RESEARCH ARTICLE

Phylogenetic diversity and structure of sebacinoid fungi associated with plant communities along an altitudinal gradient

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Abstract

To study the diversity and phylogenetic structure of Sebacinales communities from eight vegetation communities along an altitudinal gradient in the Bavarian Alps (Germany), we analysed 456 thalli or roots of plants. We detected 264 sebacinoid sequences, spanning the intergenic transcribed spacer region, 5.8S and D1/D2 regions of the nuclear rRNA gene, mostly using a nested PCR approach. Based on 97% sequence similarity, 73 Sebacinales molecular taxonomic units were found from 70 host species belonging to 44 plant families. Twenty-six molecular taxonomic units represented singletons, the most frequent of these being restricted exclusively to either wooded or grassland habitats. Although Sebacinales appear to occur in low abundance in plant roots, these microorganisms are phylogenetically diverse and widely spread in the ecosystems studied. Ordination analyses showed that land use, pH and humus content strongly influence the diversity and assembly of Sebacinales. In most cases, Sebacinales communities in ecosystems with extreme soil conditions or intensive land use exhibited significant phylogenetic clustering, whereas in undisturbed plant communities no trend was observed. These results suggest that ecosystem disturbance and environmental forces have an influence on the diversity and structure of Sebacinales community assembly over local and spatial scales.

Introduction

The vast majority of plants establish mycorrhizal associations, symbiotic interactions that are especially beneficial under stressful environmental conditions. Many factors and complex interactions influence the structure and composition of mycorrhizal communities. For example, distributional patterns in mycorrhizal communities may arise as the result of change during ecosystem succession (e.g. Jumpponen, 2003), soil factors (e.g. Nantel & Neumann, 1992; Toljander *et al.*, 2006), plant community structure (Molina *et al.*, 1992; Johnson *et al.*, 2005) or disturbance (Lekberg *et al.*, 2012). Colonization patterns and relative abundance of mycorrhizal fungi may (Haselwandter, 1979; Haselwandter & Read, 1980; Väre *et al.*, 1992) or may not (Bjorbaekmo *et al.*, 2010) decrease with increasing latitude and altitude, and the underlying causes for this variation are still not well understood.

Molecular phylogenetic analyses based on environmental samples have demonstrated that associations between Sebacinales (Basidiomycota: Agaricomycetes) and land plants are common and widespread. Weiß *et al.* (2004) distinguished two major groups within Sebacinales. Group A includes species that build fruiting bodies and occur in ectomycorrhizal association with trees (e.g. Selosse *et al.*, 2002a; Richard *et al.*, 2005), ericaceous plants from the subfamily Arbutoideae (Richard *et al.*, 2005), achlorophyllous (e.g. Selosse *et al.*, 2002b) and green orchids (e.g. Suárez *et al.*, 2008). Group B comprises species for which no macroscopically visible basidiomes are known and which are involved in mycorrhizal associations with Ericaceae (e.g. Setaro *et al.*, 2006; Selosse *et al.*, 2007), symbiotic interactions with liverworts (Bidartondo & Duckett, 2010; Newsham & Bridge, 2010) and herbaceous plants (Selosse *et al.*, 2009; Weiß *et al.*, 2011). Observations so far suggest that patterns of geographical distribution of this group are a

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result neither of co-evolutionary processes nor of host specificity. Therefore, analysing contemporary interactions among co-existing species may provide new meaningful insights into the processes of modelling diversity and the natural composition of species assemblages.

The diverse mycorrhizal abilities and ubiquity of the sebacinalean fungi provide a great opportunity to examine how Sebacinales communities are structured in terrestrial ecosystems. In the present study, we chose an altitudinal transect in the Bavarian Alps (Germany) including montane and subalpine ecosystems for the following reasons: the high topographic and soil heterogeneity, resulting in a mosaic of different vegetation types over short distances; an altitudinal gradient; the presence of relatively undisturbed plant communities as well as the presence of plant communities under various degrees of disturbance. We then used these characteristics to address the following specific questions:

(1) What is the diversity of Sebacinales associated with plants in these communities?

(2) What patterns in phylogenetic structure characterize the Sebacinales communities in the studied plant communities?

(3) Do edaphic factors, altitude and/or plant communities affect the phylogenetic structure of the Sebacinales communities?

We addressed the hypothesis that the sebacinoid communities would differ across montane and subalpine ecosystems, which could be correlated with changes in some abiotic and biotic environmental variables such as altitude and vegetation structure.

Material and methods

Study area

The study area is located near Oberjoch village (47°31′ N, 10°24′ E) in the Northern Bavarian Alps, Germany. Because of its topography, the area contains a range of montane to subalpine habitats that include boulder fields, slopes, peat bogs, dry and wet meadows, scrubland and forests. All soils are derived from dolomite, a sedimentary carbonate rock, and another mineral, both of which are composed of calcium magnesium carbonate [$CaMg(CO_3)_2$; Freudenberger & Schwerd, 1996]. These soils are grouped under the rendzic leptosol type according to the Food and Agriculture Organization classification (Buol *et al.*, 1997). The annual average air temperature is 5.9 °C at the altitude of Oberjoch and the total annual rainfall is *c.* 1800 mm (1961–1990), falling mostly in the warm season (*c.* May to September). The warmest month (July) has a mean temperature of 14.4 °C and the coldest (January) a mean temperature of −1.8 °C. The study area is covered by snow from October until around mid-April, and flowering starts in late May.

Vegetation communities and sampling strategy

To cover the plant compositional variation in each vegetation type as described by Schubert *et al.* (2001), we surveyed plots in the core areas of each vegetation type of the study sites. Plant specimens were sampled from 15 plots representing eight different plant communities distributed along an altitudinal gradient ranging from 1020 to 1830 m above sea level (a.s.l.) during the autumn of 2009: ravine forest (RF) is located at 1020 m a.s.l.; grazing meadow (GM), hay meadow (HM), peat bog (PB), spruce forest (SF) and wet meadow (WM) are distributed at elevations between 1100 m and 1500 m a.s.l.; and Alpine rose rock community (AR) and Krummholz formation (KF) are distributed at elevations above 1600 m up to 1830 m a.s.l. Distances between the collection sites ranged from 50 m to 2.1 km. We sampled two plots for each vegetation type, except for SF, where only one plot was sampled. In general, plots within the same vegetation community were separated by no more than 20 m, but plots from GM sites were separated by 300 m. As this study is part of an ongoing project, a further 10 replicate plots with identical vegetation, geological formation and soil type to plot SF, are being analysed. The typical vegetation found in each sampling site is summarized in Supporting Information, Data S1.

Aiming at including representative species from the vegetation communities in SF and RF, which are dominated by trees, we used sampling plots of 10 × 10 m; for the remaining plant communities comprising mainly herbaceous plants, the sampling plots were 2 × 2 m. We sampled four to 20 plant individuals proportional to the relative species abundance within each vegetation type. To broaden the sampling of species for each plant community, we also collected plant specimens (assigned as 'extra') occurring near the collection sites that were not sampled within the corresponding site. Herbaceous plants (including roots and above-ground parts) were dug carefully from the soil and stored separately with a portion of soil; trees were sampled by following the lateral roots from the stem base and collecting fine roots.

Processing of root samples and soil parameter analyses

After collection, plant roots were immediately separated from adhering soil by washing carefully with tap water, and then cleaned several times with sterilized water. For each species, healthy thalli or root fragments were fixed in 2% glutaraldehyde in a 0.1 M sodium cacodylate

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buffer (pH 7.2) at room temperature for transmission electron microscope (TEM) scanning. Plant specimens were pressed flat and dried between sheets of blotting paper as soon as possible after collection using a plant press at 38 °C with air circulation.

For each sampling site, three randomly selected soil samples from the upper 5–15 cm below the surface were taken with a shovel. Soil samples were taken from near the plant roots, pooled into a composite sample per plot and analysed for pH, total C and N contents, heavy metal contents, and nutrients. A portion of fresh soil from each sample was used to measure pH using a pH-electrode SenTix 61 (WTW GmbH, Weilheim, Germany) with a methodology modified from Schlichting *et al.* (1995). Humus content was calculated as described by Ad-hoc-Arbeitsgruppe Boden (1994).

DNA extraction, PCR, cloning, sequencing and sequence editing

The root systems and thalli from dried plants were examined under a dissecting microscope. Three to five fine roots of *c.* 0.5 mm from herbaceous and ericaceous plants per sample were removed with tweezers from different parts of each root system, placed in a 1.5-mL sterile reaction tube and ground with a sterile plastic pestle in liquid nitrogen several times. For ectomycorrhizal plants, three to five root tips per morphotype and sample were pooled together and used for the molecular analysis. As there is little information on morphotypes of sebacinalean ectomycorrhizas in the plant species analysed in this study, we selected morphotypes from pale-coloured to white ectomycorrhizal systems (Tedersoo *et al.*, 2003). For mosses, small portions of thalli with rhizoids were subjected to DNA extraction. In total, we extracted genomic DNA from 456 thallus or root samples (Table S1) using the standard protocols provided with a DNAeasy Plant Mini Kit (Qiagen, Hilden, Germany) or an InnuPREP Plant DNA Kit (Analytik Jena AG, Jena, Germany).

To amplify the 3′ region of the 18S region, ITS1 and ITS2, the 5.8S ribosomal subunit and the D1/D2 regions of the nucLSU of Sebacinales, we used sets of specific and universal fungal primers (Table S2). In direct PCR, by using the NSSeb1 and NL2R primers, it was possible to amplify a ~2200–2500-bp DNA fragment (Fig. S1). The 25 μL PCR reaction included 2.5 μL of total genomic DNA extract, 10.0 μL 5× Phusion GC buffer (Finnzymes Oy, Keilaranta, Finland), 1.0 μL of each primer (25 pmol μL^{-1}), 2.0 μL of dNTP-Mix (Invitrogen, Carlsbad, CA; 5 mM) and 0.5 μL Phusion High-Fidelity DNA polymerase (0.02 U μL^{-1}) (Finnzymes Oy). Subsequently, weak or visually negative amplicons were used as a template for a nested PCR (first nested PCR) using the Sebacinales-specific NSSeb2 and NLSeb1.5R primers. Thermocycling consisted of initial heating at 95 °C for 60 s, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for the first PCR and at 60 °C for the first nested PCR for 30 s, and extension at 72 °C for 45–90 s, followed by a final extension at 72 °C for 10 min. Weak or visually negative amplifications of the first nested PCR were used as templates for a second nested PCR with Taq DNA polymerase (Invitrogen), HotStar HiFidelity polymerase (Qiagen) or Mango-Taq DNA polymerase (Bioline, London, UK) (for details see Table S1). Reactions for the second nested PCR contained 10 μL CG buffer, 0.5 μL (2 Um μL^{-1}) Mango-Taq, 1.5 μL $MgCl_2$ (50 mM), 2.0 μL dNTP mix (5 mM), 25 pmol of each primer μL^{-1} and 5 μL DNA extract. All of the second nested round PCRs were carried out with the universal fungal primers ITS1F and NL4. For Taq and Mango-Taq polymerase PCRs, the initial DNA denaturation and enzyme reaction steps were performed at 94 °C for 3 min; 10 cycles with temperatures ranging from 60 °C in the first cycle to 51 °C, with each cycle decreasing by 1 °C; followed by 25 cycles with an annealing temperature of 50 °C, with each cycle consisting of an annealing step of 0.5 min; an elongation step of 72 °C for 1 min; a denaturation step of 94 °C for 0.5 min and a final extension phase at 72 °C for 7 min. The HotStar HiFidelity polymerase PCR mix included 5 μL 5× PCR buffer (containing 15 mM $MgCl_2$ and 1.5 mM dNTPs), 10 μL Q 5× solution, 2 μL (25 pmol μL^{-1}) of each primer, 0.5 μL Hotstar HiFi Taq and 2.5 μL template. The PCR profile included initial activation of the enzyme at 95 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 15 s, a denaturation step primer annealing at 56 °C for 1 min, and a final extension step at 72 °C for 10 min. Good results were achieved using 1 μL from a 1 : 10 dilution of the second PCR reaction. As nested PCR is very sensitive to contamination, we repeated experiments for a subset of samples to show the same pattern of negative and positive PCR results. Negative controls containing all reagents except the DNA template, were used in all PCR arrays. The presence and yield of PCR products were monitored by agarose gel electrophoresis followed by visualization with ethidium bromide staining and UV illumination. The amplified PCR products were cleaned using an ExoSAP-IT reagent (USB Corporation, Cleveland, OH) diluted 1 : 20 according to the manufacturer's instructions. Both DNA strands were cycle-sequenced with the primers ITS1F, ITS4, LROR and NL4 (or, in some cases, using the additional primers ITS1, ITS5, ITS2, 5.8SR, ITS3seb or LR3; Table S2) using a 1 : 6 diluted dye terminator sequencing kit (Big Dye 3.1; Applied Biosystems, Foster City, CA) on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). PCR products that could not be sequenced directly were cloned into One Shot® TOP10 chemically competent *Escherichia coli*, using the TOPO TA Cloning Kit (Invitrogen) and the pCR®4-TOPO vector

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(Invitrogen). From each cloning sample, small portions of 16 selected colonies were used directly as a template for a PCR with Taq DNA polymerase (Invitrogen) and the M13 forward and reverse primers. Clones containing an insert were sequenced using the ITS4, LROR and M13 primers. Forward and reverse sequence fragments were assembled and edited using SEQUENCHER (Gene Codes Corporation, Ann Arbor, MI). Plant vouchers that yielded Sebacinales sequences were deposited in the Herbarium Tubingense (TUB).

Sequence identity, chimera checking, alignments and phylogenetic analysis

To detect contaminant non-Sebacinales sequences, a multiple sequence alignment was generated in MAFFT version 5.7 using the E-INS-i algorithm (Katoh *et al.*, 2005). Inserts and all nucleotide segments that were highly divergent and/or difficult to align were submitted to BLAST searches (Altschul *et al.*, 1997) against the GenBank nucleotide database (www.ncbi.nlm.nih.gov). A further search for detecting chimeric sequences was done with UCLUST version 3.0 (Edgar, 2010) using the UCHIME algorithm (www.drive5.com). After exclusion of potential chimerae, sequences were aligned using MAFFT, as above, and POA version 2 (Lee *et al.*, 2002). The most consistent alignment was selected using TRIMAL version 1.2 (Capella-Gutiérrez *et al.*, 2009). Subsequently, ambiguously aligned positions from our POA multiple sequence alignment were eliminated using TRIMAL with the parameter option *–automated1*. The data matrix (TreeBASE: S11238), including 264 Sebacinales sequences and 1269 nucleotide positions, was analysed by maximum likelihood (ML) as implemented in RAXML version 7.0.3 (Stamatakis, 2006). A combined rapid bootstrapping and ML search under the GTRCAT model was computed from 1000 runs (Stamatakis *et al.*, 2008; final optimization of branch lengths using the GTRGAMMA model). To test the robustness of the phylogenetic tree, we also performed a Bayesian Markov Chain Monte Carlo (MCMC) analysis as implemented in MRBAYES (Ronquist & Huelsenbeck, 2003), using four Mio generations, the GTRGAMMA model of DNA substitution with all model parameters sampled during MCMC, four incrementally heated Markov chains, two replicate analyses, and random starting trees. Every 100th generated tree was stored, resulting in a total of 40 000 trees in each replicate analysis. The first 5000 trees sampled in each run were deleted and the remaining trees were pooled to compute a majority-rule consensus to estimate branch support. No outgroup sequences were included in the phylogenetic analyses because the ITS region shows too great a divergence between Sebacinales and taxa outside this group. The phylogenetic trees were midpoint-rooted and were displayed using FIGTREE version 1.3.1 (Rambaut, 2009). These trees derived with maximum likelihood were used for phylogenetic community structure analyses. The sequences obtained in this study are available from GenBank under accession nos. HQ180269–HQ180351 and JQ420939–JQ421001.

Community analyses

From our alignment, an uncorrected pairwise distance (*p*-distance) matrix was computed in PAUP* version 4.0b10 (Swofford, 2002). Sebacinales sequences with at least 97% similarity were defined as one molecular operational taxonomic unit (MOTU) using the program OPTSIL (Göker *et al.*, 2010; Setaro & Kron, 2011). The MOTU richness of the Sebacinales community was estimated using ESTIMATES version 8.2 (Colwell, 2009) by calculating mean sample accumulation curves per plot. The sample order was randomized 100 times. We used a sampling without replacement protocol to estimate local richness estimators and diversity.

Furthermore, Sebacinales community structures (phylogenetic clustering vs. overdispersion) were analysed using PHYLOCOM version 3.41 (Webb *et al.*, 2008, 2009). We measured Faith's index of phylogenetic diversity (PD; Faith, 1992; Faith & Baker, 2006), the Net Relatedness Index (NRI) and the Nearest Taxon Index (NTI) (Webb *et al.*, 2002) by pooling samples from plots of the same plant community. As plots from the grazing meadows (GM) were spatially separated from each other, these were analysed when pooled as well as separately. NRI and NTI indices indicate the degree to which MOTUs in a community are phylogenetically clustered or overdispersed by their deviation from zero, the expectation of random assembly. Thus, NTI and NRI values between -2 and $+2$ indicate random, values > 2 clustered, and values < -2 overdispersed phylogenetic communities. Basically, NRI provides a measure of phylogenetic 'clumpedness' of taxa over the whole phylogeny, including nodes in the backbone of the phylogeny, whereas NTI indicates whether taxa within particular terminal clades are clustered, irrespective of the relationship among those clades (Webb, 2000). We considered Sebacinales communities to be significantly clustered if both NRI and NTI were > 2 and both *P*-values were < 0.05 (see Gotelli & Rohde, 2002). First, the global phylogenetic distribution was tested using null model 0. This null model shuffles species labels across the entire phylogeny (Webb *et al.*, 2008). Secondly, more complex null models as implemented in PHYLOCOM's *comstruct* module were used, and the results did not differ from the simplest model. The significance of these statistics was determined from 9999 randomly assembled communities. To examine for potential bias created by differences in the number of sampled sequences in the estimation of NRI and NTI, we per-

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formed Mantel tests in CADM (Legendre & Lapointe, 2004). In addition, to characterize the spatial variation of Sebacinales communities, we calculated MOTU richness (S), evenness (E), Simpson's (D) and Shannon's (H) diversity indices.

Finally, canonical correspondence analysis (CCA) as implemented in the program CANOCO version 4.53 (ter Braak & Smilauer, 2002) was used to assess potential correlations between some environmental factors in the Sebacinales communities. CCA represents a direct gradient analysis where the community data matrix is constrained by multiple linear regression on a second matrix containing environmental variables. MOTU abundance was coded as the frequency of occurrence in the thallus or root samples. The environmental variables tested were gradient of altitude, soil moisture, C : N, pH and humus content. Furthermore, the influence of land use (e.g. pasture and tourism) and ectomycorrhizal (ECM) forest cover on the Sebacinales community was examined. The influence of singleton MOTUs was minimized using the 'downweighting of rare species' option. The statistical significance between community data and habitat parameters was analysed with a Monte Carlo test, using 9999 unrestricted permutations.

Transmission electron microscopy analyses

A total of 47 selected samples yielding sebacinoid sequences were used for TEM investigations, following the methodology described by Bauer *et al.* (2006). TEM analyses were used to provide morphological evidence for the presence of Sebacinales.

Results

Phylogenetic diversity of Sebacinales associated with plant roots

A total of 456 samples from 51 plant families from eight vegetation communities were investigated, of which 212 samples (46.5%) from 44 plant families produced 264 non-chimeric complete ITS + 5.8 + D1/D2 sebacinoid sequences (Table S1). For some Sebacinales belonging to Group B, PCR products using specific primer sets were characterized by the presence of a ~350-bp intron at the end of the 18S region (Fig. S1). We found molecular evidence for multiple root colonization by Sebacinales in trees (*Abies alba*, *Acer platanoides*, *Acer pseudoplatanus*, *Betula pubescens* and *Pinus mugo*), in shrubs (*Daphne striata* and *Vaccinium myrtillus*) and in perennial herbs (*Astrantia major*, *Bistorta vivipara*, *Campanula scheuchzeri*, *Globularia nudicaulis*, *Lamium* cf. *montanum*, *Pinguicula alpina*, *Poaceae* sp., *Polygala* cf. *alpestris*, *Trifolium badium*, *Trifolium pratense*, *Trifolium repens*, *Soldanella alpina*, *Urtica dioica* and *Viola reichenbachiana*). In these cases, PCRs produced single, weak products that were not directly sequenceable. Forty-two sequences from cloned samples and 11 from directly sequenceable samples were classified as chimeric and omitted from further analyses. Almost all of the junctions between sequence fragments that formed chimerae were located in the ITS1 region, but one occurred at the end of the D2 region. In a few cases, chimeric sequence fragments could be identified as the DNA of the host plant. Sixteen non-sebacinoid fungal sequences were detected, belonging to the genera *Calyptella* (*Trifolium* sp.), *Clavulina* (*Veratrum album*), *Cortinarius* (fern, *Picea abies*), *Cyphellostereum* (*Trifolium repens*), *Mycena* (*Blechnum spicant, Oxalis acetosella*, *Trifolium* sp.), *Lactarius* (*Abies alba*), *Piloderma* (*Pinus sylvestris*) and *Russula* (*Betula pubescens*). Most of them contained a single, weak PCR signal of similar lengths as the PCR products from sebacinoid fungi.

In this study, most bands obtained in the first PCR and first nested PCR proved too weak to be sequenced. Therefore, most assays used a two-step nested PCR to increase the amount of template DNA and reduce amplification inhibition. Successfully amplified sequences were obtained from the first PCR (three sequences; 1%), from the first nested PCR (65 sequences; 25%) and from the second nested PCR (196 sequences; 74%; Table S1). The 264 sequences were grouped into 73 MOTUs using a 97% similarity cut-off level. A high proportion of the MOTUs (~39%) represented singletons. The most abundant MOTUs in grassland habitats were 25 and 54, whereas MOTUs 61, 65 and 71 were the most common in wooded ecosystems.

The MOTU accumulation curve did not reach an asymptote (data not shown), indicating that the sampling effort was not sufficient to cover the complete biodiversity of thallus- or root-associated Sebacinales in the study area. Maximum likelihood and Bayesian analyses yielded consistent results. Groups receiving high ML bootstrap support were also supported in Bayesian analysis, and vice versa (Fig. 1), with minor exceptions which proved not significant for the community analyses. The phylogenetic analyses revealed the diversity of sebacinoid MOTUs derived from the plant roots or thalli, which clustered into Sebacinales subgroups A and B. The phylogenetic diversity of Group B Sebacinales tended to be greater than that observed in Group A Sebacinales. A great proportion of the terminal groups and deeper nodes received moderate to high branch support (Fig. 1).

Patterns of Sebacinales community structure

Differences in the phylogenetic structure of Sebacinales communities were observed among vegetation units.

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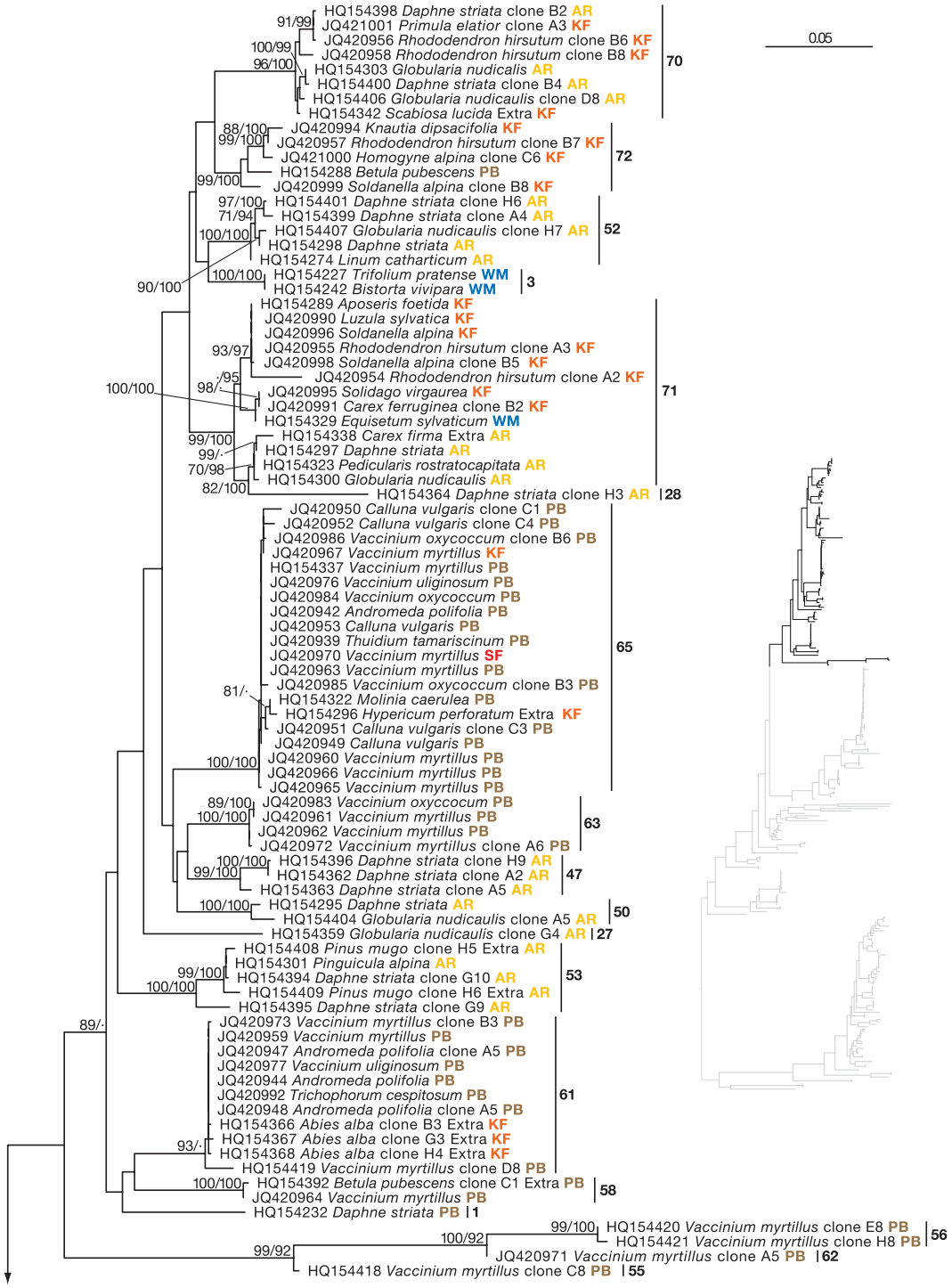

Fig. 1. Phylogenetic diversity and relationships of Sebacinales communities associated with plant species reconstructed by the maximum likelihood method for 264 ITS1 + 5.8S + ITS2 + D1/D2 rDNA sequences (alignment 1269 bp length). The midpoint-rooted phylogram was obtained from a RAxML analysis under the GTRCAT model of DNA evolution. Numbers are maximum likelihood bootstrap values based on 1000 replicates (values $\geq$ 70% are shown)/Bayesian estimates of posterior probabilities (values $\geq$ 90% are shown) inferred with MRBAYES. Bold numbers designate MOTUs inferred using OPTSIL. Acronyms for collection sites (printed in bold): AR, Alpine rose rock community; GM, grazing meadow; HM, hay meadow; KF, Krummholz formation; PB, peat bog; RF, ravine forest; SF, spruce forest; WM, wet meadow. See Data S1 for a description of plant communities. Clone = multiple Sebacinales colonization within a single root sample. Numbers indicate the MOTUs. Sebacinoid sequences are designated by the name of the corresponding host plant.

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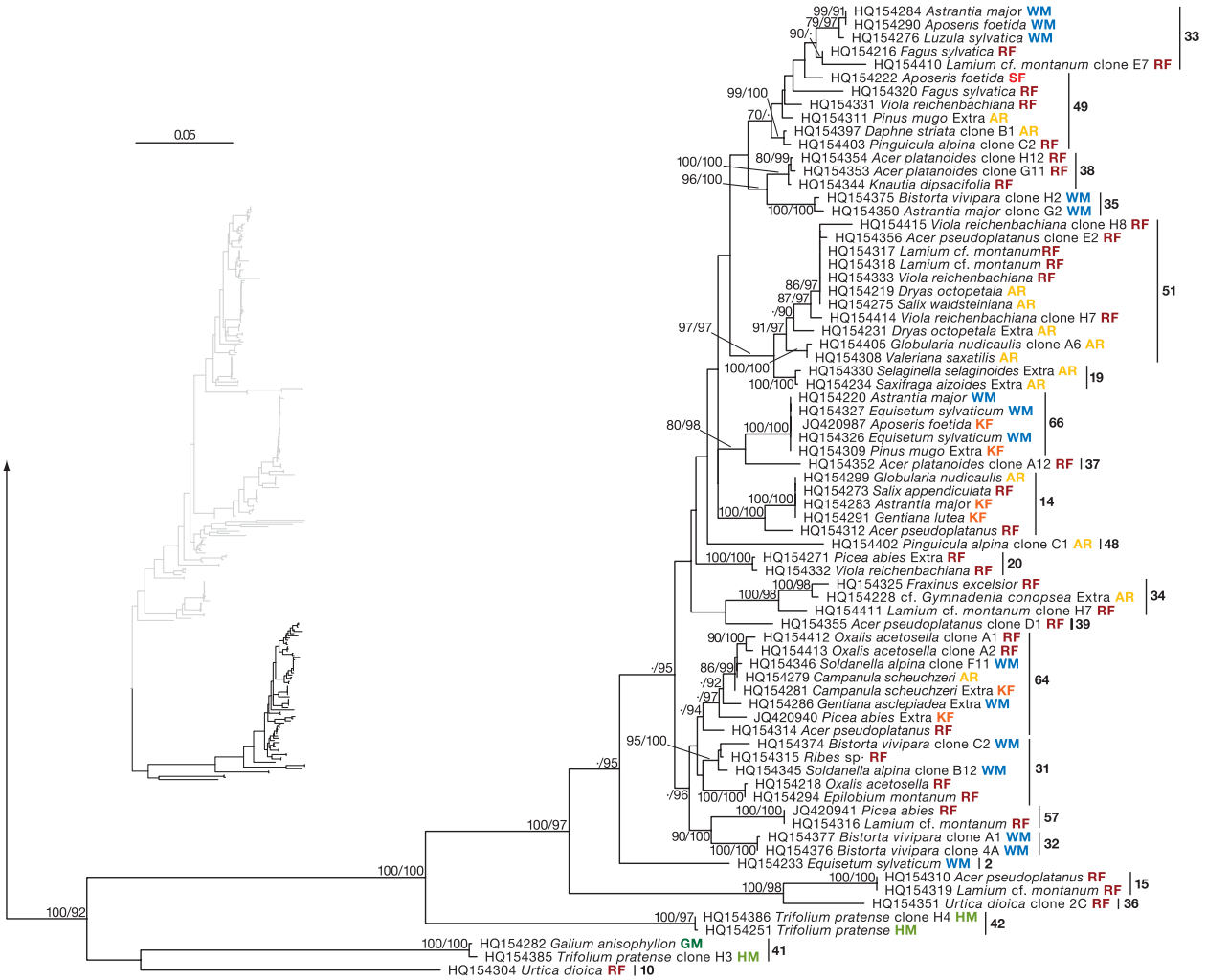

Fig. 1. (Continued)

Sebacinales communities from grazing meadow (GM), hay meadow (HM), Krummholz formation (KF) and peat bog (PB) were phylogenetically clustered to a significant degree (Table 1). Similarly, when plots of GM were analysed separately, they showed similarly significant clustering (data not shown). For Sebacinales associated with Alpine rose rock community (AR) and wet meadow (WM) there was no clear trend in the phylogenetic structure. Phylogenetic patterns in sebacinoid communities from spruce (SF) and ravine (RF) forests were not significantly different from random expectations. Significant phylogenetic clustering was observed when all grassland sites (GM, HM and WM) were pooled together. When the tree-dominated sites were pooled together with the Ericaceae-dominated sites (AR, KF, PB, RF, SF), Sebacinales communities had random phylogenetic structure. We verified the robustness of the phylogenetic community analyses by repeating the PHYLOCOM analyses using the consensus tree inferred from Bayesian MCMC analysis instead of the maximum likelihood tree, which yielded the same conclusions.

Host plant and/or environmental effects on the Sebacinales community structure

Molecular operational taxonomic units 41 and 42 were associated with herbaceous plants (e.g. *Galium anisophyllon* and *Trifolium pratense*) collected from meadows completely lacking trees (HM, GM). These sebacinoids clustered within Sebacinales Group A, which contains species forming ectomycorrhizae on tree roots, as did several MOTUs associated with herbaceous plants collected from meadows at least 20 m from the forest edge (*Bistorta vivipara* in WM), immediately beside the forest

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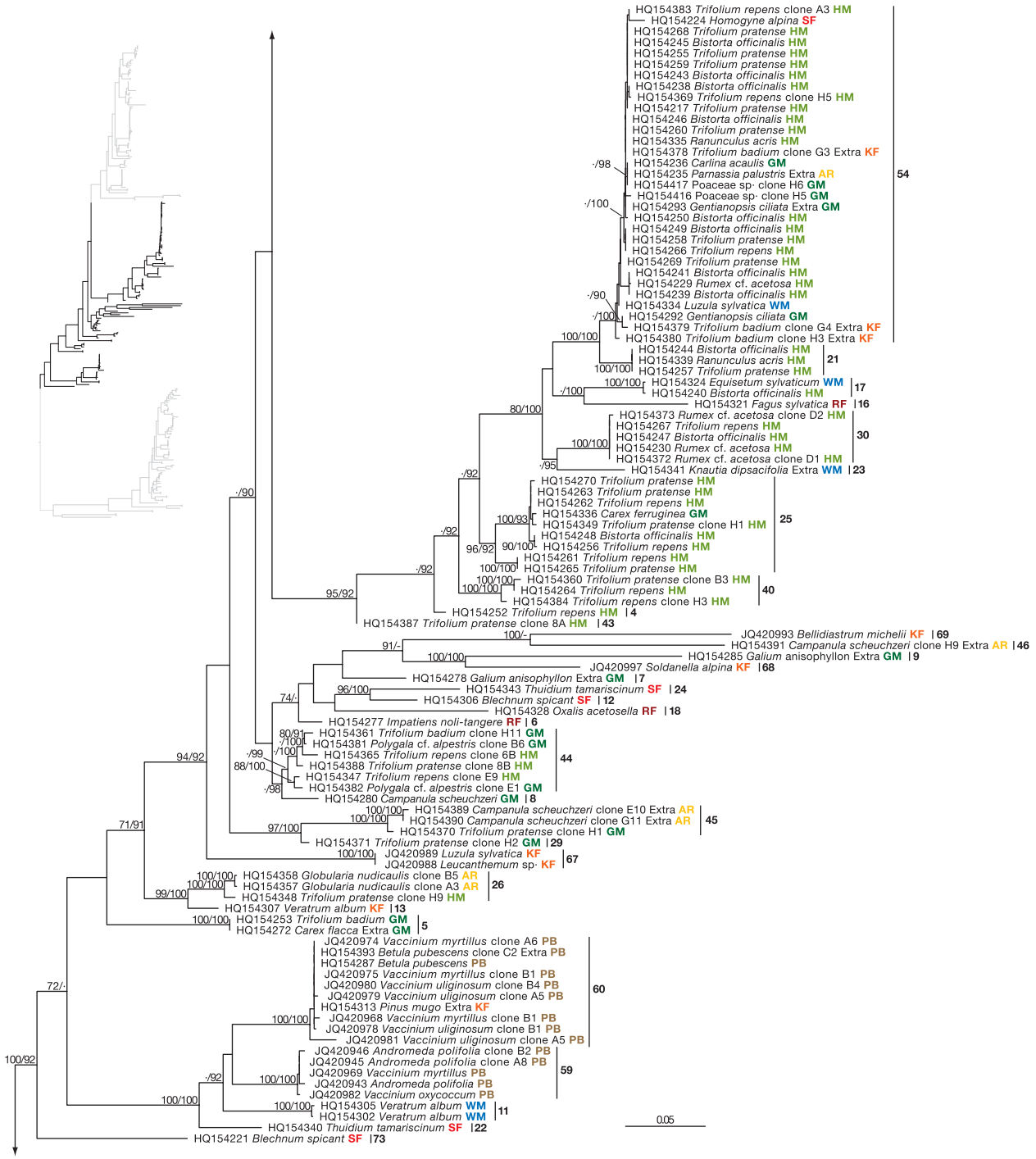

Fig. 1. (Continued).

edges (e.g. *Soldanella alpina* and *Gentiana asclepiadea* in WM) or co-occurring with trees (e.g. *Astrantia major*, *Gentiana lutea*, *Lamium* cf. *montanum*, *Oxalis acetosella* and *Viola reichenbachiana* in AR, KF, RF). However, some MOTUs containing ectomycorrhizal sequences clustered within Group B (*Abies alba*, *Betula pubescens*, *Fagus sylvatica* and *Pinus mugo* from KF and PB; Fig. 1).

In many cases, we found MOTUs with completely identical sequences from hosts belonging to different plant families at the same site, e.g. *Picea abies* and *Viola reichenbachiana* in RF (MOTU 20); *Astrantia major* and *Gentiana lutea* in KF

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Table 1. Phylogenetic diversity and structure of Sebacinales communities associated with plants along an altitudinal gradient in the Northern Bavarian Alps

Plant community	N[†]	S[‡]	E[§]	H[¶]	D[††]	PD[‡‡]	NRI[§§]	NTI[¶¶]	Pattern
Hay meadow (HM)	49	12	0.78	1.94	0.80	0.19	10.80*	10.80*	Clustered
Grazing meadow (GM)	17	10	0.91	2.08	0.90	0.14	2.45*	2.45*	Clustered
Wet meadow (WM)	23	13	0.97	2.49	0.95	0.28	–2.33	–2.33*	No trend
Overall	89	28	0.83	2.76	0.89	0.49	5.04*	2.78*	Clustered
Alpine rose rock (AR)	46	20	0.93	2.80	0.95	0.38	1.23	1.49	Random
Krummholz (KF)	36	14	0.92	2.44	0.92	0.24	3.26*	3.26*	Clustered
Peat bog (PB)	50	10	0.82	1.89	0.82	0.12	7.47*	7.47*	Clustered
Ravine forest (RF)	36	18	0.95	2.74	0.95	0.39	–3.84	–3.84*	No trend
Spruce forest (SF)	07	07	1.00	1.95	1.00	0.15	–0.57	–0.57	Random
Overall	175	52	0.89	3.52	0.96	0.75	0.24	1.16	Random

[†]Number of MOTUs.
[‡]MOTUs richness.
[§]Evenness.
[¶]Shannon's diversity index.
[††]Simpson's diversity index.
[‡‡]Faith's index of phylogenetic diversity.
[§§]Net Relatedness Index, $*P < 0.05$.
[¶¶]Nearest Taxon Index, $*P < 0.05$.
NRI and NTI are indicators of phylogenetic clustering (values > 2) or phylogenetic overdispersal (values < −2) inferred under PHYLOCOM null model 0. S, E and H values were calculated using CANOCO, and D after Simpson (1949).

(MOTU 14); *Astrantia major* and *Equisetum sylvaticum* in WM (MOTU 66); *Bistorta vivipara*, *Ranunculus acris* and *Trifolium pratense* in HM (MOTU 21); *Bistorta vivipara* and *Trifolium pratense* in WM (MOTU 3); *Carex flacca* and *Trifolium badium* in GM (MOTU 5); or from different plots or sites, e.g. *Pinus mugo* in KF and *Betula pubescens* in PB (MOTU 60); *Astrantia major* and *Equisetum sylvaticum* in WM, and *Pinus mugo* in KF (MOTU 66); *Globularia nudicaulis* in AR, *Salix appendiculata* in RF, and *Astrantia major* and *Gentiana lutea* in KF (MOTU 14); *Trifolium badium* in KF, *Gentianopsis ciliata* in GM and *Trifolium pratense* in HM (MOTU 54). The widely sampled fabid and knotweed families were associated with particularly diverse MOTUs of Sebacinales (Fig. 1).

Soil analyses revealed some differences across the plant communities, PB showing a low pH value, and HM, WM and GM tending to have lower C and N contents (Table S3). In the CCA (Fig. 2), the communities of Sebacinales were distributed into two distinctive groups: a 'meadow group' containing MOTUs associated with herbaceous plants and an 'acidophilic group' occurring on sites with low pH and high humus content. The remaining MOTUs (below first axis) are restricted to sites dominated by ectomycorrhizal trees. The total variance in the taxa data was 4.340 and the eigenvalues of the first and second axes were 0.913 and 0.875, respectively. The first axis accounted for 20.8% of the explained variance and the second axis for 40.7%. The Monte Carlo test was significant for all canonical axes ($F = 1.124$, $P = 0.0292$). Land use/presence of trees

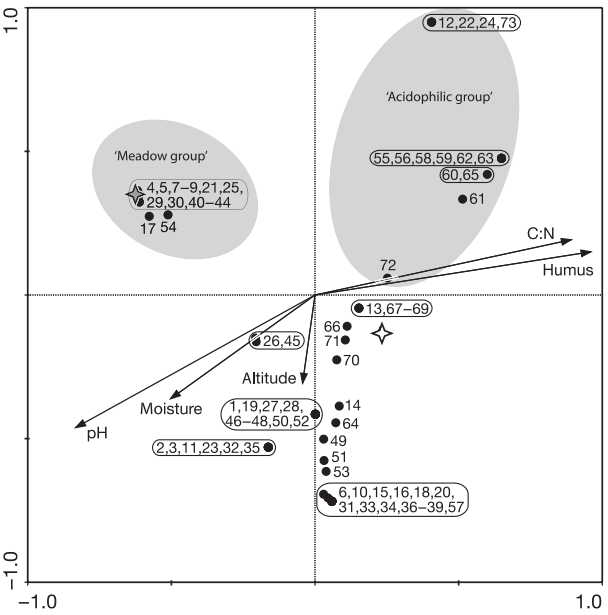

Fig. 2. Biplot of canonical correspondence analysis (CCA) of Sebacinales communities along an altitudinal gradient in the Northern Bavarian Alps. Points represent Sebacinales MOTUs. Because sampling in site K was underrepresented, weight 0.10 was given to these samples. Vectors indicate quantitative parameters. Closed star represents high disturbance and non-EMC trees. Open star indicates ECM forest cover and low land use. Mapping of the ecological parameter categories are as follows: land use, low and high disturbance; soil moisture (plant genus indicator) after Ellenberg *et al.* (1992); absence (grassland, lacking ectomycorrhizal trees within a radius of 50 m) or presence of ECM forest cover.

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($F = 1.55$, $P = 0.0002$), humus content ($F = 1.55$, $P = 0.0002$) and pH ($F = 1.59$, $P = 0.0466$) were the only significant parameters.

Of the 47 root samples analysed by TEM, Sebacinales could be detected in three samples (6%). Hyphae with sebacinoid dolipores were found in roots of *Dryas octopetala* (TUB 019323), *Salix waldsteiniana* (TUB 019367) and *Potentilla anserina* (TUB 019329) (Fig. 3). Analysis of *S. waldsteiniana* roots revealed typical ectomycorrhizae with hyphal sheaths and Hartig nets (Fig. 3a). Fungal hyphae with typical sebacinoid dolipores were observed adjacent to the root surface of *D. octopetala* (Fig. 3b), in the Hartig net in *S. waldsteiniana* (Fig. 3c) and in the external cortical cells of *P. anserina* (Fig. 3d). Hyphae were 2.0–4.7 μm in diameter and had dolipores with imperforate parenthesomes.

Discussion

Phylogenetic diversity and host specificity of Sebacinales

Although sebacinoid fungi were detected in at least 60% of the plants analysed (including samples yielding chimeric, incomplete and complete sequences), their laborious detection – mainly using a two-step nested PCR – suggests that these microorganisms occur in low abundance in the montane and subalpine ecosystems studied. These results agree with previous studies by Setaro *et al.* (2006), Setaro & Kron (2011) and Weiß *et al.* (2011), who also used a nested PCR approach for the detection of Sebacinales in plant roots. In our study, this observation is reinforced by ultrastructural evidence, where sebacinoid hyphae forming typical ectomycorrhizae or endophytic interactions were detected via TEM in a relatively low number of root samples and fine roots of herbaceous plants. We cannot, however, rule out that the TEM methodology used allowed us to examine only very tiny portions of the root systems, and this could have hampered the detection of sebacinoid fungi. There was no decrease in the diversity of MOTUs with increasing altitude, which contrasts with some mycorrhizal colonization patterns observed in other plant groups (Haselwandter, 1979; Haselwandter & Read, 1980). Long-term fruit-body based surveys (SG and FO) indicate that *Sebacina epigaea* and *Sebacina incrustans* are common ectomycorrhizal elements associated with spruce; however, no sebacinoid fruiting body structures were recorded in the sampled sites. This might indicate that sebacinoids only rarely – or perhaps never – develop fruiting bodies, or, alternatively, that the inconspicuous sebacinoid fruiting structures are overlooked in fruit body-based surveys. These observations point to the importance of studies based on root samples in alpine ecosystems as suggested by Ryberg *et al.* (2009).

Our study suggests the possibility of the host plants being linked in a common mycelial network within the same site (Fig. 1). Mycelial networks have been suggested by Ryberg *et al.* (2009), who found that dominating ecto-

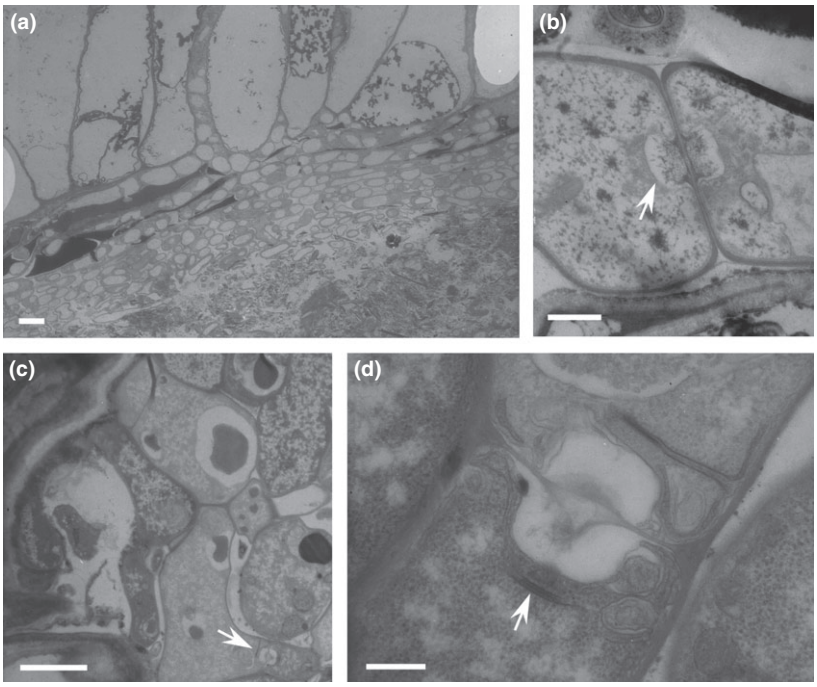

Fig. 3. Transmission electron micrographs showing the presence of Sebacinales associated with plant roots. (a) Sebacinoid ectomycorrhiza in a root of *Salix waldsteiniana* (TUB 019367). (b) Sebacinoid hypha adjacent to the root surface of *Dryas octopetala* (TUB 019323). The dolipore is covered with imperforate parenthesomes (arrow). (c) Overview of hyphae in cortical cells of *Potentilla anserina* (TUB 019329). The arrow indicates a hyphal septum. (d) Magnification of the hyphal septum illustrated in (c); sebacinoid doliporus with imperforate parenthesomes (arrow). Scale bars: (a,c) 10 μm, (b) 0.5 μm, (d) 1 μm.

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mycorrhizal fungi – including members of the Sebacinales – were shared by *D. octopetala* and *Salix reticulata* in an alpine cliff ecosystem in Northern Sweden. The observation that sebacinoids with identical sequences occurred in both ECM of trees and fine roots of herbs within the same site may support the hypothesis that the mode of cellular interaction between the Sebacinales and their host plants is host-dependent. However, *in vitro* experiments are needed to clarify the ability of individual sebacinoids to form both mycorrhizal and endophytic associations, as is the case in other fungal groups (e.g. Taylor & Bruns, 1997; Vincenot *et al.*, 2008).

We agree with Mühlmann *et al.* (2008), who found Sebacinales to be one of the ectomycorrhizal elements frequently associated with the roots of *Bistorta vivipara*. We investigated *B. vivipara* plants collected from a meadow devoid of ectomycorrhizal trees. All the sebacinoids associated with *B. vivipara* clustered in Group A, suggesting that these plants maintained their ectomycorrhizal mycobionts. In contrast, Väre *et al.* (1992) postulated that *B. vivipara* forms ectomycorrhizae only when it co-occurs with other ectomycorrhizal host plants.

Effect of plant community and/or environmental factors on Sebacinales community structure

One of the primary factors shaping fungal niche diversity is plant community composition (Molina *et al.*, 1992; Johnson *et al.*, 2005), especially because mycorrhizal species often exhibit host specificity (Bruns *et al.*, 2002). In addition, edaphic factors were shown to play a crucial role in the distribution patterns of mycorrhizal fungi (Nantel & Neumann, 1992; DeBellis *et al.*, 2006). We observed significant phylogenetic clustering of sebacinoid communities in peat bog, hay and grazing meadows and Krummholz formation, which indicates that MOTUs in these communities were more closely related than would be expected by chance (Table 1). Peat bogs include vegetation that is highly specialized to very acidic and low-nutrient soils; hay meadows are characterized by soils with high water content and are regularly affected by human activities (e.g. fertilization and mowing), and grazing meadows on the slopes are relatively dry in the summer season and have been used as ski slopes for more than three decades. Interestingly, although grassland sites differ substantially in their plant communities, Sebacinales communities in these areas exhibited significant phylogenetic clustering. Several factors are likely to be responsible for this: frequent disturbances, mineral soils and similar modes of interaction (endophytic) between fungi and herbaceous hosts. Therefore, it seems unlikely that the observed patterns of Sebacinales community structure in montane and subalpine ecosystems are determined by plant community type alone. These observations agree with those of Vamosi *et al.* (2009) and Fattorini & Halle (2004), who postulated that a 'stressful' determinant (e.g. extreme soil pH, low mineralization or frequent disturbances) would lead to more phylogenetic clustering (Fig. 2). By contrast, Sebacinales communities associated with plant roots in wet meadow, ravine and spruce forests and Alpine rose rock vegetations contain sebacinoids that are phylogenetically more diverse and exhibit no significant phylogenetic structure (Table 1). This may be because the assembly processes are dominated by competitive interactions in these Sebacinales communities. It is possible that balanced and weak environmental forces (e.g. low levels of disturbance and high availability of resources) dominate the means of community assembly. This could lead to phylogenetic structures that are indistinguishable from random (Kembel & Hubbell, 2006).

Thus, the different interaction modes of Sebacinales with herbs (Selosse *et al.*, 2009; Weiß *et al.*, 2011), Ericaceae (e.g. Selosse *et al.*, 2007) and trees (e.g. Selosse *et al.*, 2002a), and habitat spatial heterogeneity (Tilman & Pacala, 1993) could by themselves result in differences in the community structure.

There was no altitudinal trend in the ordination plot among the sites, suggesting that factors other than those associated with altitudinal gradient structure the associated Sebacinales community of plants in these ecosystems (Fig. 2). The strong interrelation among the soil parameters suggests that there is no single general determinant explaining much of the variation in the Sebacinales communities across these ecosystems, supporting the proposal by Toljander *et al.* (2006). It is therefore likely that complex inter-relationships of environmental determinants, rather than a single determinant, are responsible for the diversity and structure of Sebacinales communities. Land use was a significant factor explaining the majority of MOTU distribution. Interpretation of these patterns in terms of environmental processes suggests that recently disturbed plant communities appear to have more phylogenetically clustered Sebacinales communities than would be expected by chance, whereas 'undisturbed' old plant communities exhibit a more random phylogenetic structure. Dinnage (2009) found that disturbed plant communities proved to be more clustered than expected, whereas 'undisturbed' old plant communities tended to be more overdispersed.

In summary, we found that Sebacinales represent a phylogenetically diverse and widely distributed group of microorganisms, but these appear to be rather of low abundance in plant roots. The diversity and structure of the Sebacinales communities differed across montane and

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subalpine ecosystems, with land use constituting an important factor, whereas soil parameters may play a crucial role in extreme habitats.

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Author contribution

Sigisfredo Garnica and Kai Riess contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Description of plant communities of the study area.

Fig. S1. Schematic illustration of the DNA target regions and the location of the primers used for PCR amplifications.

Table S1. List of plant species containing sebacinoid fungi sampled in this study, ordered by family.

Table S2. Sebacinales-specific primers and fungal universal primers used in this study.

Table S3. Average soil chemical composition of the sampling sites (except for SF).

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