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Plant-fungal interactions in hybrid zones: Ectomycorrhizal communities of willows (*Salix*) in an alpine glacier forefield

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ABSTRACT

Ectomycorrhizal (EcM) fungi are essential for the establishment of woody perennial plants in the European Alps. From continental to local scales, environmental conditions and plant host characteristics can predict EcM community structure and composition. However, it is unclear whether EcM communities of congeneric host species and their hybrids are differentially structured at local scales. We aimed to i) characterize EcM communities of *Salix helvetica*, *Salix purpurea* and their hybrids and ii) elucidate the abiotic and biotic factors affecting EcM communities in hybrid zones. We analysed the EcM communities associated with willows in a glacier valley by combining molecular identification of fungi from individual ectomycorrhizas and from soil. We detected diverse EcM fungi forming non-modular and unnested networks, but we did not find significant differences in the overall EcM fungal community richness or composition among parental species and hybrids. Nevertheless, individual fungi differed regarding host preference. Our results demonstrate that in a sub-alpine hybrid zone with heterogeneous geomorphology, host genotype was not a strong predictor of overall EcM fungal community, but it influenced the occurrence of particular fungi.

1. Introduction

The ectomycorrhizal (EcM) symbiosis enhances plant nutrient and water uptake in exchange for photosynthates (Smith and Read, 2008). Furthermore, EcM fungi can influence plant diversity, nutrition, productivity and community composition in terrestrial ecosystems (van der Heijden et al., 2008; Tedersoo and Bahram, 2019). To understand and predict these plant-fungal interactions under future ecosystem changes, it is necessary to know what controls them in the environment. Ectomycorrhizal fungal communities are known to respond to abiotic factors like soil pH and nutrient availability across local, regional and continental scales (Cox et al., 2010; Suz et al., 2014; van der Linde et al., 2018) and to temperature and precipitation at global scales (Tedersoo et al., 2012, 2014). In addition, EcM fungal communities can also be affected by biotic factors like plant community composition, diversity and productivity (Waldrop et al., 2006; Bahram et al., 2012). Moreover, plant host genotype and competition with other fungi can play important roles in structuring EcM communities (Dickie, 2007; Ishida et al., 2007). In general, plant host influences EcM fungal community structure (Dickie, 2007; Tedersoo et al., 2012; van der Linde et al., 2018) and more closely related hosts share more similar EcM communities (Ishida et al., 2007; van der Linde et al., 2018), but in some cases, congeneric hosts may show

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significant differences in EcM community composition by providing distinct ecological niches for EcM fungi (Morris et al., 2008). However, the relationships among these biotic and abiotic drivers and their influence on EcM fungal communities across host hybridization zones are still poorly understood, especially in harsh environments, such as alpine glacier forefields.

In alpine ecosystems, where many dominant plants like willows are EcM, environmental change may have strong effects on EcM plant hosts and their associated fungal communities (Donhauser and Frey, 2018). At a regional scale, the richness of alpine EcM fungal communities has been shown to be positively correlated with plant species richness, suggesting that EcM fungal richness is an important driver of ecosystem functioning (Pellissier et al., 2013, 2014). Several studies have addressed this question at host family level (Ishida et al., 2007; Tedersoo et al., 2008; Smith et al., 2009), but our understanding of the interaction network between closely related plant hosts (i.e. at genus or intraspecific level) and their associated EcM communities is still very limited. Host identity in willows, when different willow species co-occurred in similar environmental conditions, had little influence on soil fungal community composition (Erlandson et al., 2016, 2018). Arctic and alpine willows have also shown low host specificity compared to other co-occurring EcM plants (Ryberg et al., 2009, 2011; Botnen et al., 2014). Consequently, the variability of EcM fungal communities across the distribution of these shrub willows has been linked predominantly to abiotic factors.

In addition to host species richness and identity, plant-host genetic variability may also affect EcM fungal communities, although studies show varied results. For instance, EcM fungal community composition in differently drought-tolerant genotypes of *Pinus edulis* was strongly influenced by host plant genetics (Gehring et al., 2017), while host genotype in *Populus* clones had little effect in determining the structure and composition of their fungal symbionts (Karliński et al., 2013). These contradictory and context-dependent results highlight our restricted understanding of how plant host genetic structure interacts with the environment to shape EcM fungal communities.

Ectomycorrhizal fungal communities may be influenced by host hybridisation, but this has been rarely tested, even though increasing plant genetic variation and its associated phenotypes may influence species interactions and ecosystem processes (Crutsinger et al., 2006; Bailey et al., 2009). Host genotypic variation can result in different phenotypes that putatively favour specific associated organisms, while in return these organisms feedback differently on the plant host (Whitham et al., 2012). The combination of different host genotypes in hybrids has the potential to generate much greater genetic variation than that found in the parents (Whitham et al., 1999), and genetically based variation in the phenotype of hybrids (e.g. leaf surface, root structure or density) can influence belowground communities. In a study of cottonwoods and their hybrids, controlling for environmental effects, host genotype played a minor role in mycorrhizal colonization compared to environmental factors (Gehring et al., 2006). Nevertheless, natural hybridisation and introgression create a genetic continuum between the two parental species that is ideal for examining changes in EcM community structure and composition. Thus, studying EcM fungal communities associated with congeneric hosts and their hybrids in alpine systems could provide further insights into host specificity across plant hybrid zones and a deeper understanding of the role of EcM fungi in the resilience of these habitats to environmental change.

Network analysis at the population and genotype levels enables identification of key interactions in plant-fungal mutualistic relationships in an ecological framework. Species may show varying levels of preference and/or specialization, resulting in certain network structures in which some species are more or less frequently connected than expected from random interactions. Nestedness and modularity indices are commonly used to characterize plant-fungal networks (Toju et al., 2013; Bahram et al., 2014; Toju et al., 2014, 2015, 2016). Nestedness measures the tendency of specialist nodes in one level of the network (plants or fungi) to interact with generalist nodes in the other level to infer the generalist-specialist balance in the community. Modularity allows inferring the existence of groups of species that form more closely interacting communities within the entire network and whether interactions within these groups are more common than among groups (Guimerà and Nunes Amaral, 2005; Almeida-Neto and Ulrich, 2011). Thus, network analysis can generate new insights into the structure of complex fungal-host communities complementing and expanding our knowledge about descriptive measures of alpha and beta diversity (Barberán et al., 2012). Extensively applied to the study of community structure in plant-fungal interactions (Caruso et al., 2012; Bennett et al., 2013; Toju et al., 2013; Bahram et al., 2014; Põlme et al., 2018), network analyses could provide information on fungal niche preference and its role on plant establishment in harsh and heterogeneous alpine habitats.

The assessment of EcM fungal community diversity and dynamics has been transformed by new high-throughput sequencing (HTS) techniques targeting the ITS1 or ITS2 regions. Despite generating millions of DNA sequence reads across numerous samples, the use of these data has limitations, such as the inability to discern between dead, dormant or active sources of DNA template, which combined with the PCR-based nature of these techniques, can lead to potentially biased observations (Lindahl et al., 2013; Nguyen et al., 2015; Hawksworth and Lücking, 2017; Wutkowska et al., 2019). Moreover, the use of HTS on bulk soil samples does not generate direct evidence of plant-fungus associations. Therefore, comparing direct sequencing of ectomycorrhizas with HTS of soil samples potentially offers robust and in-depth complementary views of EcM diversity and allows to infer host specialization regarding the available EcM inoculum in soil.

Alpine willows (*Salix* spp.) offer an excellent opportunity to compare the EcM communities in congeneric species and their intermediate individuals in hybrid zones given their genetic and ecological differentiation. In this study, we focus on *Salix helvetica*, a shrub that occurs naturally in the sub-alpine to alpine zone, and *Salix purpurea*, a widespread lowland species, able to colonize higher elevations due to global warming and subsequent glacier retreat (Gramlich et al., 2016, 2018). Using plant microsatellite markers, Gramlich et al. (2016) analysed the composition of two hybrid populations of *S. purpurea* and *S. helvetica* in the Swiss Alps and found evidence for a recent origin of the hybrids. The fine-scale environmental variation common in alpine ecosystems offered many unoccupied niches for hybrids establishment. The hybrids seemed to have a broad ecological amplitude and were able to grow under more extreme conditions, regarding soil pH, moisture and nutrient supply, than either parental species, enabling the coexistence of both parental species and the hybrids in a patchy habitat. Therefore, we designed this study to i) characterize the EcM communities that associate with *S. helvetica*, *S. purpurea* and their hybrids in one of the valleys included in Gramlich et al. (2016) and ii) elucidate the main abiotic and biotic factors that affect the structure and composition of EcM communities in a hybrid zone. Furthermore, given their ability to colonize different niches in a very restricted geographical area, we hypothesized that (1) hybrids and parental willows associate with different EcM fungi, and (2) within the same valley, host genotype explains most EcM community variability.

2. Materials and methods

2.1. Sampling

The sampling location was in the Rhône glacier valley in southern Switzerland (46°34′03.0″ N, 08°22′12.3″ E). Soil geological properties at the valley are mainly dominated by granite and granodiorite parent materials (Oberhänsli et al., 1988), formed from unconsolidated glacier and colluvial deposits building eutric dystric regosols (FAO-UNESCO, 2007). The sampling area was *ca* 0.14 km^2 with a maximum distance between sampling sites of one km (Fig. 1) ranging between 1,775 and 1,800 m a.s.l. The EcM plant community included other *Salix* spp., *Alnus viridis, Larix decidua*, *Picea abies* and *Betula* spp.

Previously genotyped individuals of *S. helvetica*, *S. purpurea,* and their first-generation hybrids (*S. helvetica x purpurea*) were selected based on Gramlich et al. (2016). In total, roots of 97 individual adult plants (*S. helvetica*: 31, *S. purpurea*: 33, hybrids: 33) distributed across 38 sites were sampled. The hybrid populations were 20–30 years old, with plants about 50–200 cm high, and distributed over the valley in a mosaic-like pattern. *Salix purpurea* grows at the more alkaline, nutrient-rich and warm sites, while *S. helvetica* occupies more acidic, nutrient-poor and colder sites. Their hybrids occur at the most extreme, acidic and nutrient-poor sites (Gramlich et al., 2016). Sites were defined as locations where individual(s) from the same or different host co-occurred within a distance of 8 m.

At least 16 roots from each individual plant were tracked from the stem, where the rocky ground permitted, carefully excavated, and stored in plastic bags at 4 °C for up to seven days until further processing. Approximately 100 cm^3 of soil was collected with a spade under each individual sampled plant for chemistry and bulk soil EcM community analyses. Soil samples for DNA community analysis were stored at 4 °C for up to 7 days, then stored at −80 °C, and freeze-dried before processing.

2.2. Environmental data

For soil chemistry analyses, we collected soil samples down to 10 cm beneath each plant. Samples were dried at 40–60 °C and sieved with a 2 mm mesh. Soil pH was measured potentiometrically in 0.01M $CaCl_2$. Total carbon (C_{total}) and total nitrogen (N_{total}) contents were measured in ground samples by dry combustion using a C/N analyser NC 2500 (CE Instruments, Italy). All soil chemistry analyses were conducted at the Swiss Federal Institute for Forest, Snow and Landscape Research, Birmensdorf (WSL). Geographic coordinates and EcM plant community composition were recorded within a radius of 10 m from each sampled individual plant.

2.3. Mycorrhizal root assessment

Root samples were rinsed in water and 16 EcM tips from 16 different roots traced from the stem were selected from each individual plant when possible. The presence of hyphae and/or rhizomorphs was recorded (Agerer, 2001, 2006) before DNA extraction. Genomic DNA was extracted from individual ectomycorrhizas using Extract-N-Amp (Sigma-Aldrich, Darmstadt, Germany) and the ITS region of the rDNA was amplified using the fungal-specific primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). Amplicons were purified using ExoSAP-IT (USB, Cleveland, OH, USA) and sequenced bidirectionally using BigDye v. 3.1 in an ABI 3730 (Applied Biosystems, Foster City, CA, USA).

Sequences were first analysed with KB Basecaller v1.1.1 (Applied Biosystems) and bases called with minimum quality value (QV) of 20 (i.e. 99% of base call accuracy). Forward and reverse sequences from each ectomycorrhiza were assembled using *phrap* v1.090518 (de la Bastide and McCombie, 2007). When the assembly of both sequences was not possible due to the poor quality of one of the sequences or to uncertainty determining their overlapping region, a high quality base-pair (QV ≥ 20) per sequence length ratio was applied and the longest sequence (>200 bp) with ratio scores ≥85% was selected for further analyses. To avoid misleading results in the DNA sequence identification process and to facilitate direct comparison with the fungal community in the soil, adjacent conserved regions (18S, 5.8S and 28S) were removed using ITSx v1.0.11 (Bengtsson-Palme et al., 2013) and the ITS2 region was selected for further analyses. Sequences were clustered in OTUs at a 97% similarity threshold using the UPARSE algorithm implemented in USEARCH v9.2 (Edgar, 2013), while simultaneously excluding chimeric sequences. The taxonomic affiliation of each OTU was inferred by blasting each OTU centroid sequence to the UNITE fungal ITS sequence database v7.2 as a reference for assignment of a species hypothesis (Nilsson et al., 2011; Kõljalg et al., 2013). For centroids with the best blast hit below 97% we used the SINTAX algorithm (Edgar, 2016) with a 0.8 cut-off to predict OTU taxonomy. The ecological functions of each OTU (i.e. trophic level and type of

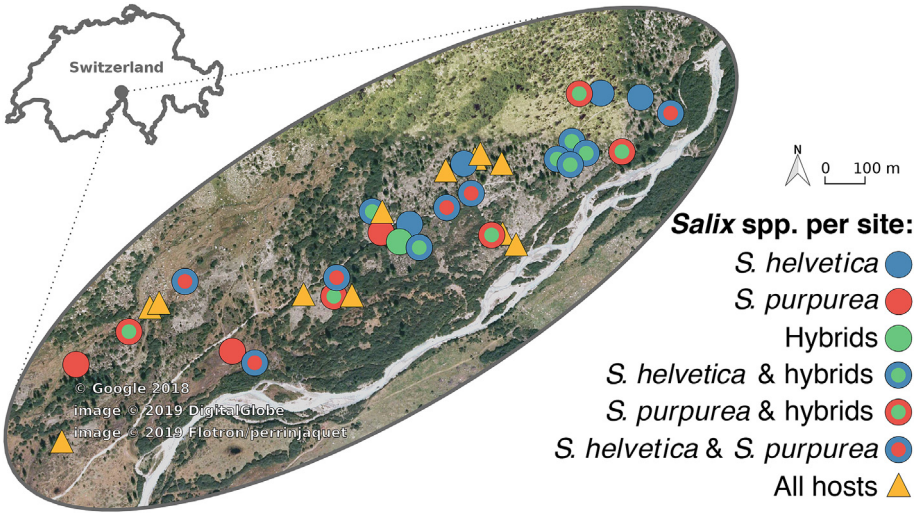

Fig. 1. Sampling sites included in this study, based on Gramlich et al. (2016).

mycorrhizal association) were assigned according to UNITE (Kõljalg et al., 2013) and only EcM fungi were used in downstream analyses. Representative sequences of each EcM OTU were deposited in NCBI under accession numbers MK838121-MK838189.

2.4. Soil DNA analysis

To analyse the soil fungal communities, we collected soil adjacent to the roots. Samples were sieved (2 mm mesh) and genomic DNA was extracted from 25 mg of 97 freeze-dried soil samples using the DNeasy PowerSoil Kit (Qiagen, Valencia, CA) and quantified using the Qubit 2.0 fluorometric system (Life Technologies, Paisley, UK). Amplifications of the ITS2 region using the primer set fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) were run in triplicates and pooled for library preparation. The PCR, library preparation and paired-end sequencing on the Illumina MiSeq v3 platform (Illumina Inc., San Diego, CA, USA) were conducted by Génome Québec Innovation Center at McGill University (Montréal, Canada).

Quality filtering and clustering into OTUs was performed using a customized pipeline based on UPARSE implemented in USEARCH (Edgar, 2013), but with some additional modifications as follows. Paired-end reads were merged using the USEARCH fastq mergepairs algorithm (Edgar and Flyvbjerg, 2015) allowing staggered alignment constructs in order to accommodate potentially short ITS2 amplicons. The PCR primers were detected and trimmed using Cutadapt (Martin, 2011) allowing for one mismatch. Reads not matching the primers or with lengths below 150 bp were discarded. Trimmed reads were quality-filtered using the USEARCH fastq filter function with a maximum expected error threshold of one. Sequences were de-replicated to retrieve information on abundance distribution, and singleton reads were removed prior to clustering in order to avoid artificial OTU inflation. Sequences were clustered into OTUs at 97% sequence identity using the USEARCH cluster_otu function that includes an 'on-the-fly' chimera detection algorithm. The OTU centroid sequences were subjected to an additional round of chimera filtering by running UCHIME against the uchime version of the UNITE database. The remaining centroid sequences were tested for the presence of ribosomal signatures using ITSx (Bengtsson-Palme et al., 2013) and 33 out of 2708 centroid sequences with the ribosomal origin not sufficiently supported were discarded. Finally, all quality-filtered reads were mapped against the final set of centroid sequences using the usearch global algorithm with the most comprehensive search criteria (maxrejects 0, maxaccepts 0 and top hit only). The taxonomic affiliation of each OTU was inferred by blasting each OTU centroid sequence to the UNITE fungal ITS sequence database v7.2. For centroids with the best blast hit below 97% we used the SINTAX algorithm (Edgar, 2016) with a 0.8 cutoff to predict OTU taxonomy. Representative sequences of each EcM OTU were deposited in NCBI with accession numbers MK838190-MK838412 while HTS reads were deposited in the Sequence Read Archive under the accession number PRJNA575244.

2.5. Alpha and beta diversity of EcM fungi

Analyses were carried out using R version 3.5.1 (R Development Core Team, 2009). Sequence and read numbers from both root and soil community matrices were normalized using the cumulative sum scaling (CSS) method as implemented in the *metagenomeSeq* package (http://www.cbcb.umd.edu/software/metagenomeSeq). Observed OTU diversity and the abundance-based estimators Chao1 and ACE were calculated using the *estimateR* function of the *vegan* package (Oksanen et al., 2019). Differences between observed and estimated values among hosts were assessed by one-way ANOVA and Tukey's HSD *post hoc* tests. The percentage of OTU richness recovered was calculated taking the proportion of OTUs observed from the estimated number of OTUs (ACE) per host. Normal distribution of the residuals and homogeneity of variance were evaluated by analysing *qqnorm* plots and significant deviations of Shapiro and Levene test routines implemented in R. Data with non-normal distribution were square root or log transformed when necessary.

To assess the proportion of shared OTUs between root and soil datasets, we performed a local blast using the *blastn* algorithm with the soil OTU centroids as reference and the root OTU centroids as queries. The OTUs with blast hits superior or equal to 97% and with congruent taxonomy (i.e. equal Species Hypothesis number) were considered equivalent.

The local contribution for beta diversity (LCBD) was calculated to measure the degree of uniqueness of each sample to the variation in community composition following the method described by Legendre and De Cáceres (2013) and implemented in the *microbiomeseq* package, grouping OTUs by their corresponding EcM lineage according to UNITE. Non-metric multidimensional scaling (NMDS) ordination was used to visualize dissimilarities in EcM communities across different plant hosts and in both root and soil datasets using Bray-Curtis dissimilarity distances. Permutation analysis of variance (PERMANOVA) was used to assess differences between groups (3 hosts x 2 datasets, roots or soil) using the *adonis* function and significant differences among pairwise homogeneity of group dispersions (variances) were calculated using *betadisper.*

2.6. Plant-fungal network analysis

To explore the interaction network between EcM fungi and their host plants, we computed three ecological network indices (Modularity, Nestedness and C-score) using the *bipartite* package for R (Dormann et al., 2008). Modularity and nestedness were calculated for both binary and weighted matrices. We computed modularity using the QuaBiMo algorithm (Dormann and Strauss, 2014) to identify aggregated sets of interacting OTUs (i.e. modules). To further investigate the architecture of the bipartite network, we calculated nestedness using NODF and weighted NODF indices (Almeida-Neto and Ulrich, 2011) for the binary and weighted matrices, respectively. In bipartite networks, nested communities (i.e. with values towards 1) are characterized by specialist nodes in one level of the network (e.g. EcM fungi) that connect to generalist nodes of the other level of the network (e.g. plant hosts) but never with other specialists and *vice versa* (Bascompte et al., 2003). To analyse co-occurrence patterns among all EcM fungal OTUs, we computed the average number of checkerboard units (C-score) (Stone and Roberts, 1990) for each unique fungal OTU pair in both soil and root datasets. The higher the C-score, the less co-occurrence, on average, among all of the fungal OTU pairs. A relatively large C-score indicates a more segregated matrix (i.e. with a checkerboard pattern where the distribution of particular fungal OTUs is influenced by the presence of other fungal OTUs), and a relatively small C-score indicates a more aggregated matrix (i.e. where this influence among particular OTUs is not found).

To assess the significance of all network indices calculated, we generated 1,000 matrices from a conservative fixed-fixed null model constraining row and column marginal sums as implemented in *vegan*::*nullmodel* and computed *p*-values and standard scores (*z*-scores) over the deviations of the observed values with those predicted by the null models. The C-score was calculated using only the binary matrix. Bipartite networks from both root and soil datasets were visualized using the *igraph* package.

2.7. Hierarchical modelling of EcM communities

To assess the dependence of each fungal OTU on environmental conditions, we applied a hierarchical joint species distribution model approach (Ovaskainen et al., 2017). This framework allows the assessment of how much variation in each OTU occurrence is due to environmental filtering, biotic interactions and random processes. Furthermore, to allow this statistical framework to calculate community-level synthesis of how species respond to the environment, it is assumed that the overall OTU responses to environment adhere to a multivariate normal distribution.

To model the distribution of EcM fungal OTUs, we used soil pH, total soil nitrogen (N_{total}) and total soil carbon (C_{total}), and to account for biotic interactions we used Chao1 estimates of soil total fungal community ($S_{fungi.total}$) and soil EcM community ($S_{fungi.EcM}$). As random effects, we used sampling site and plant host identity. We used the *bestNormalize*:*bestNormalize* function (Peterson, 2017) to determine and apply the ordered quantile normalizing transformation to N_{total} and C_{total}, and the square root to $S_{fungi.EcM}$ covariates. We ran two independent models for each of the datasets (roots and soil) using the binary (presence/absence) or the relative abundance matrix. Model priors were set to default and family distribution to probit for the binary models and lognormal Poisson distribution for the abundance variants. Parameter estimation was achieved by Markov Chain Monte Carlo (MCMC) posterior sampling for 100,000 iterations, 10,000 burning and 10 thinning. Parameter convergence was checked by trace visualisation. To assess the level of statistical support for whether the probability of an OTU abundance increased or decreased with the increasing value of a given environmental covariate, we defined the 95% central credible interval by computing the 0.025 and 0.975 quantiles of each parameter. The explanatory power of the models was calculated using the coefficient of discrimination Tjur R^2 (Tjur, 2009) for each individual fungal OTU and its average at community level.

To assess how much of the variability in fungal OTU occurrence was due to biotic, abiotic or random processes, we partitioned the variance of the explained portion of the model with higher explanatory power by grouping covariates as abiotic (soil pH + N_{total} + C_{total}), biotic ($S_{fungi.total}$ + $S_{fungi.EcM}$) and for each covariate independently using the *variPart* function of the *HMSC* package.

3. Results

3.1. Soil chemistry

Values of soil pH ranged from 3.58 to 5.59, with significantly lower values observed under *S. helvetica* than *S. purpurea* ($P = 0.019$; Fig. 2a). Total soil nitrogen across all sampling sites ranged from 0.028% to 2.4% and soils under *S. purpurea* showed significantly higher values than those under hybrids ($P = 0.012$; Fig. 2b). Total soil carbon values ranged from 0.46% to 42.5% with soils collected under *S. purpurea* showing significantly higher values than hybrids ($P = 0.047$; Fig. 2c).

3.2. Alpha and beta diversity of EcM fungi

A total of 1,233 ectomycorrhizas were sampled from the roots of 82 out of 97 individual plants (*S. helvetica*: 27, *S. purpurea*: 27, hybrids: 28) distributed across 37 of the sampling sites. We retrieved 1,076 DNA sequences, from which we kept 1,044 high quality sequences belonging to EcM lineages (*sensu* Tedersoo et al., 2010; Tedersoo and Smith, 2013). After clustering and filtering, we identified 69 OTUs (average per individual 3.42 ± 1.52) across 14 phylogenetic lineages. Approximately 90% of the EcM fungi in roots belonged to Basidiomycota and 10% to Ascomycota. The EcM lineages with higher OTU richness were *Tomentella-Thelephora* (28 OTUs), *Cortinarius* (16 OTUs), *Inocybe* (5 OTUs) and *Hebeloma* (5 OTUs).

From soil samples, a total of 9,036,820 raw DNA reads were generated, from which we retrieved 3,099,112 high quality ones. In total, we identified 2,078 fungal OTUs from which we kept 223 (average per soil sample 9.30 ± 6.32) belonging to 32 EcM fungal lineages after removing OTUs from non-EcM lineages and 22 EcM OTUs known to be specialists of conifers s.l. or *Alnus* spp. (Tedersoo et al., 2009; Nilsson et al., 2019) and that were not detected in the roots. The EcM lineages with higher OTU richness found in the soil were *Cortinarius* (51 OTUs), *Tomentella-Thelephora* (47 OTUs), *Inocybe* (22 OTUs), *Sebacina* (15 OTUs), *Russula-Lactarius* (13 OTUs) and *Laccaria* (12 OTUs).

Fifty-five EcM OTUs were detected in both roots and soil, whilst 14 were found only in roots and 168 only in soil (Table S1). Across all sites, we observed 40 EcM OTUs in roots of *S. purpurea*, 44 in roots of hybrids, and 48 in *S. helvetica* roots. In the soil, we found 142 EcM OTUs under *S. purpurea*, 143 under hybrids, and 141 under *S. helvetica*. No significant differences were observed when comparing observed and estimated OTU richness (ACE) among hosts (data not shown). The dominant EcM fungal lineages forming ectomycorrhizas were *Tomentella-Thelephora*, *Cenococcum* and *Cortinarius*, whilst in the soil the most abundant reads belonged to *Tomentella-Thelephora*, *Cortinarius* and *Russula-Lactarius* (Fig. 3).

Ordination results showed no significant dissimilarities among hosts in the EcM communities found in roots or in soil (Fig. 4). However, differences in community composition were observed when comparing root with soil datasets within hosts (Fig. 3). These results were corroborated by *adonis* ($F = 3.337$, $R^2 = 0.097$, $P = 0.001$) and by pairwise *betadisper* analysis (Table S2).

3.3. Plant-fungal network analysis

Using the QuaBiMo algorithm we identified three network compartments (i.e. modules) both in the soil EcM fungi (Fig. 5a, soil) and in the roots (Fig. 5b, ectomycorrhizas) using presence-absence and abundance association matrices respectively. Modularity likelihood observed values (Q) in the network between willows and EcM fungi in roots did not show significant differences from the null models ($Q_{root.bin} = 0.396$, $P = 0.112$; $Q_{root.weighted} = 0.242$, $P = 0.073$). In the association network between willows and EcM fungi from soil samples, significant differences from the null models were observed when using the weighted matrix ($Q_{soil.bin} = 0.388$, $P = 0.129$; $Q_{soil.weighted} = 0.329$, $P < 0.001$, z-score = 6.167) (Table S3).

The network nestedness (NODF) values observed in the soil and roots datasets were similar but only the roots *vs* willows network showed significant differences from the null models ($NODF_{roots} = 59.2$, $P = 0.032$, z-score = −0.061; $NODF_{soil} = 58.5$, $P = 0.420$). Using the weighted matrices, the observed nestedness (WNODF) values were lower and only the soil observed value in the soil fungi *vs* willows was significantly lower than expected by the null models ($WNODF_{roots} = 37.7$, $P = 0.315$; $WNODF_{soil} = 39.2$, $P < 0.001$, z-score = −2.994).

The C-score analysis revealed significant deviations from expected by the null models in the co-occurrence patterns of the EcM fungi found in the soil dataset, but not in the EcM fungi found in the roots ($C\text{-score}_{roots} = 0.502$, $P = 0.404$; $C\text{-score}_{soil} = 0.511$, $P = 0.003$, z-score = 2.127).

3.4. Hierarchical modelling of EcM communities

We observed no significant improvements in the hierarchical

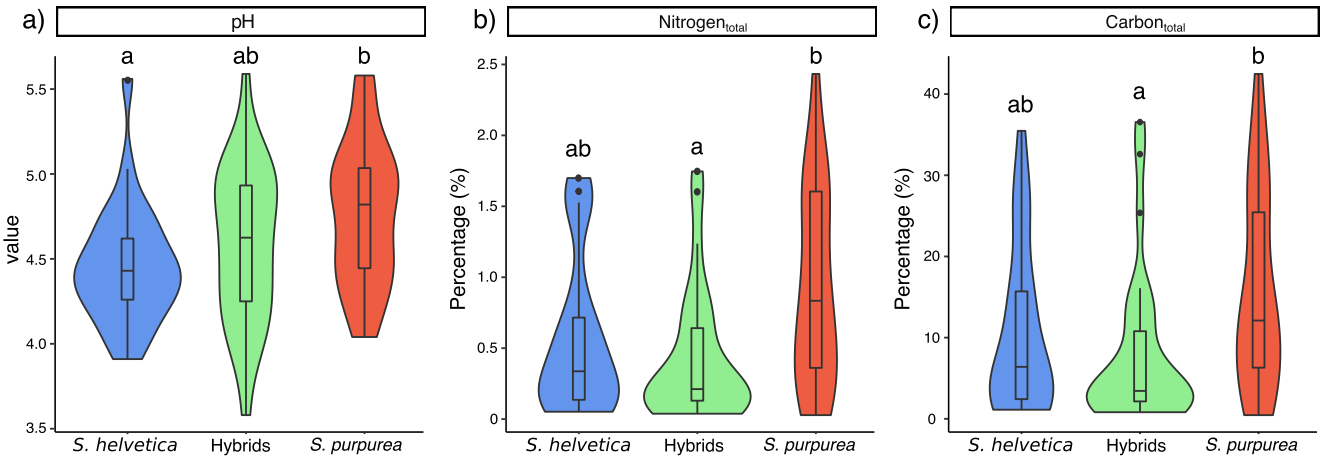

Fig. 2. Soil chemistry parameters measured under *Salix helvetica* (n = 27), *S. purpurea* (n = 27) and their hybrids (n = 28) across the sampling area. Each violin plot represents the distribution and the probability density of the non-normalised data of each parameter observed for each host. Different letters indicate significant differences after ANOVA at *p*-value < 0.05.

models of EcM fungal OTU occurrences using the same environmental predictors for roots and soil datasets. The explanatory power (R^2) of the models using the soil EcM community data was on average 0.06 (95% CI: 0.05–0.07) and 0.07 (95% CI: 0.04–0.10) for the presence-absence and abundance data matrices, respectively. In the case of the root EcM community data, R^2 was on average 0.06 (95% CI: 0.05–0.07) and 0.05 (95% CI: 0–0.09) for the presence-absence and abundance data matrices, respectively. Due to lack of reliability of the models based on the abundance data, we only considered the results of the presence-absence models.

The partitioning of the overall explained variance in the presence-absence models revealed that sampling site and plant host species (random effects) explained the majority of the variation in both datasets (Fig. 6). In the root dataset, on average, 51% of the explained variation in the data was accounted for by plant host (41%) and site (10%), whilst the abiotic variables accounted for 35% (C_{total} = 14%, N_{total} = 13%, and soil pH = 8%) and the biotic variables 14% ($S_{fungi.total}$ = 6% and $S_{fungi.EcM}$ = 8%). In the bulk soil dataset, approx. 46% of the explained variation in the data was accounted for by plant host = 32% and site = 14%. The proportion of the explained variance accounted, on average, by fixed effects in this dataset was 36% by abiotic variables (C_{total} = 15%, N_{total} = 15% and soil pH = 6%) and 18% by biotic variables ($S_{fungi.total}$ = 13% and $S_{fungi.EcM}$ = 5%).

4. Discussion

We aimed to explore whether host intra-specific genetics through hybridization could influence EcM fungal communities. However, we did not find a strong influence of host identity in richness or composition of the EcM fungal communities associated with *S. helvetica*, *S. purpurea* and their hybrids in the Rhône glacier valley, despite their previously observed different habitat/niche preferences (Gramlich et al., 2016). Lack of host specificity among willows has been previously reported beyond the Alps (Ryberg et al., 2009; Botnen et al., 2014; Erlandson et al., 2016, 2018). Ryberg et al. (2011) found similar EcM communities associated with *Salix polaris* and *Salix herbacea* in a Swedish alpine tundra. In a study comparing *Salix viminalis* growing in arable soils versus adjacent natural or naturalized stands in Sweden, Hrynkiewicz et al. (2012) detected site and host identity effects on EcM root colonization, but no effects on EcM fungal abundance and diversity. However, in other ecosystems and plant host species, host genetics has been found to directly or indirectly influence EcM communities, for instance, long-term studies of *P. edulis* populations showed an effect of host plant genetics on EcM community composition (Gehring et al., 2014, 2017) and individual spruce trees (*P. abies*) were found to be partly responsible for the high diversity and patchy distribution of EcM communities in boreal forests (Korkama et al., 2006; Velmala et al., 2013). A lack of host specificity in arctic and alpine ecosystems might be a mechanism that favours plant hosts to more rapidly and easily colonize newly available habitats, favoured by the establishment of symbiotic relationships with fungi with different physiological attributes (Botnen et al., 2014). Nevertheless, we cannot disregard the overall role EcM fungi may play on the colonization and establishment of hosts in these habitats.

The high soil heterogeneity characteristic of alpine ecosystems might also influence EcM fungi and mask differential host effects on EcM composition and structure. In our study we observed variation in soil pH, C and N content, leading to the presence of many microhabitats with distinct edaphic characteristics. The same soil heterogeneity was also highlighted by Gramlich et al. (2016) reporting that despite its recent emergence, the hybrid population occupies ecologically distinct sites in the studied forefield with respect to the parent species, scattered over the whole area of the alluvial plains in a mosaic-like spatial pattern. Our results show however that the dominant EcM fungi colonising roots were shared among parents and hybrids; these fungi may be better adapted to the extreme environmental characteristics in sub-alpine habitats than host-specialists, thus explaining the lack of plant-fungal specificity or preference observed across willows. Nara and Hogetsu (2004) compared the growth rate of *Salix reinii* seedlings growing next to already established willow shrubs and observed that EcM fungi associated with the latter were essential in facilitating seedling establishment of later-successional plant species. When comparing EcM fungal community composition in roots (ectomycorrhizas) versus fungi in soil, Goldmann et al. (2016) observed that plant host neighbour effects were stabilizers of fungal community composition. They showed less distance decay in root-associated fungal communities compared to the soil fungal communities in a beech-dominated forest, suggesting that host

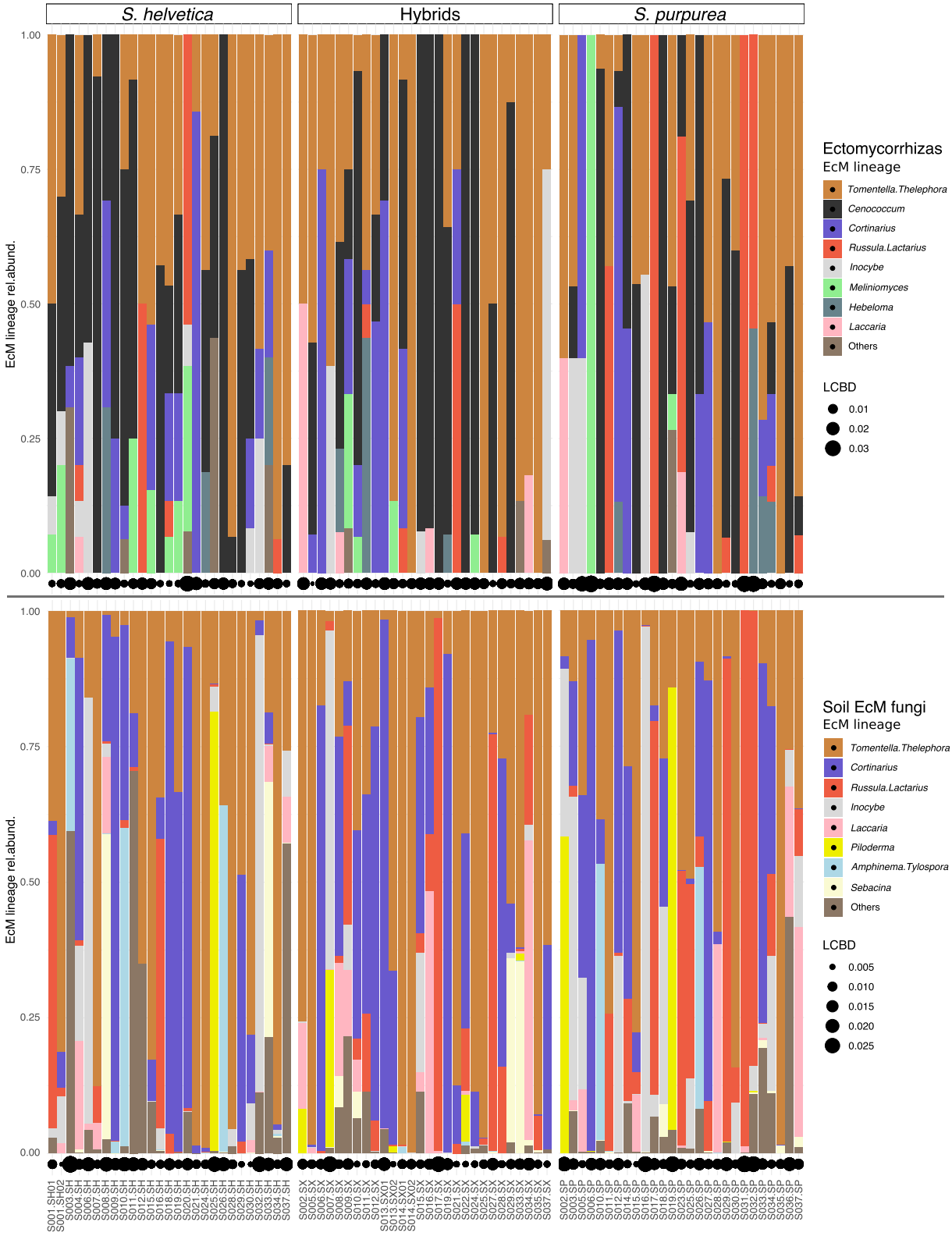

Fig. 3. Relative abundance and local contribution for beta diversity (LCBD) of the most abundant EcM lineages found as ectomycorrhizas (top) and in the soil (bottom) in each individual willow.

trees could buffer the effects of changes in microclimatic and environmental conditions that could directly influence fungal community composition in soil. In our study, other EcM hosts, including other willow species present in the valley, may have prevented EcM community differentiation among hosts by being a permanent source of diverse generalist inoculum.

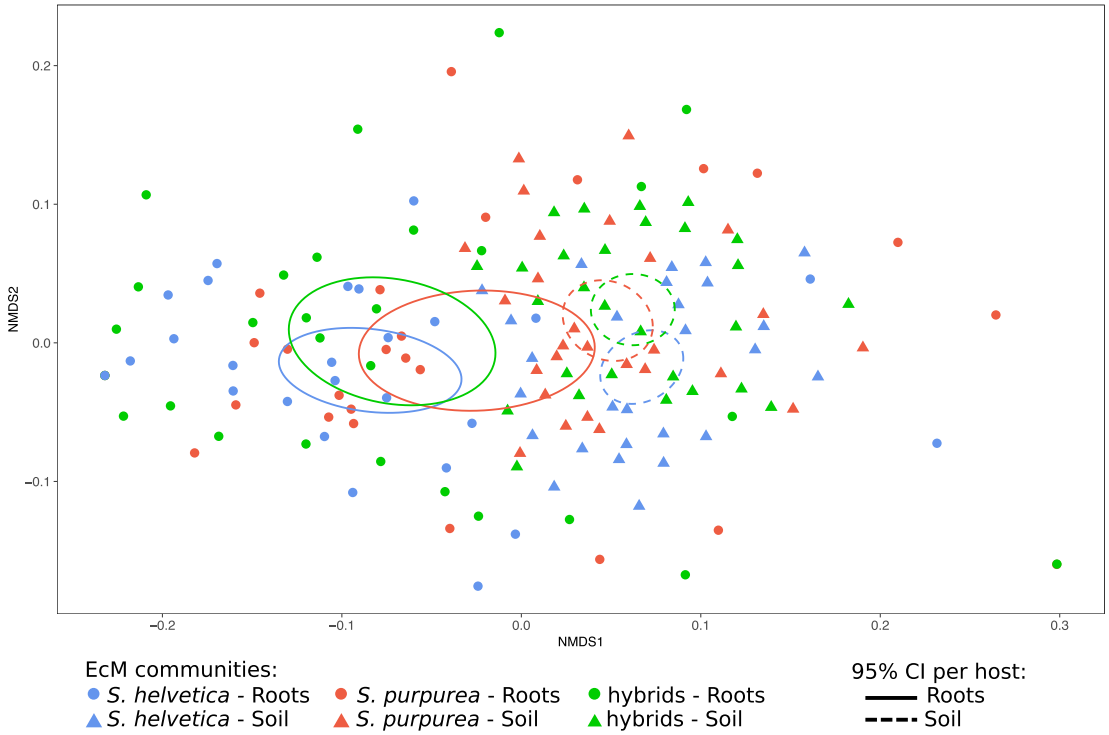

Fig. 4. Nonmetric multidimensional scaling (NMDS) of community dissimilarities using Bray–Curtis distances. Circular points represent EcM communities associated with roots (ectomycorrhizas) and triangular shape points represents EcM communities in soil under each individual plant. Circumferences represent the 95% confidence interval of samples for each plant host. Stress of the ordination was 0.19.

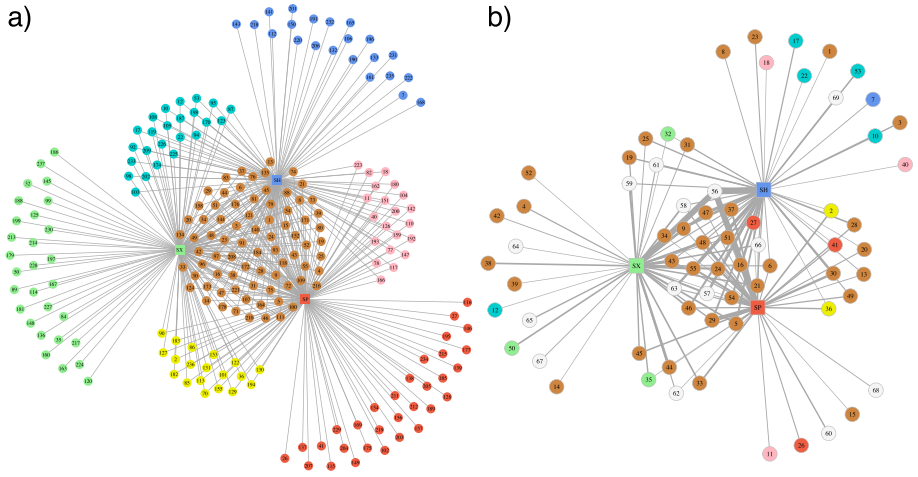

Fig. 5. Visualisation of EcM networks in a) presence-absence of soil EcM fungi *vs* willows and b) abundance of ectomycorrhizas *vs* willows, based on the Fruchterman Reingold algorithm. Host plants and fungal OTUs are represented by squares and circles, respectively. Host plant abbreviations are: SH – *Salix helvetica*, SX – Hybrids and SP–*S. purpurea*. Network edge thickness on plot b represent the strength of the association (i.e. the number of times that particular association was observed). Coloured vertices on b) represent the shared OTUs between soil EcM fungi and ectomycorrhizas and derive from their relative position in plot a). White vertices represent OTUs only present as ectomycorrhizas.

4.1. Fungal community richness and composition

On average, we recovered 89% of the estimated EcM richness (ACE) in willow roots (87% in *S. helvetica*, 90% in hybrids and 91% in *S. purpurea*). Despite the harsh environmental conditions in sub-alpine habitats, these willows harbour a considerable number of EcM fungal species within a relatively small geographical area. As suggested by previous studies, the large number of EcM fungal species found in alpine and sub-alpine habitats might be linked to the high richness of EcM plant hosts in these habitats (Krpata et al., 2007; Pellissier et al., 2014), moreover, hybrid zones are considered centers of biodiversity for many organisms (Whitham et al., 1994).

As expected, due to the presence of other EcM hosts in the valley and probably to the methodological bias from using rDNA in bulk soil samples that does not discriminate active, dormant or dead organisms, or intra- versus extra-cellular DNA template sources (Carini et al., 2016; Wutkowska et al., 2019), the EcM OTU richness found in the soil was significantly higher than in ectomycorrhizas (142 OTUs under *S. purpurea*, 143 under hybrids and 141 under *S. helvetica* compared to 40, 44 and 48 in their roots, respectively).

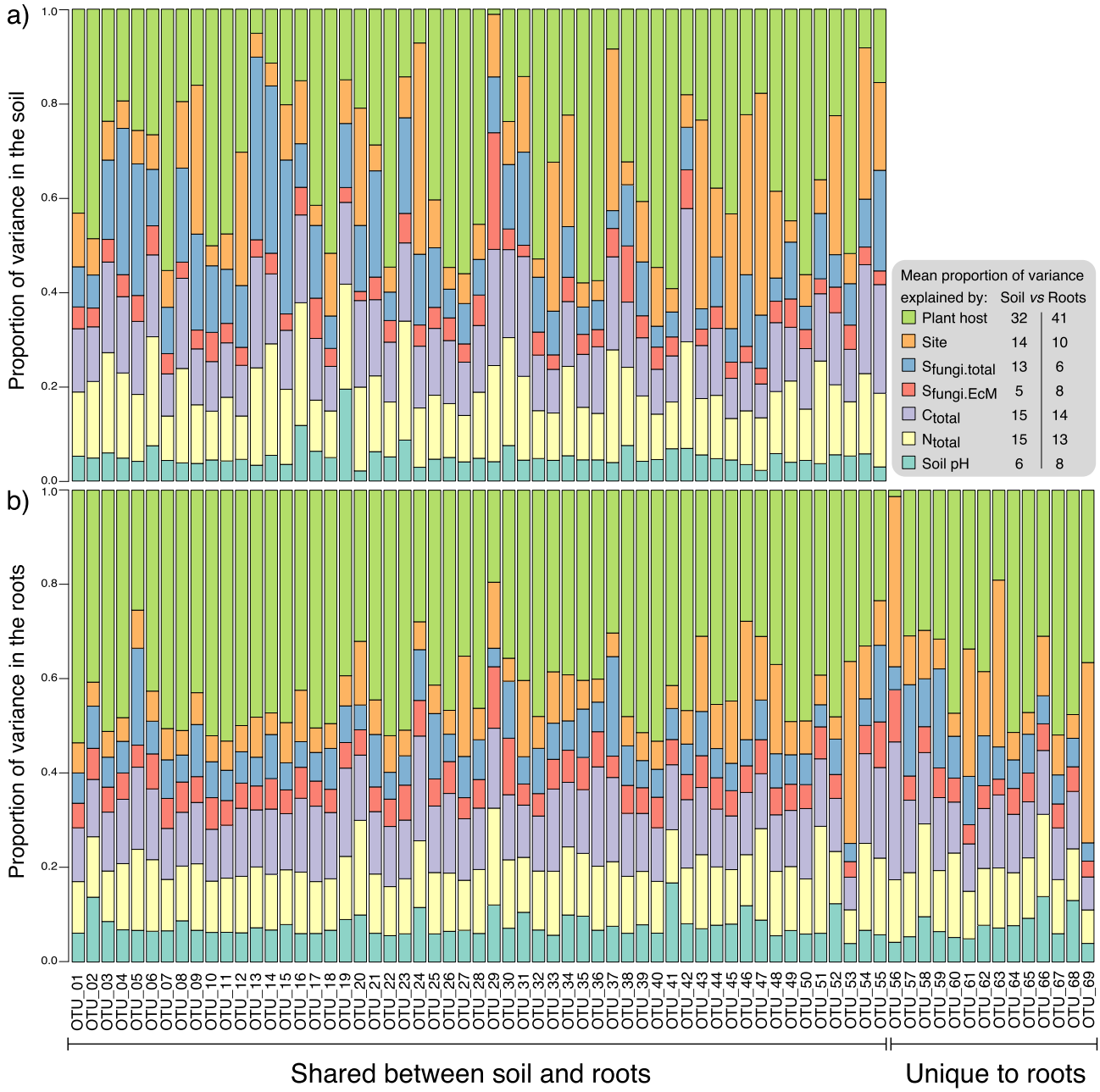

Fig. 6. Variance partitioning in EcM fungal communities in a) soil and b) roots (ectomycorrhizas). Variance partitioning was calculated using the presence-absence of fungal OTUs in response to site, plant host, soil pH, total C, total N, estimated total fungal community in the soil ($S_{fungi.total}$) and estimated soil EcM community ($S_{fungi.EcM}$). Only shared OTUs between soil and root datasets are illustrated in a).

Nevertheless, the EcM community richness and composition observed did not differ significantly across hosts, following the same pattern observed in roots. Collecting data from both ectomycorrhizas and from soil allowed testing for fungal specificity or preference of the three hosts from the potential fungal inoculum available. Our results indicate that a small proportion of the full potential inoculum (approx. 25%) was actually recruited by the plants. Similar rates of recruitment (27%) were also inferred by Goldmann et al. (2016) in a temperate beech forest. In contrast, approximately 80% of the OTUs observed as ectomycorrhizas were detected in the soil.

Soil has been suggested as a good proxy for estimating fungal richness at regional scales (Landeweert et al., 2005); however, the inability to detect in soil some dominant fungi associated with roots (e.g. *Cenococcum geophilum*, *ca.* 32%) reveals the bias and risk of using only bulk soil data as a source of fungal community information. *Cenococcum geophilum* is an anamorphic complex of species that associates with a vast range of host plants globally, forming abundant black sclerotia in soil (Obase et al., 2017) and increasing host plant drought tolerance (Pigott, 1982; Jany et al., 2003). Studying bulk soil communities without accounting for autecology, potential methodological biases, target limitations of genetic markers and bioinformatic challenges, can lead to biased views of EcM communities, artificial results and misleading conclusions (Lindahl et al., 2013). In this study, three OTUs of *C. geophilum* were among the most abundant and conspicuous fungi observed in roots (Fig. 3), as in other studies sampling ectomycorrhizas in alpine glacier valleys (Krpata et al., 2007;

Mühlmann et al., 2008; Mühlmann and Peintner, 2008). However, our results are also congruent with Pellissier et al. (2014), Rime et al. (2015) and Frey et al. (2016) where *C. geophilum* was not detected in soil. This might be due to primer bias, inability of DNA extraction methods to break thickly melanized cell walls, removing larger sclerotia through sample sieving before DNA extraction, and/or ephemeral or sporadic hyphal growth in soil from a stable population of ectomycorrhizas. To examine whether some of these issues could have contributed to the non-detectability of *C. geophilum* in our soil dataset, we i) manually verified the identity of the binding region of the fITS7 primer in all *C. geophilum* OTUs found in roots and the respective UNITE reference sequences; ii) verified that no hits of *C. geophilum* were observed using Blast on all centroid sequences from the soil dataset against the UNITE+INSD v8.0 database, and iii) verified the detection of *C. geophilum* in other studies using the same DNA extraction kit and similar analyses in soil (Kirker et al., 2017), roots (Evans et al., 2015), mesh bags (Ning et al., 2019) and cultures (Peter et al., 2016). Thus, our findings may reflect limited extraradical growth by a dominant EcM fungus.

4.2. Plant-fungal network structure

Network modularity, which can be directly attributable to partner selectivity, measures how aggregated some sets of interacting species can be in a community (Dormann and Strauss, 2014) while nestedness measures the degree of interaction of specialists in one guild with generalists in the other guild (Bascompte et al., 2003). In the Rhône Glacier valley, plant-fungal networks of willow hosts *vs* EcM fungi are non-modular and unnested. The networks of soil fungi *vs* willows and root fungi *vs* willows revealed low likelihood values of modularity and no significant deviations from the null models, except when using the weighted matrix of soil fungi *vs* willows (Table S3). The lack of modularity in plant-EcM fungi networks has been previously suggested and inferred to be context-dependent, varying with species identity and potentially with the phylogeny of both partners (Bahram et al., 2014; Põlme et al., 2018). Moreover, modularity indexes are known to be sensitive to network size, as networks with many species and links allow for more possible combinations of species-in-modules, leading to higher values of modularity (Allesina and Pascual, 2009). Similarly to our study, Põlme et al. (2018) also found weak modularity values in EcM fungal networks due to a low number of hosts.

When using the weighted version (i.e. abundance data) of the soil fungi *vs* willows matrix instead of the binary one (i.e. presence-absence), we observed significantly higher modularity than expected by the null models. We believe this can be an artefact due to difficulties in the process of generating null models for abundance matrices. We generated our null models constraining matrix row and column sums, but due to the high discrepancies in read numbers (even though CSS-normalized) and to the small number of hosts (i.e. reduced matrix size), the randomization scheme was very constrained, thus increasing the probability of type I errors (Lavender et al., 2016).

In accordance with other studies (Bahram et al., 2014; Toju et al., 2014; Põlme et al., 2018), we found that the network of plant hosts *vs* EcM fungi was unnested. We detected higher values of nestedness when analysing the binary matrices and more significant differences than expected by the null models using the binary matrix from the roots and the weighted matrix from the soil. Despite these differences, none of the indexes were indicative of nestedness in our networks (i.e. values towards 1 indicate nestedness). Similarly to modularity, measuring nestedness in plant-EcM fungal networks is challenging due to specific matrix properties (e.g. matrix size) (Bascompte et al., 2003; Põlme et al., 2018).

In a competitively structured community, the observed C-score is significantly higher than that generated by null models. Lack of significance suggests that species co-occur randomly, and significant lower C-score than expected from null models indicates species aggregation (Gotelli and Entsminger, 2001). In our case, the C-score analyses did not show significantly higher values than the null models in the ectomycorrhizas suggesting that overall, the EcM community is not competitively structured. However, since the C-score only measures the average number of checkerboard units (Stone and Roberts, 1990), we cannot rule out that unseen co-occurrence patterns among particular pairs of EcM OTUs could occur in willow roots. We found however that C-scores were significantly higher than the null models for the EcM fungi detected in soil, suggesting in this case a non-random distribution of species meaning that these communities could be competitively structured. Other hidden processes, however, such as differentiation on fungal OTU niche preference or other historical or evolutionary processes also may have led to less coexistence than expected. Non-random distributions of EcM communities were previously described by Koide et al. (2004) who found that some EcM fungal species occurrences were negatively correlated, suggesting that these negative interactions among species at small scales could affect community structure. Using the same C-score analysis, Pickles et al. (2012) found that EcM communities in roots of Scots pine in a forest plantation in Scotland were strongly structured by competitive interactions, or ecological processes generating a similar spatial pattern, rather than neutral processes. We only obtained significantly higher observed C-scores than expected in the soil dataset; however, describing the dynamics of soil fungal communities based on environmental DNA may be biased as it does not discriminate between metabolically active cells, dead biomass, or dormant structures such as spores, potentially masking any competition pattern by generating false positive fungal co-occurrences.

4.3. Biotic and abiotic drivers of EcM communities

The joint species distribution modelling revealed that the occurrences of EcM fungi in the Rhône glacier valley were shaped by a complex grid of biotic and abiotic drivers. Despite the number of environmental variables measured, the majority of the variability in EcM fungal community composition remained unexplained. The high soil heterogeneity, the effects of the abiotic factors across fine-scale microhabitats characteristic of alpine ecosystems and the dominance of generalist fungi might partly explain this. In microbial communities, habitat generalists respond mostly to spatial variables rather than to local environmental variability (Pandit et al., 2009; Luo et al., 2019). Thus, given the relatively small scale of the present study and the dominance of EcM fungi that are habitat generalists, the small amount of explained variability may still be indicative of the role of these biotic and abiotic factors in the EcM fungal community in this glacier valley.

We found a weak effect of host on EcM fungal communities associated with willows across the valley. However, when exploring the factors influencing the explained variability of the overall EcM fungal community distribution models ($R^2 = 0.06$), host identity emerged as the best predictor of EcM community composition. On average, plant host explained the larger fraction of variance on the occurrence of each EcM OTU in both soil and roots. However, the effect of host identity on EcM community structure might be masked by the different characteristics of the ecological niches in which these plants occur (Fig. 2). In contrast, when using relative abundance in the models instead of presence/absence (Fig. S1), site emerged as a better predictor of OTU relative abundance than host, indicating high spatial variation of EcM

communities. Using relative abundance data from HTS read counts, Collins et al. (2018) observed that fungal diversity and relative abundance had high spatial variation, overwhelming the predictive power of other abiotic factors. Moreover, Feinstein and Blackwood (2012) found high spatial variation in forest floor fungal communities and little explanatory power of plant traits or plant species identity. Even though using relative abundance data from communities is becoming more frequent than using presence-absence data, without reliable abundance data from HTS technologies, understanding the drivers of fungal community composition and function will remain limited (Friedman and Alm, 2012; Taylor et al., 2016).

Variation partitioning revealed that host and site effects were the main predictors of the explained variation in the overall EcM fungal community composition. However, the presence-absence of some OTUs in particular was mainly explained by soil chemistry and soil fungal community. Individual OTU responses to environmental factors help to better understand EcM fungal community resilience to environmental change, in particular, across the European Alps where complex geomorphology and an array of microclimates contribute to a wide variety of habitats and high levels of biodiversity. For instance, the occurrences of *Russula emetica* (OTU_029) and *Tomentella* sp. 11 (OTU_053) ectomycorrhizas were predominantly but differently explained by soil chemistry and soil fungal community (Fig. 6b), revealing that the relative importance of environmental and stochastic effects varies sharply among taxa.

5. Conclusions

Overall, our results indicate that alpine willow EcM communities are highly diverse and dominated by generalist fungi. These communities are non-modular, unnested and they are not competitively structured. Pairing two detection approaches for EcM fungi, we identified their strengths and weaknesses; caution is needed when analysing and extrapolating results from soil DNA that can miss dominant fungi in the roots. Hybrids did not associate with significantly different EcM communities than their parental species despite their ability to colonize different ecological niches. When accounting for the effects of environmental variables, we found a differential effect of host on EcM composition, that seems to be masked by the high soil heterogeneity in the valley. Further studies increasing sampling effort to additional valleys along a wider gradient of environmental variables, and including manipulative experiments, are now needed to disentangle the biotic and abiotic factors shaping EcM fungal communities in alpine zones.

Author contribution

R.A.C, M.I.B and L.M.S conceived the study and sampling design. R.A.C, S.G and L.M.S conducted the fieldwork. R.A.C, M.I.B and L.M.S processed and analysed samples. S.Z. performed the soil chemistry analysis. R.A.C and L.M.S analysed the data with support of B.F for NGS data and T.N. for OTU clustering pipeline. R.A.C drafted the manuscript, M.I.B. and L.M.S contributed extensively and provided chief contributions.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2020.100936.

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