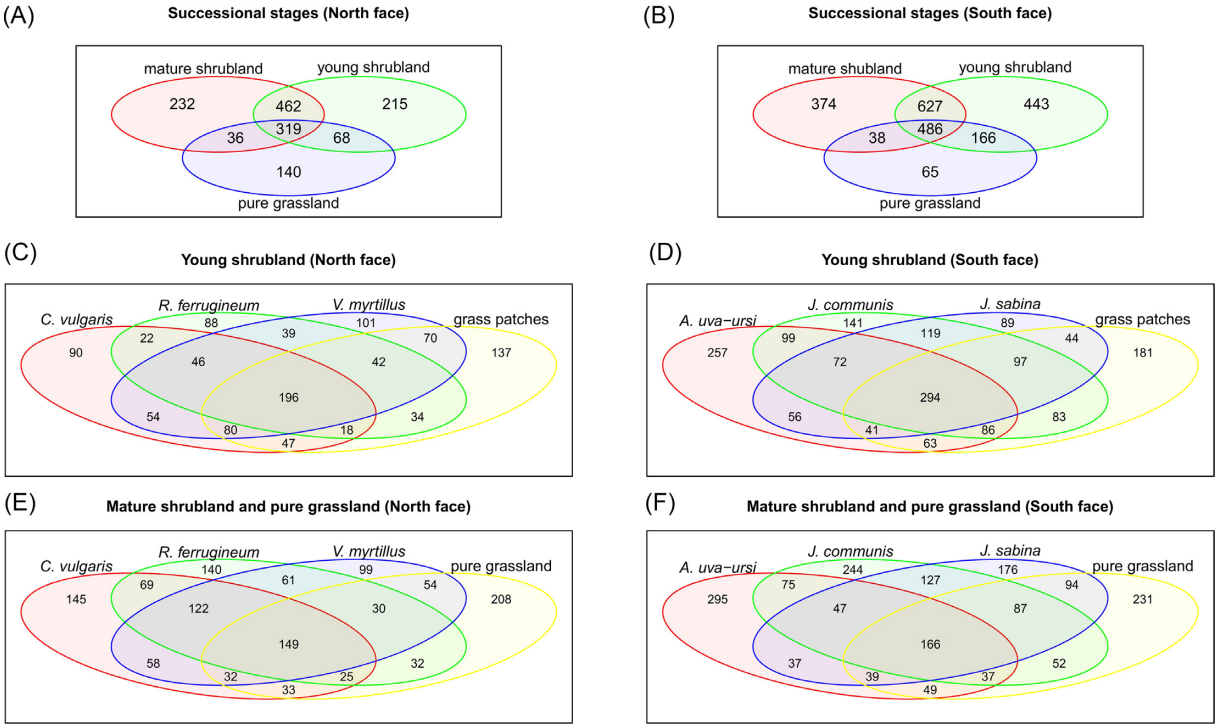

Figure 2. Venn diagrams of the number of OTUs exclusive to (a) and (b) each successional stage, (c) and (d) the young shrubland, and (e) and (f) the mature shrubland and the pure grassland (North face, left figures; South face, right figures). Non-overlapping colours represent OTUs found only in a single vegetation type, and overlapping colours represent OTUs that are only shared by two or more vegetation types.

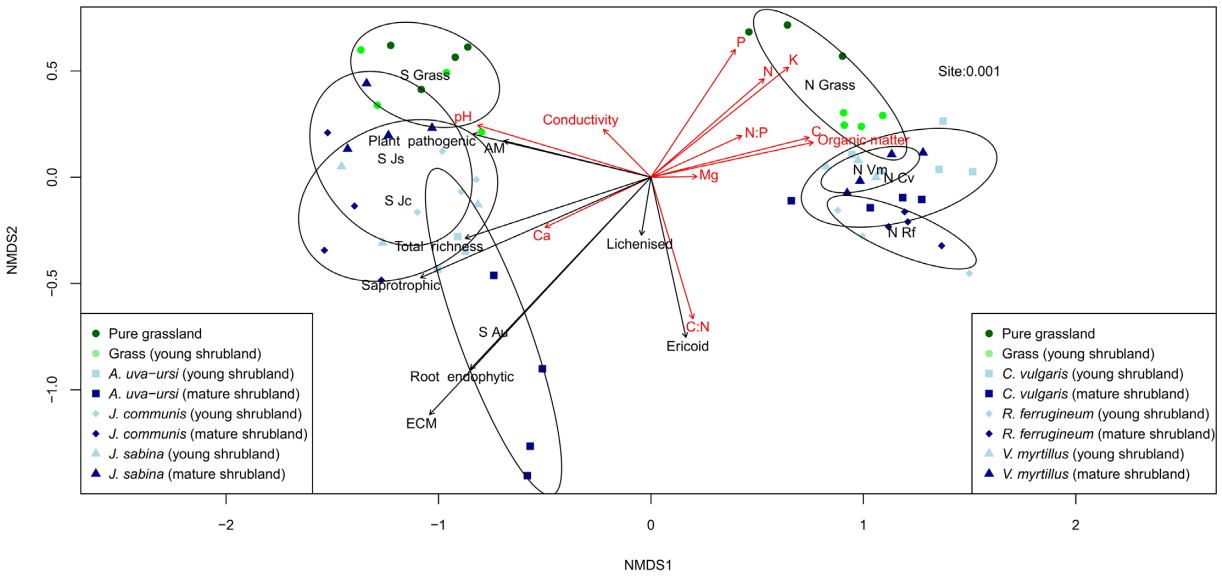

Figure 3. Non-metric dimensional scaling analysis of the presence or absence of OTUs (community composition) along the succession, from the pure grassland (dark green) to the grass patches in the young shrubland (light green), the shrubs in the young shrubland (light blue), and the shrubs in the mature shrubland (dark blue) at South face (group on the left, left legend) and at North face (group on the right, right legend) (stress 0.104). The presence–absence data are based on all high-quality sequences. The total richness and the richness of all functional groups (black arrows) and the soil variables (red arrows) fitted on the NMDS axes are also shown. Most groups and soil variables were strongly correlated, either positively or negatively, with at least one of the two axes (see Table S3, Supporting Information, for the regression weights and r^2 of the variables fitted in the NMDS). The abbreviations in the plot indicate the centroid of the ellipses that group each vegetation type in the NMDS: N, North face; S, South face; Grass, pure grassland or grass patches in the young shrubland; Cv, *C. vulgaris*; Rf, *R. ferrugineum*; Vm, *V. myrtillus*; Au, *A. uva-ursi*; Jc, *J. communis*; Js, *J. sabina*. The *P* value (significant at <0.05) in the figure was obtained by PERMANOVA with the 'adonis' R function for analysing the differences in composition of the soil fungal communities between the two sites. The uneven number of points for some vegetation types indicates that some soil samples were not sequenced (4 of 64).

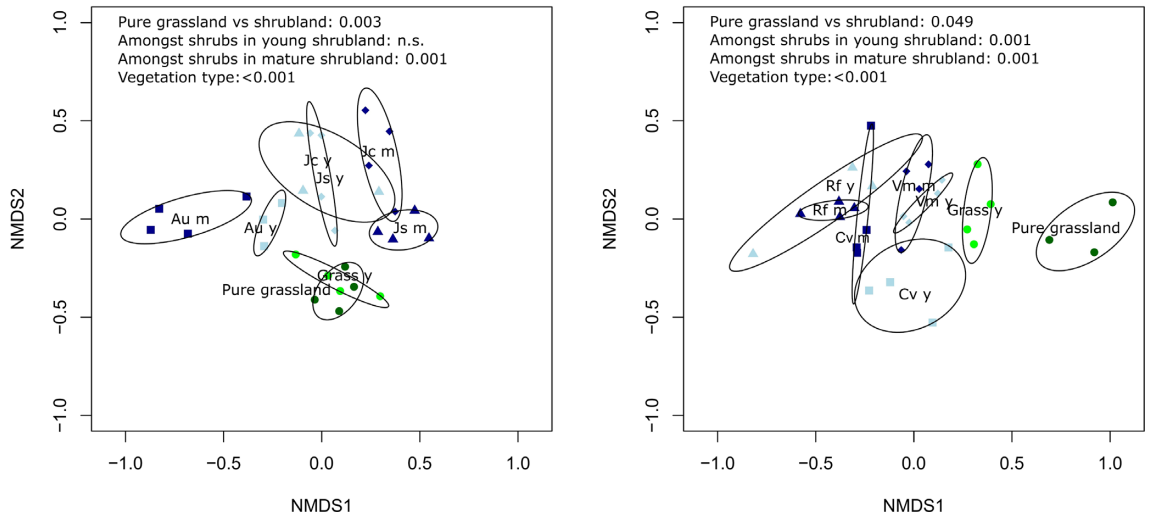

Figure 4. Non-metric dimensional scaling analyses of the presence or absence of OTUs (community composition) along the succession, from pure grassland (dark green) to grass patches in the young shrubland (light green), young shrubland (light blue) and mature shrubland (dark blue), where the soil fungal communities from the two sites have been analysed separately (South face on the left, stress 0.190; North face on the right, stress 0.158). Grass, grassland; Cv, *C. vulgaris*; Rf, *R. ferrugineum*; Vm, *V. myrtillus*; Au, *A. uva-ursi*; Jc, *J. communis*; Js, *J. sabina;* y: in young shrubland; m: in mature shrubland. The significances (*P* values) of the changes in community composition are indicated (i) between the pure grassland and the shrubland, (ii) amongst vegetation types (grass, shrub 1, shrub 2 and shrub 3) and (iii) amongst shrubs within the young or the mature shrubland. The *P* values (significant at <0.05; m.s., n.s., not significant) were obtained by PERMANOVA using the 'adonis' R function.

between the young and mature shrublands for *J. communis* at South face and for *C. vulgaris* and *R. ferrugineum* at North face (Fig. S6, Supporting Information). The composition between the young and mature shrublands differed marginally significantly in *A. uva-ursi* and *J. sabina* and did not differ significantly in *V. myrtillus*.

The dispersion of the composition of OTUs was higher in the young and mature shrublands than the pure grassland at both sites (Fig. 5). The dispersion in the young and mature shrublands did not vary significantly at North face, whereas dispersion was significantly higher for the mature than the young shrubland at South face.

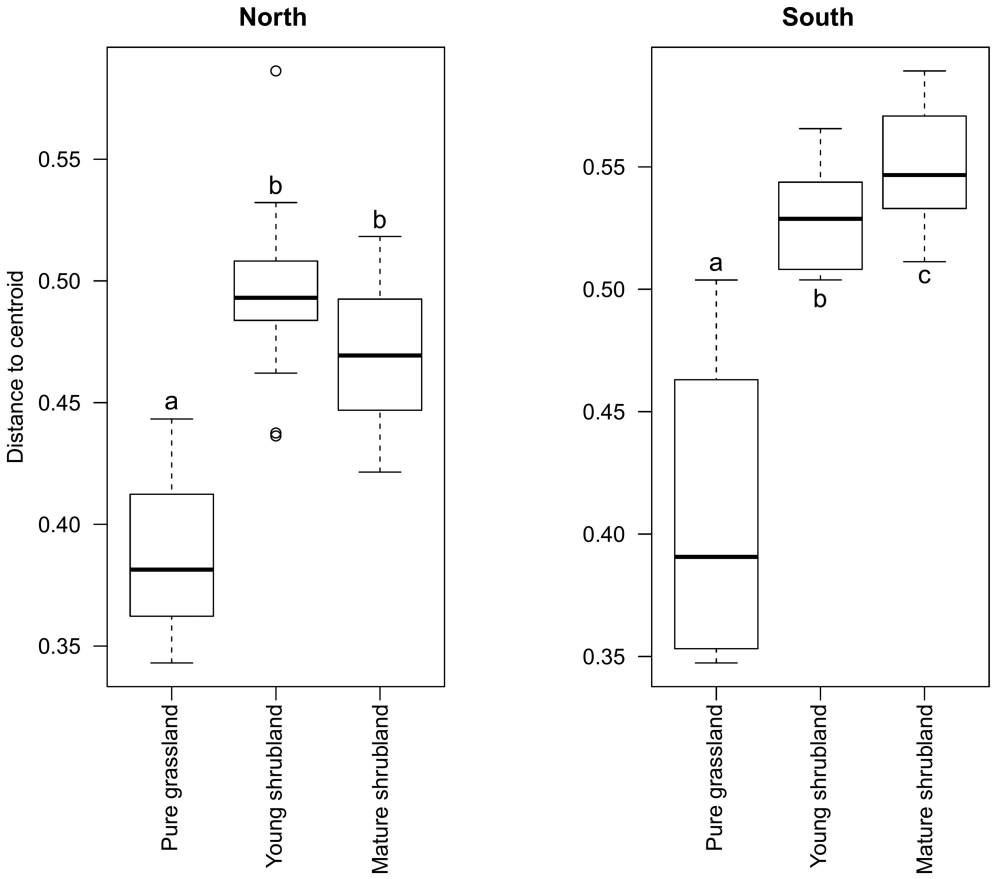

Figure 5. Dispersion of the composition of fungal OTUs (expressed as the distance to the centroids of each successional stage) when North face and South face were analysed separately (see Fig. 4a). The letters indicate significant differences obtained from ANOVA-like permutational tests between the pure grassland and the young shrubland or the mature shrubland.

The composition of all functional groups differed significantly between North face and South face (PERMANOVA, $P < 0.001$ for all groups; Table 2), similar to the pattern when all fungal OTUs were analysed together (Fig. 3). Compositional turnover along the succession within each site was significant or marginally significant within most functional groups, except for the ericoid and the root endophytic fungi at North face (Table 2). Compositional changes along the succession were consistent in the communities of saprotrophic and plant pathogenic fungi, regardless of the shrub species along the succession. The compositional changes of the other functional groups, however, were sensitive to the identity of the shrub species along the succession. AM fungal composition differed for only two shrub species at North face (*R. ferrugineum* and *V. myrtillus*); ericoid fungal composition differed only for the shrub species at South face; root endophytic fungal composition differed only along the succession to *C. vulgaris* or *A. uva-ursi* shrubland; ECM fungal composition differed in the succession to the *C. vulgaris*, *R. ferrugineum* or *A. uva-ursi* shrubland; and lichenised fungal composition differed in all cases, except along the succession to the *J. communis* shrubland. Globally, the number of functional groups with compositional changes was higher along the succession where *C. vulgaris*, *R. ferrugineum* or *A. uva-ursi* occurred in the succession than in the *V. myrtillus*, *J. communis* or *J. sabina* succession. Several functional groups also had significant or marginally significant compositional differences between the young and mature shrublands when each shrub species was analysed separately; these differences occurred in more functional groups and for more shrub species at South face than North face.

Relationship between the soil properties and the richness and composition of soil fungal communities

Soil C, N, P, K and organic-matter contents and the C:N and N:P ratios (on a mass basis) were significantly higher at North face than South face (Fig. 6), whereas Ca, pH and electrical conductivity had the opposite pattern. At North face, the C:N ratio was higher in the young and mature shrublands than the pure grassland and C tended to be higher in the young shrubland for all shrub species and in the mature shrubland for *V. myrtillus*. The organic-matter content also increased towards the mature shrubland, particularly for *R. ferrugineum* and *V. myrtillus*, K tended to be lower in the mature shrubland, and soluble-P content increased in the young shrubland for *V. myrtillus* but decreased in the mature shrubland for *R. ferrugineum*. At South face, total N, P and K contents were lower in the mature shrubland than the pure grassland, the soil C:N ratio was significantly higher in the mature shrubland for *A. uva-ursi* and *J. communis* than the pure grassland and pH was particularly low in the mature shrubland for *A. uva-ursi*.

We found strong site-specific correlations between some soil variables and the richness of some functional groups (Table 3). P content was correlated with the richness of more functional

Table 2. Significance (*P* values) of the compositional changes in the functional groups of soil fungi (i) between sites (top row), (ii) along the entire succession within each site (pure grassland, young shrubland and mature shrubland; unshaded rows) and (iii) between the young and mature shrublands within each site (excluding the pure grassland and the grass patches in the young shrubland; shaded rows). The significance shown in each cell is from a PERMANOVA where only a chosen subset of the presence–absence data was included each time (a subset filtered by site, successional stage, shrub species and functional group). The *P* values in bold indicate significant results (<0.05); m.s., marginally significant; n.s., not significant.

Site	Successional stage	Shrub species in the analysis	AM	ECM	Ericoid	Root endophytic	Saprotrophic	Lichenised	Plant pathogenic
North vs South	Entire succession	All species	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
North face	Entire succession	All species	**0.002**	**0.015**	n.s.	n.s.	**0.001**	**0.001**	**0.001**
North face	Entire succession	*C. vulgaris*	n.s.	**0.037**	n.s.	**0.007**	**0.001**	**0.014**	**0.001**
North face	Entire succession	*R. ferrugineum*	**0.011**	**0.003**	n.s.	n.s	**0.001**	**0.001**	**0.001**
North face	Entire succession	*V. myrtillus*	**0.036**	n.s	n.s.	n.s.	**0.001**	**0.002**	**0.003**
North face	Young vs mature shrubland	*C. vulgaris*	n.s.	n.s	n.s.	**0.037**	**0.035**	n.s	0.052 (m.s.)
North face	Young vs mature shrubland	*R. ferrugineum*	0.067 (m.s.)	n.s	n.s.	n.s.	n.s	**0.027**	n.s
North face	Young vs mature shrubland	*V. myrtillus*	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s
South face	Entire succession	All species	**0.017**	**0.045**	**0.009**	0.08 (m.s.)	**0.001**	0.074 (m.s.)	**0.002**
South face	Entire succession	*A. uva-ursi*	n.s.	**0.002**	**0.001**	**0.001**	**0.001**	**0.027**	**0.001**
South face	Entire succession	*J. communis*	n.s	n.s	**0.033**	n.s.	**0.001**	n.s.	**0.026**
South face	Entire succession	*J. sabina*	n.s.	n.s	**0.003**	n.s.	**0.001**	0.064 (n.s.)	**0.001**
South face	Young vs mature shrubland	*A. uva-ursi*	n.s.	0.086 (m.s.)	n.s.	**0.011**	0.053 (m.s.)	n.s.	0.053 (m.s.)
South face	Young vs mature shrubland	*J. communis*	n.s.	n.s	n.s.	n.s.	**0.029**	n.s.	**0.027**
South face	Young vs mature shrubland	*J. sabina*	n.s.	n.s	0.067 (m.s.)	0.058 (m.s.)	**0.037**	n.s.	**0.034**

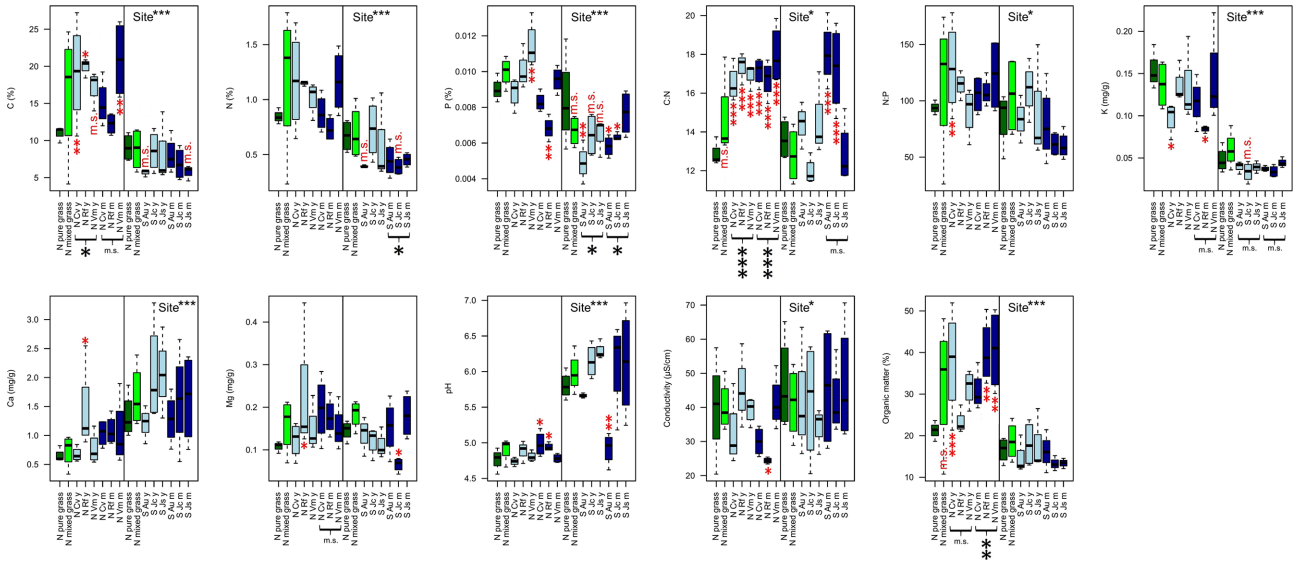

Figure 6. Characterisation of soil nutrient and organic-matter contents, pH and electrical conductivity. N, North face (left half of each panel); S, South face (right half of each panel); pure grass, pure grassland (dark green); mixed grass, grass patches in the mixed, young shrubland (light green); y, shrub in the mixed, young shrubland (light blue); m, shrub in the monodominant, mature shrubland (dark blue); Cv, *C. vulgaris*; Rf, *R. ferrugineum*; Vm, *V. myrtillus*; Au, *A. uva-ursi*; Jc, *J. communis*; Js, *J. sabina*. The black asterisks in the panels indicate significant differences between the two sites. The significant differences between the pure grassland (determined as the reference level in the models) and the other combinations of vegetation type × stage are marked in red; the significances are based on the Akaike information criterion selection of the best of the three models tested (see Methods). The curly brackets below the panels indicate the significances between successional stages (between the pure grassland (dark green) and the shrubs in the young shrubland (light blue) or between the pure grassland and the shrubs in the mature shrubland (dark blue)). *** or ***, $P < 0.0001$; ** or **, $P < 0.001$; * or *, $P < 0.05$; m.s., marginally significant.

groups: it was negatively correlated with the richness of AM fungi at North face and with the richness of ECM, ericoid and root endophytic fungi at South face. N content was correlated negatively with total and AM fungal richness at North face and positively with plant pathogenic fungal richness at South face. The C:N ratio at South face was correlated positively with ECM fungal richness and negatively with plant pathogenic fungal richness.

The fungal community compositions at North face and South face in soils with inherently different properties, indicated by the first NMDS axis in Fig. 3 (Table S3, Supporting Information), illustrated the compositional differences between sites and the main differences in soil variables. The compositional changes were in turn associated with changes in soil variables along the succession at both sites. The C:N ratio was strongly

Table 3. Pearson's product-moment correlations between soil variables and total fungal richness and the richness of each functional group of fungi at North face and South face. Bold values indicate significant correlations ($P < 0.05$); non-bold values indicate marginally significant correlations ($P < 0.09$); n.s., not significant.

Site	Soil variable	Total richness	AM	ECM	Ericoid	Root endophytic	Saprotrophic	Lichenised	Plant pathogenic
North face	C	**−0.392**	**−0.42**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	N	**−0.419**	**−0.385**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	P	n.s.	**−0.408**	n.s.	0.341	n.s.	n.s.	−0.354	n.s.
	K	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**0.47**	n.s.
	Ca	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Mg	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	C:N	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	N:P	−0.337	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	pH	n.s.	n.s.	n.s.	n.s.	n.s.	0.348	n.s.	n.s.
	Conductivity	n.s.	−0.34	n.s.	n.s.	n.s.	n.s.	**−0.531**	n.s.
	Organic matter	n.s.	n.s.	0.346	n.s.	n.s.	n.s.	0.318	n.s.
South face	C	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	N	0.31	n.s.	n.s.	n.s.	n.s.	0.32	n.s.	**0.362**
	P	n.s.	n.s.	**−0.488**	**−0.586**	**−0.502**	n.s.	n.s.	n.s.
	K	n.s.	n.s.	n.s.	n.s.	n.s.	−0.32	n.s.	n.s.
	Ca	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Mg	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	C:N	n.s.	n.s.	**0.396**	n.s.	n.s.	n.s.	n.s.	**−0.501**
	N:P	**0.43**	n.s.	n.s.	n.s.	n.s.	**0.415**	n.s.	n.s.
	pH	n.s.	n.s.	−0.318	−0.343	n.s.	n.s.	n.s.	n.s.
	Conductivity	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Organic matter	0.333	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.307

Table 4. *P* values obtained by Mantel tests to assess the correlation between soil properties (including all variables) and the composition of the fungal communities along the succession from the pure grassland to the mature shrubland. The first two columns include all shrub species along the succession from the pure grassland to the mature shrubland at North face or South face. The other columns show the results for each shrub species separately. The first row includes all fungal OTUs, and the remaining rows are for one functional group only. Bold values indicate significant correlations ($P < 0.05$); non-bold values indicate marginally significant correlations ($0.05 \leq P < 0.1$); n.s., not significant.

	North face	South face	*C. vulgaris*	*R. ferrugineum*	*V. myrtillus*	*A. uva-ursi*	*J. communis*	*J. sabina*
All fungi	**0.004**	**<0.001**	**0.001**	**<0.001**	n.s.	**<0.001**	**<0.001**	**0.003**
AM	0.072	n.s.	n.s.	**0.0041**	n.s.	**0.048**	n.s.	n.s.
ECM	**0.002**	**0.001**	n.s.	**0.012**	n.s.	**0.005**	**0.049**	0.087
Ericoid	n.s.	**0.001**	n.s.	n.s.	n.s.	n.s.	**0.009**	n.s.
Root endophytic	n.s.	0.067	n.s.	n.s.	n.s.	**0.024**	n.s.	n.s.
Saprotrophic	0.098	<0.001	0.099	n.s.	n.s.	**0.018**	**0.007**	**0.005**
Lichenised	**0.0136**	**0.001**	**0.029**	n.s.	n.s.	n.s.	0.132	**0.012**
Plant pathogenic	**<0.001**	0.081	0.087	**0.022**	0.095	**0.026**	**0.046**	**0.034**

negatively correlated with the second NMDS axis, which encompassed the compositional turnover along the succession; high C:N ratios were associated with the fungal communities in the mature stages. In contrast, N, P and K contents were significantly positively correlated with the second NMDS axis and were positively associated with fungal composition in the pure grassland. The link between the composition of soil fungi and the soil data was supported when we ran Mantel tests to determine the correlation between the changes in soil properties, including all variables, and the compositional turnover of the fungal communities along the succession within each site (first row in Table 4). The correlation was highly significant when all fungi were included, regardless of the shrub species along the succession (except for *V. myrtillus*).

Compositional turnover was also correlated with the changes in soil properties at the level of functional group. Within-group variability was significantly correlated with the changes in soil properties in ECM, lichenised and plant pathogenic fungi at North face and in ECM, ericoid, saprotrophic and lichenised fungi at South face (Table 4). The identity of the shrub species along the succession was also key for understanding the strength of the correlation between each functional group and soil property.

DISCUSSION

Changes in soil properties

The encroachment of shrubs into subalpine grasslands promoted abiotic heterogeneity by modifying soil properties along the succession at North and South face sites. Both sites were characterised by an increase in the C:N ratio along the succession, despite the marked difference in soil properties between North face and South face (Fig. 6). This ratio integrates information on the capacity of the soil to store and recycle energy and

nutrients (Gregorich *et al.* 1994). The C:N ratio in the pure grassland at North face was about 13, similar to or slightly higher than the ratio in agricultural soils, where it generally ranges from 10 to 12, whereas it ranged between 16 and 20 in the mature shrubland. This increase in the C:N ratio was likely due to the expansion of ericaceous shrubs along the succession at this site; these shrubs produce recalcitrant litter, which has a high C:N ratio and high contents of polyphenolic compounds that bind to organic material in the soil (Wurzburger and Hendrick 2007), which reduces the activity of soil enzymes and the rate of nutrient mineralisation (Bloom and Mallik 2006; Horton *et al.* 2009). The increase in the C:N ratio was also observed along the succession in the *J. communis* or the ericaceous *A. uva-ursi* shrubland at South face; in this site, though, there was also a decrease in essential nutrients such as N, P and K towards the mature stage. The decrease in essential nutrients was possibly due to the decrease in excrement deposition from domestic herbivores and to the internalisation of nutrients in the biomass of the shrubs. Internalisation has been well documented for ericaceous shrubs (Horton *et al.* 2009), but it may also occur in many other shrubs in cold, nutrient-limited ecosystems (Berendse and Jonasson 1992) and may be a mechanism by which shrubs control nutrients in an ecosystem (Chapin *et al.* 1997), enabling them to outcompete grass species and advance the succession.

We also found differences in soil properties amongst vegetation types within the young and mature shrublands that increased abiotic heterogeneity in the soil at the microhabitat scale, possibly due to species-specific feedbacks between plants and soil (Bezemer *et al.* 2006).

General patterns of the structure of soil fungal communities

The differences in total richness and composition were larger between the two sites than between successional stages or amongst shrub species (Figs 1a and 3). North face and South face differed greatly in climatic, edaphic and biogeographic characteristics (Table 1, Fig. 6), suggesting that the structure of the fungal communities was primarily driven by the specialisation of the fungi to site-specific abiotic conditions and by limitations to dispersal (Talbot *et al.* 2014).

Total fungal richness did not vary significantly along the succession from grassland to shrubland, and the differences amongst vegetation types were generally small (Fig. 1a; Table S2, Supporting Information), contrary to our expectations. Fungal composition, however, varied significantly along the succession within each site (Figs 3–5), as observed in other biomes, latitudes and altitudes where shrubs are encroaching (Maestre *et al.* 2009; Hollister *et al.* 2010; Yannarell, Menning and Beck 2014; Veach, Dodds and Jumpponen 2015; Collins *et al.* 2016; Creamer *et al.* 2016; Li *et al.* 2017; Schwob *et al.* 2017; Table S4, Supporting Information). We also found that both the advance of the succession and the identity of the shrub species along the succession played a key role in the development of compositionally unique fungal communities (Figs 2–5 and 7). This development was demonstrated by the large number of unique fungal OTUs in the soil of each successional stage and vegetation type (Fig. 2) and by the compositional differences amongst co-occurring shrub species within the young or the mature stage (Fig. 4a). The factors that determine the composition of shrub species across the subalpine belt of the Pyrenees (Grau *et al.* 2012; Ninot *et al.* 2013; Illa *et al.* 2017) are thus crucial for understanding the occurrence of compositionally unique communities of soil fungi at different spatial scales.

The strong correlation of the C:N ratio and N, P and K contents with the second NMDS axis in Fig. 3 (Table S3, Supporting Information), that encompassed the compositional turnover from the pure grassland to the mature shrubland at both sites, however, illustrates that the changes in soil properties may also help to account for the compositional variability of the fungal communities along the succession (Cline and Zak 2015). The Mantel tests provided further evidence of the covariation between soil properties and the compositional turnover of the fungi along the succession within each site (first row in Table 4). The composition of soil fungal communities possibly depended on the interplay of biotic factors (e.g. occurrence and identity of shrubs along the succession) and on plant-driven abiotic changes (e.g. soil properties) (Fig. 7).

Interplay between richness of the functional groups of soil fungi and biotic and abiotic factors

The richness of all functional groups, except for the ericoid and the lichenised fungi, followed the pattern of total fungal richness and was higher at South face (Fig. 1; Table S2, Supporting Information). The differences in abiotic conditions between North and South face may therefore primarily determine richness at the level of functional groups, as for total richness. Biotic factors may also play a role in some groups, e.g. ECM richness was most probably higher at South face because the plots at this site were much closer to the individuals of *P. uncinata* and *P. sylvestris* (both ECM trees) than at North face.

Not all functional groups differed significantly in richness along the succession, but some were particularly sensitive to the occurrence and identity of shrubs. This sensitivity could be partly due to positive or negative biotic interactions between the shrubs and fungi. Previous studies, for example, have reported that the richness of AM or ECM fungi was positively correlated with the expansion of shrubs that established AM or ECM symbioses, respectively (Williams *et al.* 2013; Schwob *et al.* 2017). We similarly found that the richness of ericoid and ECM fungi increased when the ECM and/or ericoid shrub *A. uva-ursi* was present (Fig. 1c and d). This finding supports Krpata *et al.* (2007), who suggested that *A. uva-ursi* could potentially be useful for providing mycorrhizal inocula for forest regeneration and afforestation in subalpine grasslands, due to the high richness of mycorrhizal fungi associated with this shrub. The richness of most functional groups, though, was not necessarily or exclusively due to the potential establishment of symbiotic relationships with the shrub, against our hypothesis. For example, the richness of AM fungi did not change significantly along the succession to the AM *J. communis* or *J. sabina* shrublands, whereas it tended to decrease where the ericoid mycorrhizal shrubs *C. vulgaris* or *V. myrtillus* occurred (Fig. 1b). This pattern suggests that the AM hosts and their fungal symbionts may have been outcompeted by the ericoid mycorrhizal shrubs but not by the AM shrubs. We also found that the richness of lichenised fungi was sometimes lower in the pure grassland than the shrublands (Fig. 1g), probably because the lichens were more easily outcompeted by grasses (Boch *et al.* 2016) than by the shrubs.

These patterns in the AM and lichenised fungi, however, could not be generalised. These uneven responses indicated that the richness of the functional groups did probably not only result from biotic interactions between plants and fungi (e.g. mycorrhization or competition) but also from changes in abiotic conditions along the succession or amongst vegetation types within each site (Albornoz *et al.* 2016). Changes in P content were generally correlated with the richness of root-associated fungal

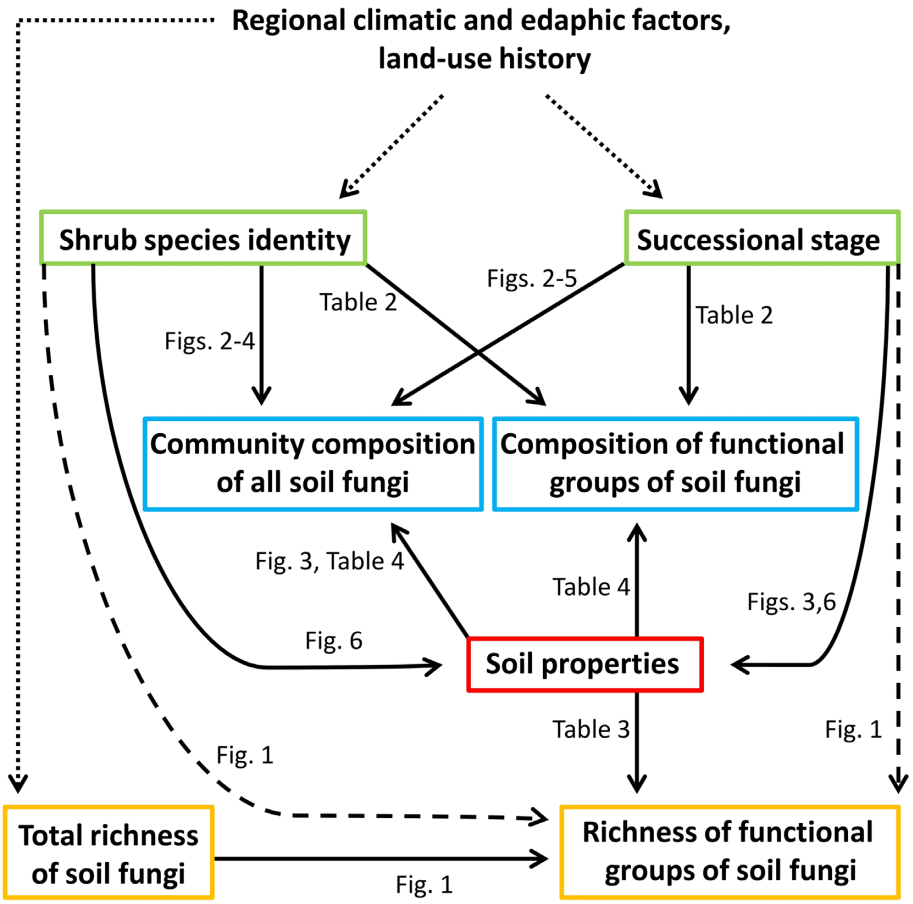

Figure 7. Conceptual diagram of the interplay between biotic factors (shrub species identity and stage of shrub expansion; green frames), soil properties (red), and the richness (orange) and composition (blue) of soil fungal communities and functional groups in our study. The figures or tables in the text that support each relationship are indicated for each arrow. Solid arrows indicate globally robust relationships; dashed arrows indicate relationships only observed in some cases; dotted arrows indicate relationships not formally tested in this study.

groups (e.g. with ECM, ericoid and root endophytic fungi at South face and with AM and ericoid fungi at North face; Table 3), most likely due to the role of mycorrhiza in the uptake of soil P (Read 2003; Albornoz *et al.* 2016; Raven *et al.* 2018). Changes in soil properties and nutrient status along the succession and amongst vegetation types, therefore, possibly influenced the richness of the functional groups of soil fungi (Fig. 7).

Interplay between the composition of functional groups of soil fungi and biotic and abiotic factors

The composition of all functional groups of soil fungi differed greatly between the two sites. This indicates that site-specific abiotic conditions and limitations to dispersal (Talbot *et al.* 2014) also be the most relevant drivers of the composition of soil fungi at functional group level. This could explain why the composition of soil fungi in the ericaceous shrub *A. uva-ursi* at South face differs much from that of the ericaceous shrubs at North face, against our initial hypothesis. Most groups also had significant within-group differences along the succession and were highly sensitive to the identity of the shrub species along the succession (Table 2; Figs 3, 4 and 7).

The compositional changes in mycorrhizal groups were particularly sensitive to the identity of the shrub species along the succession, because not all shrub species were associated with the compositional changes of these groups (Table 2). In fact, the composition of root-associated fungal groups is expected to be particularly responsive to the occurrence of their host plants (Zhang and Yao 2015). The link between the compositional changes in the mycorrhizal groups and the occurrence of hosts in our study must be interpreted with caution, because DNA was extracted from soil samples and not from root samples. We nevertheless found no evidence that the compositional changes of the AM, ericoid, or ECM fungi were necessarily due to the presence of their host shrubs, against our initial hypothesis. For example, the composition of AM fungi did not differ significantly between the pure grassland and the shrubland when the AM shrubs *J. communis* or *J. sabina* were present, but the composition was sensitive to the presence of other shrubs with which the AM fungi were not symbiotically associated. Similar incongruities were observed for the ECM and ericoid fungal communities. This suggests that, although host availability may have influenced the occurrence of a number of fungal OTUs within each mycorrhizal group, the abiotic conditions associated with each shrub species possibly played a key role in determining the composition of soil fungi at the functional-group level (Cline and Zak 2015), as with the richness patterns (Fig. 7).

The lack of clear compositional changes in ericoid fungi when ericoid mycorrhizal shrubs were present along the succession at North face (Table 2) may have important ecological implications. It suggests that the fungal OTUs that were common in the mature shrublands with ericoid shrubs at this site

were already present in the pure grassland, possibly as propagules, such as spores or explorative mycelia. A similar pattern was observed for the AM communities at South face, which did not change along the succession when AM shrubs were present, possibly as an ecological strategy of some fungal groups to drive vegetation dynamics along secondary succession (García de León *et al.* 2016) by facilitating the expansion and dominance of ericoid shrubs at North face and of AM shrubs at South face.

The composition of non-mycorrhizal groups was sensitive to the occurrence of most shrubs (Table 2). This indicated that the changes in abiotic conditions driven by the presence and identity of the shrubs was associated with the within-group compositional variability of the saprotrophic, lichenised and plant pathogenic fungi.

The Mantel tests indicated that plant-driven changes in soil properties were strongly associated with the compositional turnover of most of the fungal functional groups (Table 4). Against our hypothesis, this association was not more evident in mycorrhizal or saprotrophic fungi; lichenised or plant pathogenic fungi often showed significant correlation with changes in soil properties. Other abiotic factors that co-varied with shrub encroachment such as water availability, temperature, wind speed, or pattern of snow accumulation (Gómez-Aparicio *et al.* 2005; Van Auken 2009; Eldridge *et al.* 2011; Grau *et al.* 2013) may have also affected the structure of the fungal communities, but these factors were not analysed in our study. The Mantel tests included all soil variables, but the increase in the C:N ratio in most of the shrublands at both sites, and the decrease in N, P and K contents at South face, were the most notable changes along the succession (Fig. 6) and could be the main drivers of the changes in soil properties and compositional turnover of most of the functional groups. This could explain why *A. uva-ursi* or *J. communis* had the strongest correlations in the Mantel tests, because these two shrubs caused a particularly significant increase in the C:N ratio and decreases in N, P and K contents. We did not observe though that those functional groups of soil fungi that actively contribute to nutrient cycling, like mycorrhizal or saprotrophic fungi, were more strongly correlated with soil variables than the other groups.

Our findings therefore suggest that the within-group compositional variability in most functional groups along the succession (Table 2) is probably due to niche-based rather than stochastic processes, which are more common in less stable environments, such as early stages of primary succession (Jumpponen 2003; Blaalid *et al.* 2012). This within-group compositional variability indicated that the fungal OTUs belonging to the same functional group may have different ecological functions due to differences in strategies of nutrient acquisition or in hyphal exploration types (Bruns 1995; Dickie, Xu and Koide 2002; Grau *et al.* 2017). Niche differentiation and functional complementarity within each group relative to changes in biotic and abiotic conditions may thus favour the co-existence of a large number of fungal species along the succession (Buée *et al.* 2007; Cline and Zak 2015). This niche differentiation may occur in all stages of shrub encroachment, including pure grassland, where fungal communities may have adapted to many centuries of anthropic management.

Finally, the species of shrubs at the two sites were the most dominant plants along the succession, but the occasional presence of smaller plants in the understories of the shrubs may also favour within-group compositional variability of soil fungi (Wubet *et al.* 2012) by microsite changes in biotic and/or abiotic conditions, even though the cover of these co-occurring plants was very low.

Concluding remarks and implications for ecosystem functioning

The expansion of shrubs into the subalpine belts on the north- and south-facing slopes of the Pyrenees occurred since the mid-20th century, mostly due to changes in land use (García-Ruiz *et al.* 1996; MacDonald *et al.* 2000; Roura-Pascual *et al.* 2005; Alados *et al.* 2011; Komac, Alados and Camarero 2011; Barrio *et al.* 2013). Shrub encroachment is expected to persist in the future in areas that are less accessible to livestock grazing (Carreras *et al.* 2016; García-Pausas *et al.* 2017), unless major disturbances such as fires or intensification of land use occur. The ongoing climatic warming in the Pyrenees (Sanz-Elorza *et al.* 2003; Martín-Vide, Prohom and Busto 2016) is also expected to increase the productivity of woody vegetation (Grau *et al.* 2013; Peñuelas *et al.* 2016) and promote the expansion of shrubs in the subalpine belt.

Shrub expansion will modify soil properties and the nutrient status due to an increase in the C:N ratio and/or decrease in N, P and K contents, possibly due to the decrease in nutrient inputs from excrements of domestic herbivores and to the transfer of nutrients from the soil to the biomass of the shrubs (Horton *et al.* 2009), which may favour the further expansion of shrubs into grasslands. The identity of the shrub species along the succession and the changes in soil properties will likely affect the richness and composition of the fungal communities. These changes will also occur at the functional group level, possibly due to within-group changes in the ecological roles and functions of soil fungi. The occurrence of small patches of shrubs in grasslands may have already affected the structure of soil fungal communities, because the compositional differences were larger between the pure grassland and the young shrubland than between the young and mature shrublands (Fig. 2a and b; Figs 4 and 5). Any future changes in the composition of shrub species that encroach into subalpine grasslands are expected to greatly influence soil properties and the structure and uniqueness of soil fungal communities (Tables 2–4; Figs 1–7). As with all functional ecological studies of fungal communities, more information is needed for a large portion of fungi to improve the assignments of OTUs to functional groups so that the implications of ecosystem functionality are better understood.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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