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Soil fungal communities of grasslands are environmentally structured at a regional scale in the Alps

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Abstract

Studying patterns of species distributions along elevation gradients is frequently used to identify the primary factors that determine the distribution, diversity and assembly of species. However, despite their crucial role in ecosystem functioning, our understanding of the distribution of below-ground fungi is still limited, calling for more comprehensive studies of fungal biogeography along environmental gradients at various scales (from regional to global). Here, we investigated the richness of taxa of soil fungi and their phylogenetic diversity across a wide range of grassland types along a 2800 m elevation gradient at a large number of sites (213), stratified across a region of the Western Swiss Alps (700 km^2). We used 454 pyrosequencing to obtain fungal sequences that were clustered into operational taxonomic units (OTUs). The OTU diversity–area relationship revealed uneven distribution of fungal taxa across the study area (i.e. not all taxa are everywhere) and fine-scale spatial clustering. Fungal richness and phylogenetic diversity were found to be higher in lower temperatures and higher moisture conditions. Climatic and soil characteristics as well as plant community composition were related to OTU alpha, beta and phylogenetic diversity, with distinct fungal lineages suggesting distinct ecological tolerances. Soil fungi, thus, show lineage-specific biogeographic patterns, even at a regional scale, and follow environmental determinism, mediated by interactions with plants.

Keywords: alpine grassland, elevation gradient, environmental gradients, phylogenetic diversity, plant–fungi interactions, soil fungal community, 454 pyrosequencing

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Introduction

The spatial distribution and diversity of above-ground plant and animal macro-organisms have been well described and are known to be regulated by multiple biotic and abiotic factors such as climatic clines (Lomolino *et al.* 2005; Franklin 2010). In contrast, the structure of below-ground communities along climatic gradients remains far less understood (Martiny *et al.* 2006; Hanson *et al.* 2012; Sato *et al.* 2012), even though it is of great interest to understand the role of micro-organisms

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in ecosystem functioning (von der Heijden *et al.* 1998; Wardle *et al.* 2004; Maherali & Klironomos 2007; Pellissier *et al.* 2013a). Soil micro-organisms have already been identified as essential players in carbon, nitrogen and phosphorus cycling, and consequently in soil fertility (Whitman *et al.* 1998; Yang *et al.* 2014), as well as for the degradation and recycling of organic matter (Johnson *et al.* 2013). In particular, some soil fungi interact directly with plants (Pellissier *et al.* 2013a), by associating with them as pathogens and mutualists (Wardle *et al.* 2004; Kivlin *et al.* 2011; Sato *et al.* 2012; Põlme *et al.* 2013). Identifying the geographic and environmental factors that regulate the distribution and assemblage of fungi (i.e. fungal biogeography) is of paramount importance to have a general understanding of processes in ecosystems (Maherali & Klironomos 2007; Hanson *et al.* 2012; Hazard *et al.* 2013).

Whether free-living microbial taxa in soils exhibit biogeographic patterns (*sensu* lato, i.e. from regional to global scales, along latitude and altitude) similar to above-ground macro-organisms (i.e. limited by environmental, dispersal and biotic constraints), or whether alternatively most of them are 'found everywhere but the environment selects' (i.e. dispersed well, Baas-Becking 1934), had been identified as a key question in microbial biogeography (e.g. Martiny & Field 2005; Martiny *et al.* 2006; Lozupone & Knight 2007; King *et al.* 2010; Fierer *et al.* 2011; Sato *et al.* 2012; Hazard *et al.* 2013). While existing evidence suggests that the spatial distribution of belowground microbial communities is largely nonrandom (Horner-Devine *et al.* 2007; Peay *et al.* 2007; Hanson *et al.* 2012; Hazard *et al.* 2013), follows trends along latitudinal gradients (Tedersoo *et al.* 2012), and, in the case of bacteria, do not follow patterns of macro-organisms (Fierer *et al.* 2011), there is still limited knowledge about the way soil fungi distribute along altitudinal gradients and the relative importance of specific environmental factors that control them (Hanson *et al.* 2012). This is largely because so far comprehensive data sets are missing that are well-sampled along wide elevation gradients, comparable to those existing along latitudinal gradients (Tedersoo *et al.* 2012), or for above-ground macro-organisms (Lomolino *et al.* 2005). This is important as many conservation decisions are currently driven by patterns of above-ground organisms and could therefore fail to preserve the biodiversity of other groups of organisms.

Evaluating which factors drive fungal diversity at the landscape scale and along altitudinal gradients requires an appropriate design of the sampling strategy, along with detailed abiotic and biotic data measured in each plot. Meta-analyses of existing data sets (Tedersoo *et al.* 2012) can yield useful answers but often mix heterogeneous data with different sampling quality and density and therefore do not replace single well-designed surveys along an entire gradient. As for above-ground macro-organism communities, abiotic factors (e.g. temperature, moisture, soil pH and soil nitrogen) are expected to play an important role in shaping fungal diversity (Bahram *et al.* 2012; Hanson *et al.* 2012; McGuire *et al.* 2012). In addition, soil fungal diversity is expected to relate to plant diversity and functional diversity (Hamilton & Frank 2001; de Vries *et al.* 2012; Garnica *et al.* 2013; Peay *et al.* 2013). Finally, considering the phylogenetic context is expected to yield more detailed information on the role of abiotic and biotic filters on fungal community assembly when accounting for lineages relatedness (Cavender-Bares *et al.* 2009).

Here, we investigated patterns of soil fungal community diversity in different grasslands along a 2100 m elevation gradient using a comprehensive data set of 213 sites distributed across the Western Swiss Alps (Fig. 1a). Mountains are particularly suitable for investigating drivers of fungal diversity, because large abiotic (e.g. temperature, soil acidity) and biotic (e.g. plant diversity) gradients are represented within short distances. Focusing only on grasslands allows us to compare fungal patterns in a same habitat type along a wide gradient of environmental variation and compare these with already available plant community data. Although fungi and plants can interact in both directions (Pellissier *et al.* 2013a), here we take the fungal biogeography perspective and thus only look at plants as factors potentially driving fungal distribution, diversity and composition. We used 454 pyrosequencing (as in McGuire *et al.* 2012) to obtain >1 million ITS1 sequences that were clustered into more than a thousand OTUs, using a recently developed software (Pagni *et al.* 2013), numerically benchmarked on this particular data set. In each sampled plot, we further measured abiotic factors and inventoried plant communities, including measures of plant functional traits and a reconstructed plant phylogeny, to assess the respective influence of potential drivers of fungal diversity. Using this framework, we ask three main questions:

1 How is soil fungal diversity spatially structured at the regional scale: Does it increase continuously with increasing surface area or does it reach saturation? That is, are all fungal taxa found everywhere or do different sites show distinct fungal communities?
2 What are the abiotic factors that control alpha and beta diversity of fungal communities? In this context, are soil factors more important than climatic factors?
3 What are the biotic plant factors that control alpha and beta diversity of fungal communities? Are fungal communities related to plant communities?

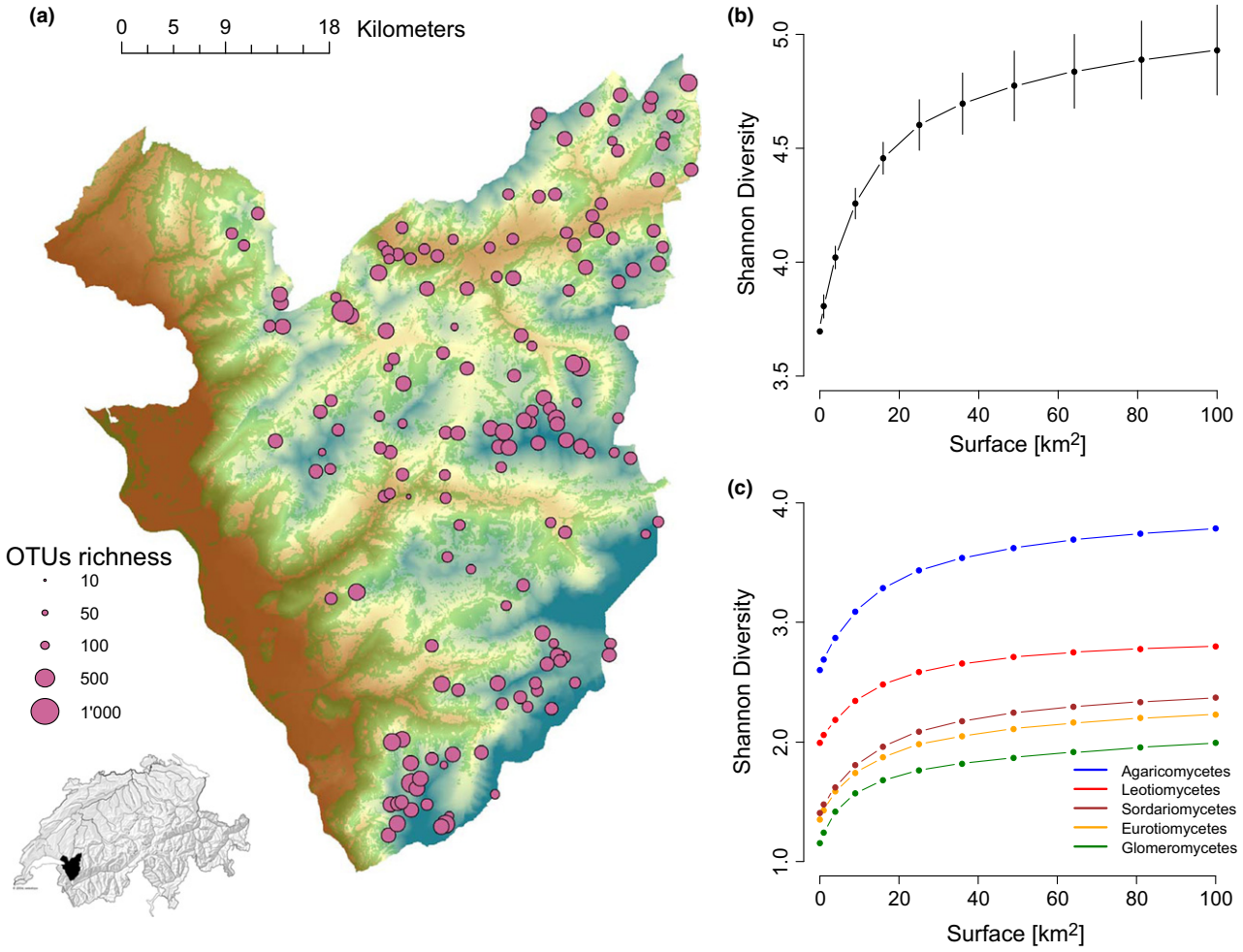

Fig. 1 (a) Study area in the Western Swiss Alps (700 km^2) spanning a 2100 m elevation gradient. The inset map shows Switzerland with the study area coloured in black. The dot size corresponds to OTU richness. The green surface represents forested areas. (b) Overall relationship between area (km^2) and the Shannon diversity index of fungal OTUs for the whole data set with bars representing the 95% confidence intervals. (c) Relationship between area (km^2) and the Shannon diversity index of fungal OTUs of the five most abundant fungal classes: blue: Agaromycetes; red: Leotiomycetes; brown: Sordariomycetes; orange: Eurotiomycetes; green: Glomeromycetes.

Materials and methods

Data collection

This study was conducted in the Western Swiss Alps (Fig. 1a). The elevation gradient of the region ranges from 400 to 3210 m. We collected soil samples, inventoried all plant species and measured different abiotic parameters in 213 plots of 4 m^2 across a variety of non-forest sites between 400 and 2535 m. The sites above this limit lacked a humic layer. We excluded forest areas because trees may determine soil fungal composition in a different way than herbs and, unlike grasslands, do not occur along the full elevation gradient. Investigating grasslands along the entire elevation gradient, thus, allowed a better comparison of the fungal composition from lowest to highest sites. The location of these plots was chosen following a random-stratified sampling design (Hirzel & Guisan 2002), that is selecting an equal number of sampling points within all possible combinations of elevation, slope and aspect strata. Five soil cores of 10 cm^3 were sampled per vegetation plot (from the four corners and one from the centre of each plot) for DNA extraction and soil chemical analysis. The samples were taken from the top 10 cm of the soil after removing the plant litter surface. This corresponds primarily to the organo-mineral horizon (Gobat *et al.* 2004).

Chemical analyses were conducted to characterize the soil samples in each plot (Table 1). To equalize eventual

Table 1 Description of abiotic and biotic environmental variables associated with spatial patterns of fungal communities, with the references to previous studies using those data. Also mentioned is whether the variable was used in alpha or beta diversity analyses. Elevation is not included here, because it has no direct effect on organisms, only an indirect one through determining temperature (degree-days here) and partially other environmental gradients (e.g. moisture index through precipitation)

Variables	α	β	Description	References
Climate				
Degree-days	x	x	Degree-days are the accumulated product temperature above a threshold defined at 0° during the year	Dubuis *et al.* (2011)
Moisture index	x	x	Moisture index is the amount of precipitation minus the potential evapotranspiration	Dubuis *et al.* (2011)
Soil				
pH	x	x	pH is a measure of the acidity or basicity of a solution and was measured with a pH meter after diluting soil in water in a 1:2.5 proportion	Dubuis *et al.* (2013a)
Nitrogen	x	x	The total nitrogen content was analysed using element analysis after combustion with a CHN analyser	Dubuis *et al.* (2013a)
Phosphorous	x	x	The total phosphorus content was determined by colorimetric analysis after mineralization at 550 °C	Dubuis *et al.* (2013a)
Granulometry	x	x	Proportion of soils of the following particle size classes: clay (CLA, <2 μm), fine silt (FSI, 2–20 μm), coarse silt (CSI 20–50 μm), fine sand (FSI, 50–200 μm), coarse sand (CSI 200–2000 μm)	Dubuis *et al.* (2013a)
Plant				
Richness	x		The sum of different vascular plant species in a given 2 × 2 m plot	Dubuis *et al.* (2011)
Taxonomic turnover		x	Turnover of plant composition among plots measured with the Jaccard index	Pellissier *et al.* (2013a)
Phylogenetic diversity	x	x	Plant phylogenetic diversity was calculated within and between communities using the mean pairwise phylogenetic distance (MPD) and mean nearest phylogenetic taxon distance (MNTD)	Pellissier *et al.* (2013b)
Functional weighted mean	x		Community-weighted mean of leaf functional traits (for specific leaf area [SLA], leaf dry matter content [LDMC], leaf nitrogen [LN], leaf carbon [LC], leaf carbon nitrogen ratio [C/N])	Dubuis *et al.* (2013b)
Functional diversity		x	Plant functional diversity was calculated between communities using the modified version of the Rao quadratic entropy presented in De Bello *et al.* (2010), for the same functional traits as above	Pottier *et al.* (2013)

intraplot variation, soil coming from the five cores per plot was mixed in equal proportions. The methods used to obtain the pH, soil texture, total nitrogen (N), total phosphorous (P) and organic carbon (C) are described in Dubuis *et al.* (2013a). Nutrient concentrations are expressed in mg/g of soil sample.

In each plot, exhaustive inventories of all vascular plant species were conducted (Dubuis *et al.* 2013a). Four functional traits, leaf dry matter content (LDMC), leaf nitrogen content (LN), specific leaf area (SLA) and leaf organic carbon (LC) were collected for the 231 most frequent plant species in the study area (Dubuis *et al.* 2013b; Pottier *et al.* 2013). Phylogenetic relationships between these 231 plant species are described in Ndiribe *et al.* (2013), based on plant material collected for all species within the vegetation plots. The time-calibrated ultrametric tree obtained for these plant species, based on DNA sequences and published fossils, is illustrated in Fig. S1 (Supporting information) (Drummond & Rambaut 2007; Ndiribe *et al.* 2013; see Pellissier *et al.* 2013b for further details).

We calculated climatic predictors over the area by averaging data on the monthly means of temperature (°C) and annual sums of precipitation (mm) recorded for the period 1981–2010 by the Swiss network of meteorological stations and interpolated using a digital elevation model at 25-m resolution. We computed degree-days as the sum of days multiplied by the temperature above 0 °C and moisture index as the difference between precipitation and potential evapotranspiration for the growing season of June, July and August (Zimmermann & Kienast 1999). Climatic interpolations showed good correlations to observed meteorological data (temperature $r^2 = 0.98$, moisture index: $r^2 = 0.86$).

Finally, we conducted pairwise regression analyses using linear models between each pair of abiotic and biotic environmental predictors that were associated

with fungi communities to calculate coefficients of determination (R^2) and assess their degree of correlation.

Sample preparation, DNA extraction, PCR and pyrosequencing

To determine the soil fungal community composition in all of the 213 locations, the five soil core samples coming from each plot were processed independently and only the obtained sequenced data were pooled for further analysis (see 'Bioinformatic analysis'). Thus, DNA has been extracted from 250 mg representative of each soil core. Each one of the 1065 soil samples (5 cores × 213 plots) was homogenized and sieved with a 2-mm sieve. From that, 3 g of soil was air-dried at 30 °C for 24 h. Drying the soil before DNA extraction results both in a better lysis (Frostegard *et al.* 1999) and in a more efficient recovery of DNA, and fungal profiles in soils show generally low alteration by air-drying (McDonald *et al.* 2008). DNA extraction was performed with the PowerSoil®-htp 96 Well Soil DNA Isolation Kit (MoBio Laboratory, Carlsbad, CA, USA) from 250 mg of dry soil. In spite of repeated attempt to extract DNA from these soils, we could not obtain genomic DNA for 23 of the 1065 soil samples. The fungal internal transcribed spacers (ITS1) were successfully amplified from 832 of the 1042 samples using the universal fungal primers ITS1F (Gardes & Bruns 1993 and ITS2 (White *et al.* 1990) associated with primers designed specifically by Roche for the Genome Sequencer FLX Titanium pyrosequencing. These amplicons were pooled in six libraries of 138 or 139 samples and sequenced in three runs in parallel on the Genome Sequencer FLX Titanium 454 System (454 Life Sciences/Roche Applied Biosystems, Nutley, NJ, USA). The run effect on the number and length of reads generated per sample has been tested by ANOVA. See Supporting Information for further details on PCR amplification conditions and library preparation for pyrosequencing. An additional half run was necessary to be carried out to reach a similar number of reads of a same length for all the six libraries. As the number of samples sequenced per plot was not systematically five and the numbers of reads varied accordingly between plots and with OTU numbers (Fig. S2–S4, Table S1, Supporting information), we tested whether these variations might introduce a bias in our analysis. We first examined the distribution of the plots with 1–5 samples on the study area, and we did not found any geographic bias (Fig. S3, Supporting information). Second, we tested whether the number of soil samples and reads per plot affected the OTU diversity per plot, and whether using the Shannon diversity index H (Shannon 1948) or a resampling rarefaction analysis (Voriskova & Baldrian 2013) could reduce the bias (see below), as recommended by Haegeman *et al.* (2013). The variation in fungal community diversity per plot, as described by the Shannon diversity index, was found to be similar between the plots represented by two to five soil samples, but was statistically lower for the plots represented by only one soil sample (Fig. S4, Supporting information). For this reason, the nine plots represented by only one sample were removed from the data set. Thus, further analyses were conducted on 204 plots.

Bioinformatic analyses

The processing of the 1 649 376 raw reads obtained, and the computation that leads to the definition of 1199 ITS1 full length OTUs (Table S2, Supporting information) has been previously described in detail (Pagni *et al.* 2013). More details are given in the Supporting Information. The full length of the variable sequence of the ITS1 region was extracted from our data set following a procedure similar to Santamaria *et al.* (2012). The clustering tool used, DBC454, also provided the assignment of the 1199 OTUs to known sequences (see Table S2, Supporting Information on Bioinformatic analyses). The corresponding taxonomy was extracted from the NCBI taxonomy database. The OTUs that were identified, at least at the family level, were placed on a tree based on the phylogenetic relationships known between the fungal families (James *et al.* 2006). Fungal sequence data are available at the NCBI under BioProject SUB278788 and BioSample SUB281720.

Spatial structure of fungal distribution

To detect spatial structuring in fungal communities, we tested whether an increase in the local area led to an increased Shannon diversity index and OTU richness (both metrics of alpha or gamma diversity of OTUs) across the study area. Shannon was estimated using the 'diversity' function of the 'vegan' package in R (R Development Core Team 2013, www.r-project.org.), and OTU richness was calculated as the sum of all distinct OTUs identified with the DBC454 tool. We overlaid grids of increasing mesh size (from 1 to 100 km^2) on the sampled area and estimated the average diversity of OTUs for each of these grids with Shannon. To avoid local effects, we shifted the origin of the grid by 200-m steps along the longitude and latitude coordinates. Hence, for a grid with a 1 km^2 mesh size, we used 25 different grid origins, while for a grid with 100 km^2 mesh size, we used 2500 different grid origins. Saturation beyond a given mesh size would suggest that the sampling allowed us to properly identify the scale at which most OTUs in the area would

be sampled. We also measured relative values of the Shannon index, corrected by the initial and maximum Shannon value per group, and measured species-area curves for noncontiguous plots (i.e. no geographic constraints; see Dengler & Oldeland 2010). Finally, we compared the relative species accumulation curves based on Shannon to a curve drawn similarly for plant species across the same plots.

Fungal alpha diversity within plots

Two facets of plot-level fungal diversity—taxonomic and phylogenetic—were investigated. OTU richness and Shannon per plot were calculated as described above. OTU phylogenetic diversity was calculated, based only on OTUs for which a correspondence in GenBank could be identified, to build the tree, using the standardized effect size (SES) of the mean pairwise phylogenetic distance (MPD) (equivalent to −1*NRI, Webb & Ackerly 2002). We built null models by reshuffling the tip label on the fungal phylogeny in a way that preserved important properties over the study area (e.g. species prevalence, species richness, Pellissier *et al.* 2013b). The correlation between these alpha diversity indices and geographic distance was first assessed with a Mantel test based on Pearson's product-moment correlation with 9999 permutations. To dissect factors associated with OTU richness and phylogenetic diversity, we then computed a number of plot-level abiotic and biotic environmental descriptors (Table 1). The two facets of plot-level fungal diversity were related to the environmental descriptors of plot conditions using a multivariate linear model with a Gaussian distribution. The best-fit multivariate model was chosen based on the minimum AIC score. From the most parsimonious multivariate model, we calculated the total explained variance as well as that of the abiotic and biotic components. To investigate how diversity extremes vary along environmental gradients, we fitted quantile regressions at the 20th and 80th percentile using the functions implemented in the quantreg package in R (R Development Core Team 2013).

Fungal beta diversity between plots

Two facets of between-plot fungal diversity were investigated: Change in fungal composition based on presence–absence of OTUs, and the turnover in the prevalence of phylogenetic lineages. Fungal compositional beta diversity was measured using the Jaccard similarity index implemented in the 'vegan' library in R (R Development Core Team 2013), and fungal phylogenetic beta diversity using the comdistnt (MNTD) and comdist (MPD) functions in the 'picante' package (Kembel *et al.* 2010). The correlation between these beta diversity indices and geographic distance was first assessed with a Mantel test based on Pearson's product-moment correlation with 9999 permutations. We then computed standardized effect size (SES) of MNTD and MPD of fungal communities to investigate whether environmental factors played a role in community assembly by filtering species based on their evolutionary history. To dissect factors associated with beta diversity of fungal communities, we computed a number of between-plot abiotic and biotic environmental descriptors (presented in Table 1). We evaluated the relationship between OTU beta diversities and environmental variables using Mantel tests with 9999 permutations. Because changes in plant functional and phylogenetic structure may explain the same variance as the baseline plant compositional turnover, we assessed the independence of these factors using partial Mantel tests.

The relationship between environmental predictors and OTU compositional differences between sites was further assessed by canonical correspondence analysis (CCA), using the variables that explained most of the variation in fungal communities. The results were visualized in a CCA plot. We labelled the communities based on phytosociological classifications (Delarze *et al.* 1998) into grassland types (Table S3, Supporting information). We assessed the significance of the correspondence using a permutation analysis as implemented in the 'ade4' package in R (R Development Core Team 2013).

Results

Pairwise correlations of abiotic and biotic predictors

Abiotic environmental and biotic vegetation predictors associated with fungal communities were reasonably uncorrelated, as the values of the determination coefficients were all below 0.6 (Table S5, Supporting information), and 0.68 for degree-days and moisture, whereas values <0.7 are considered acceptable for a joint use in correlative analyses (Dormann *et al.* 2012; see also Franklin 2010).

Overall fungal diversity

We assembled an extensive spatial data set of soil fungal communities for exhaustively sampled vegetation plots along a large elevation gradient (see Dubuis *et al.* 2013a). Across the 204 plots, 1199 OTUs were identified (Table S2, Supporting information), of which 998 matched fungal sequences in the investigated databases.

The remaining 201 nonfungal OTUs presented no similarity in GenBank with sequences of any other kingdoms, thus likely representing unknown fungal taxa. Therefore, we still considered these 201 OTUs in further analyses, but as a separate group to determine whether these would respond in the same way as the known fungal OTUs.

From the 998 OTUs identified as existing fungal sequences, 708 OTUs could be assigned to species or fungal family level (59% of OTUs). These were representative of five fungal phyla, 14 fungal classes and 56 genera (Table S2, Supporting information). The class that presents the highest richness in OTUs was that of Agaricomycetes (293 OTUs). It was followed by three Ascomycota classes: Leotiomycetes (154), Sordariomycetes (67), Eurotiomycetes (34) and the Glomeromycetes being in the fifth position (30) (Figs 1 and 2; Tables S2 and S4, Supporting information). The abundance of the OTUs in the data set varied between 0.0008% and 6%. Eleven of them corresponded to more than 1% of the sequences: three OTUs assigned to *Mortierella*, two to *Cryptococcus*, five to unknown fungi and one to which no taxonomy could be assigned.

Patterns of fungal diversity within plots (alpha)

No correlation was observed between alpha diversity and geographic distance, as measured by a Mantel test applied to species richness ($r = 0.021$, P-value = 0.163) and the Shannon index ($r = 0.021$, P-value = 0.164). Both Shannon diversity index and richness of OTUs were not evenly distributed across the study area, but showed spatial clustering at a fine scale (Fig. 1b; Figs S6–S8, Supporting information). They increased steadily up to grids of 16 km^2 mesh size, then levelled off until accumulating the majority (ca. 80%) of OTUs around 80–100 km^2 (Fig. S7, Supporting information), a phenomenon also observed in each of the five major fungal classes (Fig. 1c; Fig. S6, Supporting information). The relative values of the Shannon provided similar patterns to the untransformed ones (Fig. S6, Supporting information), as the same analysis without spatial constraints (Fig. S9, Supporting information). The Shannon diversity index also proved insensitive to the number of reads per plot (Fig. S10, Supporting information) and correlated with OTU richness derived from the rarefaction analysis to the same (minimum) number of reads per plot (Fig. S11, Supporting information). The shape of the rarefaction curve was also comparable to the one obtained for plant diversity (Fig. S6, Supporting information graph D).

In the most parsimonious multivariate model, OTU richness was higher in moister conditions with higher plant species richness, higher SLA community-weighted mean and lower phosphorus content in soils (Table 2A; Fig. 3a). Quantile regression analyses also indicated that extremes (20th and 80th percentile) of OTU richness and phylogenetic diversity varied along environmental gradients (Table S6, Supporting information). Fungal communities were more phylogenetically diverse in

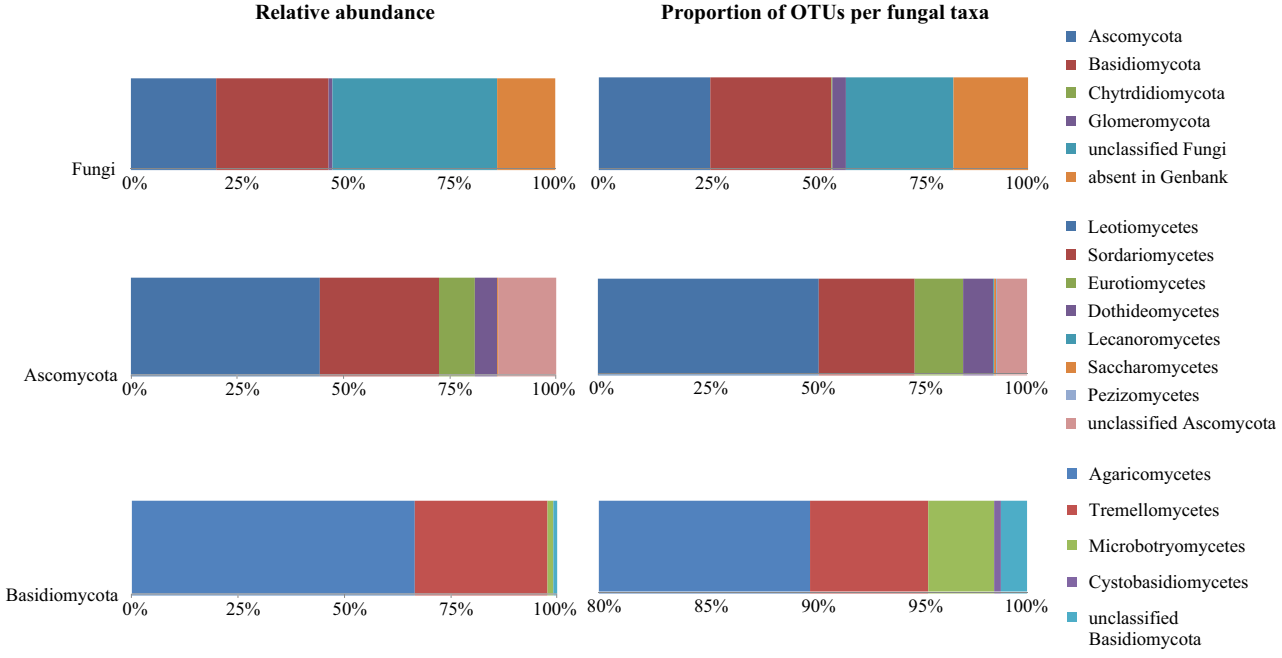

Fig. 2 The inferred taxonomic composition of fungal communities in the Western Swiss Alps and their relative abundance.

Table 2 Results of best-fit multiple regressions for (A) fungal OTU richness based on the 1199 OTUs, (B) phylogenetic diversity of communities based on the 708 OTUs with assignment using the standardized effect size of the mean phylogenetic distance (SES MPD equivalent to—minus the net relatedness index)

Parameter	Estimate	*t*	
A. OTUs richness			
Moisture index	0.08	2.08*	
Soil phosphate	−42.15	−2.66*	$R^2 = 0.098$
SLA CWM	434.32	2.25*	
Plant richness	42.15	3.03*	$R^2 = 0.038$
	AIC = 1924		$R^2 = 0.13$
B. Phylogenetic diversity (SES MPD)			
Moisture index	0.001	2.00*	
Soil pH	−0.351	−4.37*	
Soil nitrogen	−0.562	−2.14*	$R^2 = 0.20$
Plant richness, 1	2.532	2.19*	
Plant richness, 2	−3.322	−2.83*	
N CWM	−0.622	−2.1*	$R^2 = 0.16$
	AIC = 510.8		$R^2 = 0.30$

The estimate of the slope of the relationships and the associated *t* statistic are presented. Best-fitting models were chosen based on minimum AIC score. R^2 of abiotic, biotic and of the total model are provided. CWM: community-weighted mean (for traits) across the plant community.
*Significant slopes (*P*-value<0.05).

soils with higher pH and higher moisture index (Fig. 3b), but also in areas with lower temperature and lower nitrogen content, all factors found at higher elevation (Table 2B; Fig. S12, Supporting information), and where plants generally contained less leaf nitrogen (Fig. 3b; Table 2; Dubuis *et al.* 2013b). Which of these conditions is most important to explain fungal richness is difficult to establish, as these factors can be confounded along the elevation gradient. OTU phylogenetic diversity also peaked at intermediate values of plant species richness (Table 2B; Fig. 3b). Finally, all fungal communities were phylogenetically clustered, with each community representing a phylogenetically reduced subset of the regional species pool (negative SES MPD values, Fig. 3b, Table S6, Supporting information).

Investigating the richness of each class separately, we found that some taxonomic groups were positively correlated with moisture index (e.g. Eurotiomycetes; Glomeromycetes, although not significantly; Leotiomycetes), while Sordariomycetes showed a negative relationship (Table S7, Supporting information). Agaricomycetes and Eurotiomycetes were correlated with plant species richness, but surprisingly not the Glomeromycota (Table S7, Supporting information). We found a significant negative correlation of richness with soil phosphate in three classes (and nonsignificant but still negative in two others; Table S7, Supporting information), but a positive correlation with soil organic carbon only in Tremellomycetes (and nonsignificant in Glomeromycetes). Soil nitrogen was only nonsignificantly positively related with richness in Eurotiomycetes. Specific leaf area of plants had a significant positive relationship with four of the classes (and a nonsignificant effect on two more classes). As they displayed distinct responses to the abiotic and biotic environment, the shift in OTU richness of the different taxonomic classes along the abiotic and biotic gradients resulted in the overall turnover of phylogenetic diversity in fungal communities along these gradients. Interestingly, the unassigned OTUs followed the general trend of total richness (Fig. S6, Supporting information) and were more diverse in moister conditions in soil with low phosphorus.

Fungal beta diversity between plots

No correlation was observed between beta diversity and geographic distance as measured with the Jaccard similarity index (i.e. beta diversity; Mantel statistic *r*: 0.019; *P*-value: 0.220), nor between phylogenetic beta diversity and geographic distance, as measured by MNTD (*r*: 0.005, *P*-value 0.577) and MPD (*r*: 0.040, *P*-value: 0.120). The total variance explained by abiotic and biotic environmental variables was higher for OTU phylogenetic diversity than for richness (Table 3). We found that both abiotic and biotic variables explained a significant proportion of the variation in OTUs among plots (Fig. 4). Among the abiotic variables, pH, degree-days, moisture index, soil, nitrogen and phosphorus showed the strongest correlation with OTU compositional turnover in Mantel tests (Table 3A). OTU compositional turnover was also correlated with plant compositional and phylogenetic turnover and changes in plant SLA, LDMC and N (Table 3A). The effect of plant trait and plant phylogenetic turnover was still significant after baseline plant compositional turnover was accounted for, suggesting an effect of plant traits and plant phylogenetic turnover on fungal diversity that was independent from plant compositional shifts.

For fungal phylogenetic beta diversity, we found that degree-days, moisture index, soil pH, nitrogen, phosphorus, plant taxonomic and phylogenetic turnover and changes in plant traits were similarly well correlated with changes in the phylogenetic composition of fungal communities (Table 3B). Change in nitrogen, leaf content and plant phylogenetic diversity were still significant once plant taxonomic turnover was accounted for. This suggests that this information, in addition to plant taxonomic turnover, explains distinct variation among fungal communities.

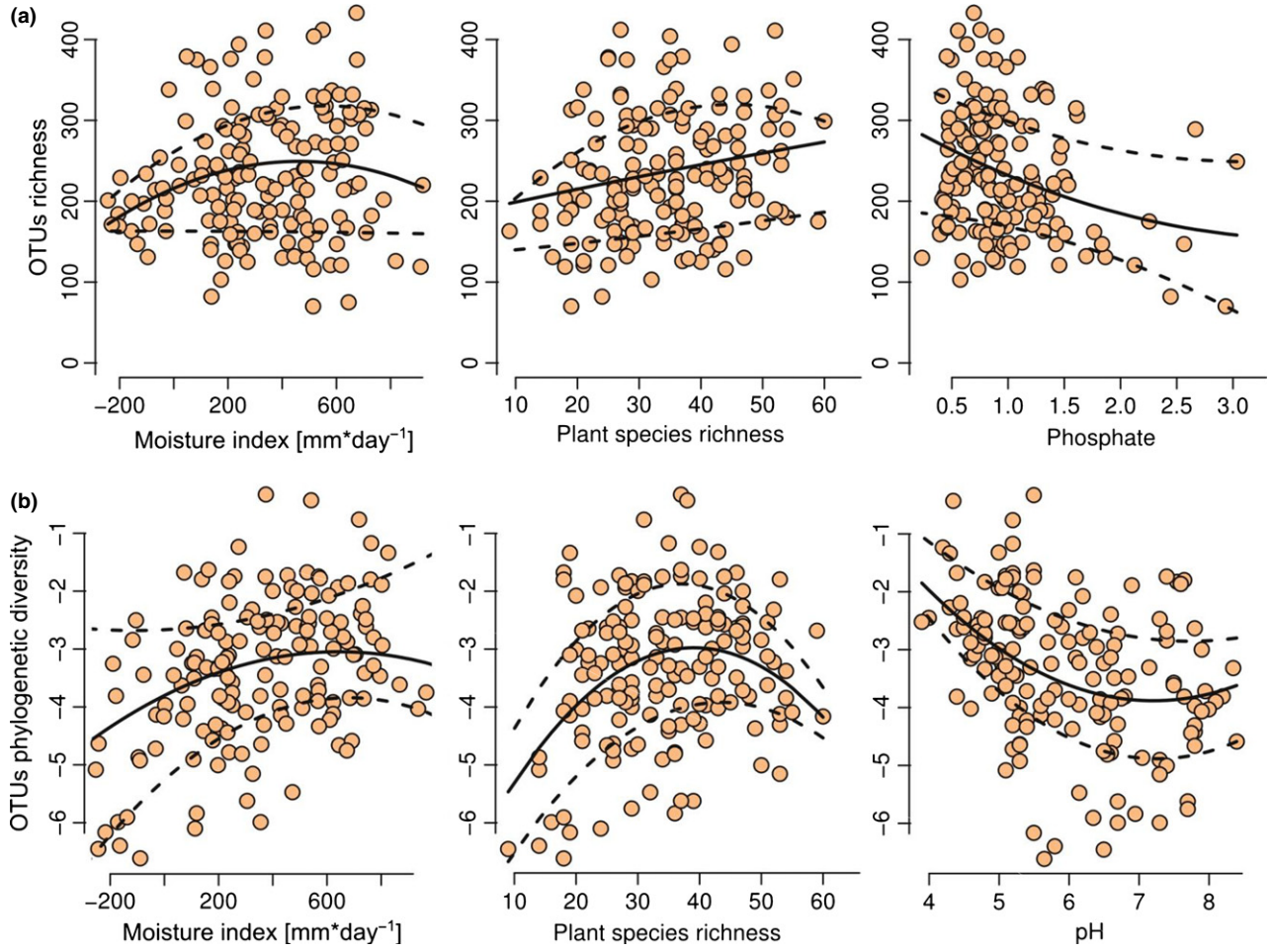

Fig. 3 Relationship between: (a) fungal OTU richness and moisture index (unitless), plant species richness and phosphorus (mg/g); (b) phylogenetic diversity of OTU measured as the standardized effect size of the mean phylogenetic distance (SES MPD) and moisture index, pH and plant species richness. The black central curve corresponds to a polynomial regression based on the mean, while the dashed lines correspond to the quantile regression (20th and 80th percentile). All relationships are significant, and the statistics are presented in Tables 2 and S6 (Supporting information).

Finally, the arrangement of fungal taxa in an environmentally constrained ordination space revealed clear co-patterning with plant communities. Permutation analysis showed a significant association between fungi composition and environmental variables ($P < 0.0001$, Fig. 4).

Discussion

Our study explored the importance of different biotic—(vegetation) and abiotic (climate and soils) drivers for the entire soil fungal diversity and phylogenetic structure on a large number of grasslands distributed along a wide elevation gradient. Our results reveal three main findings. First, soil fungal taxa–area (i.e. OTU area here) relationships show nonrandom spatial patterns, similar overall to those traditionally observed for above-ground organisms (Fig. 1; see also Peay *et al.* 2007). Second, soil fungal taxa and diversity correlate with abiotic factors, including climate (moisture index) and soil composition (pH, phosphorus and nitrogen; Fig. 3), and thus exhibit clear environmental preferences. However, intriguingly unlike most other taxonomic groups (e.g. Dubuis *et al.* 2011; Ndiribe *et al.* 2013; Pellissier *et al.* 2013b,c), fungal richness and phylogenetic diversity were found to be higher in colder conditions and higher moisture index found at higher elevations (similar to Tedersoo *et al.* 2012 showing higher ectomycorrhizal fungal richness towards higher latitudes and cooler temperatures). Third, the shared structure between soil fungi and plant community properties suggests that the biotic effect of vegetation (plant functional and phylogenetic structure) may also play a significant role in structuring fungal communities across space (Fig. 4).

Previous studies already suggested the existence of nonrandom patterns of soil fungi along geographic and environmental gradients (e.g. Martiny *et al.* 2006; de

Table 3 Results of the Mantel test correlating fungal beta diversity and various abiotic and biotic predictor variables

	Mantel *r*		Partial *r*	
A. Taxonomic diversity				
β plant taxonomic	0.33*			
β plant phylogenetic (MNTD)	0.33*		0.18*	
β plant SLA	0.23*		0.12*	
β plant LDMC	0.23*		0.14*	
β plant N	0.20*		0.10	
β plant C/N	0.20*		0.09	
β plant phylogenetic (MTD)	0.15*		0.06	
β plant C	0.12*		0.04	
ΔpH	0.40*			
Δ degree-days	0.25*			
Δ moisture	0.20*			
ΔN	0.14*			
ΔP	0.13*			
ΔFSA	0.08			
ΔCSI	0.06			
ΔCLA	0.06			
ΔFSI	0.02			
ΔCSA	0.01			
	MTD	MNTD	MTD	MNTD
B. Phylogenetic diversity (SES)				
β plant N	0.23*	0.22*	0.17*	0.19*
β plant taxonomic	0.17*	0.11*		
β plant C/N	0.17*	0.16*	0.11*	0.13*
β plant phylogenetic (MNTD)	0.15*	0.15*	0.07	0.10*
β plant SLA	0.13*	0.09	0.03	0.05
β plant LDMC	0.10	0.09	0.02	0.05
β plant C	0.08	0.07	0.03	0.04
β plant phylogenetic (MTD)	0.06	0.06	0.02	0.03
ΔP	0.19*	0.14*		
ΔN	0.16*	0.16*		
ΔCSI	0.14*	0.04		
Δ moisture	0.13*	0.19*		
ΔCLA	0.12*	0.04		
ΔpH	0.09	0.05		
ΔFSI	0.08	0.05		
Δ degree-days	0.05	0.13*		
ΔCSA	0.05	0.01		
ΔFSA	0.04	−0.04		

(A) Fungal OTU compositional turnover based on the 1199 OTUs. (B) Phylogenetic turnover of communities based on the 708 OTUs with family assignment measured using the standardized effect size of the mean nearest taxon distance (MNTD) and the mean taxa distance (MTD). When appropriate partial Mantel tests, with the plant taxonomic beta diversity as covariable, is provided. Abiotic and biotic variables are described in Table 1.
*Significant relationships at $P < 0.01$.

Vries *et al.* 2012; see review by Hanson *et al.* 2012), but many of them focused on forest sites (e.g. Nilsson *et al.* 2010; Pickles *et al.* 2010; Taylor *et al.* 2010) or plant species associations with fungi (i.e. mycorrhizal fungi; e.g. Kivlin *et al.* 2011; Tedersoo *et al.* 2012; Põlme *et al.* 2013; Peay *et al.* 2013), were restricted to particular fungal taxa (e.g. Sato *et al.* 2012 and the mycorrhiza references above) or only spanned a short elevation gradient (e.g. King *et al.* 2010; Pickles *et al.* 2010; McGuire *et al.* 2012). Our results complement and corroborate nicely these findings by showing that whole below-ground fungal communities in grasslands can also exhibit marked spatial patterns (e.g. OTU–area relationship) and turnover along wide environmental gradients; but unlike for macro-organisms, cold and wet habitats at high elevation may shelter more species. We further show that fungi exhibit clade-specific environmental specializations with distinct levels of interrelation with plant communities. In the next sections, we develop on these findings and on the methodological strength of our study.

Spatial trends in below-ground fungi

Our results reveal that soil fungi also exhibit OTU–area relationships, of general shape roughly comparable to the species–area relationships traditionally observed for macro-organisms, and thus all fungal taxa are not everywhere. In particular, the cumulated species pool (gamma diversity) reached a plateau only above a sampling window of 16 km^2, a pattern robust to random repetitions of the initial placement of the window. This suggests that sampling a large area comprising multiple heterogeneous habitats with distinct abiotic and biotic conditions is necessary to detect the full regional fungal diversity. This would not have been expected if most fungal taxa were generalists, as a plateau would be reached with a smaller window size. Fungal biogeography might therefore at least partially mirror the distribution of above-ground macro-organisms such as plants and animals. However, one aspect that differs from macro-organisms, and that is seemingly characteristic of many micro-organisms like fungi and bacteria (Bryant *et al.* 2008; Wang *et al.* 2012), is the phylogenetic clustering we observed within communities (Fig. 3). As for macro-organisms (Graham *et al.* 2009; Pellissier *et al.* 2013c), our results thus suggest that abiotic and biotic conditions imposed a strong filter on the evolution and assembly of microbial communities along biotic and environmental gradients.

Soil fungal communities also correlate with environmental factors

Another major question in microbial biogeography is which environmental factors best explain the distribution of microbial taxa across environmental gradients. The main factors assumed so far to determine soil

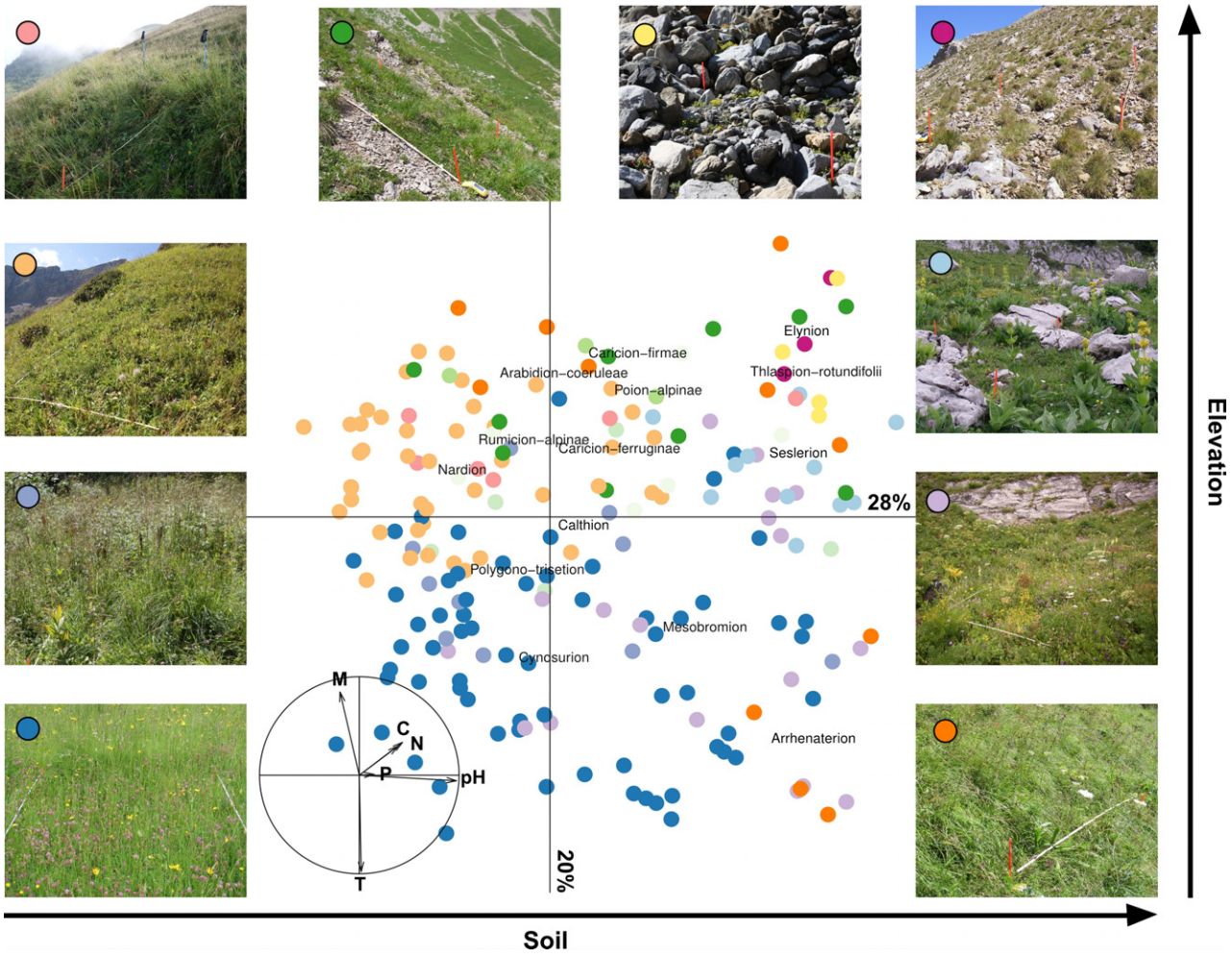

Fig. 4 Canonical correspondence analysis on fungal taxonomic composition based on the 1199 OTUs identified and constrained by the main abiotic factors. The first axis mostly represents soil chemical composition (mainly pH and P), while the second axis corresponds to temperature and moisture index (M) strongly correlated with elevation. The colours and labels in the graph (Nardion, Calthion, ...) relate to phytosociological classifications of above-ground plant communities (for a description, see Table S3, Supporting information) and highlight the shared structure between plant and fungi communities along abiotic gradients.

microbial diversity were physical and chemical edaphic properties (Cox *et al.* 2010; Rousk *et al.* 2010; Hossain & Sugiyama 2011; Zinger *et al.* 2011; McGuire *et al.* 2012; Nielsen *et al.* 2012; Põlme *et al.* 2013). Our analyses reveal that soil pH and phosphate are the most important factors explaining fungal community turnover along gradients (Fig. 3). The effect of pH was already reported in a field study on AMF fungi in *Lolium perenne*, and related to its known effect on sporulation, spore density and richness, extraradical mycelium growth and community composition (Hazard *et al.* 2013). Our study suggests that pH has a general effect on the whole soil fungal community. The negative effect of phosphate on fungal OTU richness suggests that nutrient deposition decreases soil fungal diversity (Cox *et al.* 2010), as has been documented for plants (Stevens *et al.* 2004). As a consequence, phosphate concentration in the soil may limit the potential maximum fungal diversity as found for fungi in agricultural soils (Verbruggen *et al.* 2012). This supports more specific analyses on ectomycorrhizal fungal communities showing that nitrogen availability is a primary determinant of ectomycorrhizal fungi across complex environmental gradients (Lilleskov *et al.* 2002; Cox *et al.* 2010; Kjøller *et al.* 2012). Our results also support a negative effect of soil organic carbon content on Glomeromycetes and Tremellomycetes. It is already well known that higher organic matter leads to a reduced frequency of Glomeromycota species and lower levels of colonization in roots. The arbuscular mycorrhizal symbiosis formed by Glomeromycetes is replaced by ericoid mycorrhizal fungi in some plant families when reaching more

extreme levels of organic matter (Smith & Read 2008). It is thought that this is because high-organic soils are often highly acidic and plants have more difficulty obtaining N than P. Glomeromycota are not efficient in taking up N or P from organic sources, and at high acidity, there is reduced microbial activity converting these nutrients to inorganic forms (Smith & Read 2008). Such relationships are still not well understood for the Tremellomycetes.

We found that climatic parameters, and especially moisture index, strongly correlate with some, but not all, fungal taxonomic groups. Interestingly, while we see the diversity of Eurotiomycetes, Leotiomycetes and Glomeromycetes increasing with the moisture index, the opposite effect is observed for the Sordariomycetes. However, the class that presents the most diversity in these grasslands—the Agaricomycetes—seems not to be sensitive to the moisture index. Although our data show fungal class-specific responses to moisture index, they generally support previous analyses based on spatially more restricted fungal community data sets (Drenovsky *et al.* 2010; McGuire *et al.* 2012).

Higher soil fungal diversity at higher elevations

Notably, the highest species richness and phylogenetic diversity of fungi was found in moist conditions at high elevation, where environmental filtering on fungal lineages was likely less intense (with the exception of Sordariomycetes). This observation contrasts with results observed for bacterial communities, in which species richness showed either a tendency to decrease with elevation (Bryant *et al.* 2008; Wang *et al.* 2012) or seems to be unaffected by elevation (Fierer *et al.* 2011). A first possible explanation for this sharp contrast may be that, due to the fierce competition between these two groups, a decrease in bacterial diversity with elevation could promote an increase in fungal diversity. Another reason could be that moister conditions at intermediate to high elevations may be more favourable to fungi than drier conditions at low elevations. We did not sample fungi at the highest elevations (in the nival zone) as these sites lacked a humic layer (our highest sampled point was at 2535 m, while the study area reaches 3210 m). Barren soils at these nival elevations may present extreme conditions for fungal life, leading to the dominance of a few adapted lineages (Schmidt *et al.* 2012) and, thus, a lower diversity (Lynch *et al.* 2012). A last explanation could be that more soil organic matter may be available as energy to fungi at higher elevations, due to a slower decomposition in these habitats. However, it is unlikely, as we did not observe organic-C to increase with elevation (Fig. S13, Supporting information).

Biogeographic patterns also reflect fungal–plant interactions

Empirical evidence suggests that positive and negative feedbacks between fungi and plants are major determinants of fungal community assembly (Zinger *et al.* 2011; Bever *et al.* 2013). The above-ground vegetation structure and composition is expected to influence the microbial community within the soil, via root exudates and decaying plant material (Meier *et al.* 2010; Drigo *et al.* 2012). In accordance, we found that biotic factors, including plant composition and functional structure, were correlated with fungal diversity. Particularly, turnover in the traits most linked with plant nutrient allocation and leaf palatability (LN, SLA and LDMC) was associated with turnover of fungal communities. Differences in litter composition, particularly in cellulose and lignin contents reflected in SLA and LDMC measurements, can result in differences in how readily they can be decomposed (Fortunel *et al.* 2009). Our results suggest that a shift in functional aspects of plant community may structure the distribution of fungal decomposer and saprophyte communities in space (see also de Vries *et al.* 2012).

Overall shifts in fungal assemblages along environmental gradients could be driven by changes in specific groups with particular functions. For instance, some fungi are tightly linked to plants through symbiotic and parasitic relationships (Horbach *et al.* 2011; Bever *et al.* 2013). In particular, Agaricomycete richness, a fungal class with the high ecological impact through their activities of wood-decayers and ectomycorrhizal symbiosis with trees, shrubs, perennial alpine plants (Blaschke 1991), was positively correlated with plant species richness. This finding suggests that the Agaricomycetes are important drivers of ecosystem functioning, not only in forest soil (Peay *et al.* 2007; Clemmensen *et al.* 2013), but also in grassland soils. In contrast, the Glomeromycota, that are obligatory symbionts of plants, were not found to correlate with plant species richness. This finding has to be taken with caution as the diversity of this fungal phylum is only partially explored with the primers used in this study, as the primers are more biased to amplifying the Ascomycota and Basidiomycota. Further experiments, using 18S gene sequences for OTU identification and discrimination, have to be conducted to elucidate the interaction between plant species richness and Glomeromycota diversity. At the fungal community level, we found that the composition in fungal species was correlated with plant phylogenetic turnover. Plants ability to interact with mycorrhizal fungi may be taxonomically clustered (Wang & Qiu 2006; but see Reinhart *et al.* 2012). As such, turnover in plant lineages in space may shape diversity of

mycorrhizal composition in the soil. In addition, fungal pathogens have often co-evolved with specific plants and display a high level of host specificity (Holah & Alexander 1999). Plant roots are not passive targets and produce a variety of defensive secondary metabolites that fungal species likely have to circumvent (Walker *et al.* 2003). One can, thus, hypothesize that metabolites exuded by plant roots that are specific to plant lineages may shape the distribution of soil fungi in space (Broeckling *et al.* 2008). Field experiments could be developed to test these hypotheses.

A methodologically comprehensive and robust approach

Our findings are particularly robust because they were based on (i) a specific clustering approach to define operational taxonomic units (OTUs) from a large set of sequences (Pagni *et al.* 2013), (ii) a well-designed strategy to sample a large number of plots with high spatial accuracy across a region (ca. 700 km^2) encompassing wide elevation and topographical variation, and (iii) include a large set of biotic and abiotic environmental descriptors. Concerns have been raised about the quantity of soil that should be used in metabarcoding (Ranjard *et al.* 2003; Taberlet *et al.* 2012) or unequal numbers of reads per plot in further diversity analyses (Voriskova & Baldrian 2013). The OTU diversity in a sample may be dependent on the amount of soil used to initially extract DNA; however, other factors such as DNA quality and habitat may also potentially influence OTU diversity. Although a similar bacterial diversity was recovered from a soil sample size varying between 0.125 and 4 g, changes in the fungal community structure might be observed when soil sample sizes smaller than 1 g are used for DNA extraction (Ranjard *et al.* 2003). A much higher amount of soil has to be sampled in the case of plant communities (Taberlet *et al.* 2012). Here, we show that a relatively small soil quantity of 0.5 g coming from at least two soil cores (but usually more), and the use of the Shannon diversity index to fix the problem of varying numbers of reads (performing comparably to the alternative but computationally more intensive rarefaction analysis; Fig. S11, Supporting information), allowed us to unravel the spatial patterns of fungal communities. Moreover, we demonstrate that with the specifically extracted variable region of the full ITS1 region, the dominant OTUs present in grassland soils were consistently detected using as little as 1257 sequence reads on average per plot, a value relatively lower than other pyrosequencing studies (e.g. twice as many sequences on average are reported in Clemmensen *et al.* 2013). However, in spite of this difference in the minimum number of reads, the diversity on fungal orders and families is similar to that previously described in boreal forest soils with similar tools (Clemmensen *et al.* 2013). Nevertheless, a methodological bias concerning the primers used and the data process could exist. The ITS1 region is not representative of the overall Glomeromycete diversity. The 18S region is more often used (Opik *et al.* 2009; e.g. used in Pellissier *et al.* 2013a). Consequently, only few Glomeromycete taxa have been detected in the present study with ITS1F ITS2 primer pairs. For this reason, the results presented here concerning this fungal order have to be carefully interpreted. Only a further study exploring the full diversity of Glomeromycetes with the AM1 NS31 primer pairs will give us a better view of the relationship between these symbiotic fungi and their plant hosts. The choice to retain only the reads corresponding to the full ITS1 region might exclude the fungal OTUs with an ITS1 region longer than 490 bp that are mostly represented by the fungal OTUs associated with the rDNA group-I intron meaning the Helotiales spp. as the ITS1 region of these species often contains intron. However, we obtained a good coverage of this group as Leotiomycetes was one of the most abundant groups.

Conclusion and perspectives

Despite recent developments in microbial biogeography, knowledge on elevation patterns of soil fungal communities remains scarce. Although observational and correlative, our study provides valuable data on fungal composition, richness and phylogenetic diversity across a variety of sites distributed along a wide elevation gradient and across a whole region, to answer fundamental questions in soil fungal biogeography. By showing progressive OTU–area curves, we demonstrate that soil fungal communities, such as macro-organisms, display marked spatial structuring along environmental gradients. By correlating fungal alpha and beta diversity with various environmental factors and their variation in space, we showed that both soil and climate factors are potentially important and that, surprisingly, higher diversity was found in moist conditions at high elevations. Interestingly, distinct fungal classes revealed distinct responses to environmental conditions, suggesting that different fungal lineages exhibit environmental niche separation, as similarly observed in above-ground macro-organisms. And finally, by looking at covariation between fungal and plant communities, we found that biotic factors, including plant composition, functional structure and phylogenetic diversity, were correlated with fungal diversity and turnover.

Our results indicate that soil fungi have clear environmental niches and that direct and indirect interactions with plants may shape their spatial distribution. However, because our results are correlative, we cannot disen-

tangle a direct effect of environmental variables on fungal diversity and composition from an indirect effect mediated by a shift in plant composition. Furthermore, fungi themselves can also influence plant distributions, and it remains difficult with such observational data and correlative analyses (or other mathematical approaches, such as structural equation modelling or network analysis) to untangle if plants influence fungi or reciprocally (Pellissier *et al*. 2013a). Only controlled laboratory or manipulative field experiments will ultimately allow quantifying the fundamental environmental requirements—abiotic and biotic—of these fungi and testing the links between fungal and plant distributions (see possible field experiment approaches in Pellissier *et al*. 2013a). Field manipulations of micro-organisms, in particular, are challenging, and initial observational studies, like this one, will prove valuable to help design such experiments.

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A.G., H.N.H., I.R.S., J.G., A.D., I.X. and L.P. designed the research; A.D., H.N.H., C.N., N.S. and L.P. collected the data; L.P., H.N.H., J.G., N.G. and M.P. conducted the analyses; L.P., H.N.H., A.G., I.R.S. and J.G. wrote the first draft of the manuscript, and subsequently, all authors contributed to the writing.

Data accessibility

Fungal sequences data are available at the NCBI under BioProject PRJNA253027 and BioSample SUB575526. Plant species data, the environmental data and the ITS sequences are available on Dryad (doi:10.5061/dryad.88 fm3). An OTU table is available in the supplemental material.

Supporting information

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

Fig. S1 Molecular dated phylogenetic tree of 231 angiosperm species from the Western Swiss Alps.

Fig. S2 Number of OTUs per plot as a function of the number of reads per plot.

Fig. S3 The spatial distribution of plots in the study area according to the number of samples successfully amplified and sequenced per plot.

Fig. S4 Boxplot of the Shannon diversity of fungal OTUs showing the variation in diversity according to the number of samples pooled per plot.

Fig. S5 Fungal OTUs phylogenetic tree based on the topology of published fungi phylogeny.

Fig. S6 Three variants of the species-area curves in Fig. 1c and a comparison with the curve similarly drawn for plant diversity.

Fig. S7 Variant of Fig. 1c with OTUs richness instead of Shannon diversity index.

Fig. S8 Variant of Fig. 1c without the nine plots represented by only one soil sample.

Fig. S9 Classical species accumulation curve of OTUs, not accounting for geography.

Fig. S10 Relation between Shannon diversity index and the number of reads for each plot.

Fig. S11 Shannon diversity as a function of rarefied number of OTUs.

Fig. S12 Scatterplots of degree-days and moisture index with elevation.

Fig. S13 Relationship between organic carbon and elevation.

Table S1 Number of samples pooled per plot for each location.

Table S2 List of the 1199 OTUs with their assigned annotation.

Table S3 Description of the habitat types or alliances defined from a phytosociological analysis.

Table S4 FASTA file with the consensus sequences of every OTU cluster with its taxonomic assignment.

Table S5 Coefficient of determination for the bivariate regressions between each pair of abiotic and biotic environmental predictors that were associated with fungi communities.

Table S6 Results of the quantile regressions for fungi OTU richness and phylogenetic diversity (SES MPD).

Table S7 Results of best fit multiple regressions for fungi OTU richness for each of the most frequent fungal taxonomic classes.