

Exploring structural and molecular diversity of Ericaceae hair root mycobionts: a comparison between Northern Bohemia and Patagonia

Martin Vohník (✉ vohnik@ibot.cas.cz)

Czech Academy of Sciences

M. Clara Bruzone

Centro Regional Universitario Bariloche, IPATEC (Universidad Nacional del Comahue – CONICET)

Tereza Knoblochová

Czech Academy of Sciences

Natalia V. Fernández

Centro Regional Universitario Bariloche, IPATEC (Universidad Nacional del Comahue – CONICET)

Zuzana Kolaříková

Czech Academy of Sciences

Tomáš Větrovský

Czech Academy of Sciences

Sonia B. Fontenla

Centro Regional Universitario Bariloche, IPATEC (Universidad Nacional del Comahue – CONICET)

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Abstract

Core Ericaceae produce delicate hair roots with inflated rhizodermal cells that host plethora of fungal symbionts. These poorly known mycobionts include various endophytes, parasites, saprobes, and the ericoid mycorrhizal (ErM) fungi (ErMF) that form the ErM symbiosis crucial for the fitness of their hosts. Using microscopy and high-throughput sequencing, we investigated their structural and molecular diversity in 14 different host × site combinations in Northern Bohemia (Central Europe) and Patagonia (South America). While we found typical ericoid mycorrhiza (=intracellular hyphal coils in the rhizodermis) in all combinations, we did not detect ectomycorrhiza (Hartig net) and arbuscular mycorrhiza (arbuscules). Superficial mantles of various thickness formed by non-clamped hyphae were observed in all combinations except *Calluna vulgaris* from N. Bohemia. Some samples contained frequent intercellular hyphae while others previously unreported intracellular haustoria-like structures linked with intracellular hyphal coils. The 711 detected fungal OTU were dominated by Ascomycota (563) and Basidiomycota (119), followed by four other phyla. Ascomycetes comprised Helotiales (255), Pleosporales (53), Chaetothyriales (42), and other 19 orders, while basidiomycetes Sebacinales (42), Agaricales (28), Auriculariales (7), and other 14 orders. While many dominant OTU from both Hemispheres lacked close relatives in reference databases, many were very similar to identical to unnamed sequences from around the world. On the other hand, several significant ericaceous mycobionts were absent in our dataset, incl. *Cairneyella*, *Gamarada*, *Kurtia*, *Lachnum*, and *Leohumicola*. Most of the detected OTU (623) could not be reliably linked to a particular trophic mode and only two (ca. 3% of all reads) could be reliably assigned to the archetypal ErMF *Hyaloscypha hepaticicola*. Probable ErMF comprised *H. variabilis* (4 OTU/0.8%) and *Oidiodendron maius* (1/0.5%), both detected only in N. Bohemia. Possible ErMF comprised sebacinoid fungi (42/14%) and several unnamed members of *Hyaloscypha* s. str. (14/0.5%). While *H. hepaticicola* was dominant only in *C. vulgaris* (1/32%), this model ErM host lacked *O. maius* and sebacinoid mycobionts. *Hyaloscypha hepaticicola* was absent in two and very rare (≤ 10 reads) in six combinations from Patagonia. Nine OTU represented dark septate endophytes from the *Phialocephala fortinii* s. lat. – *Acephala applanata* species complex, including the most abundant OTU (16%, the only detected in all combinations). Statistical analyses revealed marked differences between N. Bohemia and Patagonia, but also within Patagonia, due to the unique community detected in a Valdivian temperate rainforest. Ericaceous hair roots host a high diversity of mycobionts with mostly unknown functions and many novel ErMF lineages apparently await discovery. Transhemispheric differences (thousands of km) in composition of their communities can be evenly matched by local differences (scales of km, m, and less).

Introduction

The ericoid mycorrhizal (ErM) symbiosis is an endomycorrhizal type morphologically characterized by intracellular hyphal coils formed by specific fungal symbionts (= mycobionts) predominantly in the inflated one-layered rhizodermis of the delicate hair roots (typically less than 100 μm in diam.) of core Ericaceae [= members of the early anther inversion clade of Ericaceae as defined in Kron et al. (2002)] (Gimingham 1960; Read 1996; Massicotte et al. 2005; Vohník 2020). Many ericaceous plants (co-)dominate harsh, nutrient poor acidic soils and the ErM symbiosis is believed to be the key factor in their success, providing them with an access to nitrogen and phosphorus bound in recalcitrant organic substrates (Cairney and Meharg 2003; Read et al. 2004). This is due to the considerable saprobic abilities of the so far investigated ErM fungi (ErMF) that seem to be metabolically and phylogenetically closer to saprobes and pathogens than to the ectomycorrhizal (EcM) fungi (Vohník et al. 2012; Perotto et al. 2018; Martino et al. 2018; Fehrer et al. 2019). However, most of our knowledge on the ErM symbiosis is based on just two archetypal ericaceous mycobionts, namely the ascomycetes *Hyaloscypha hepaticicola* and *Oidiodendron maius* (Leake and Read 1991; Perotto et al. 2002; Rice and Currah 2006), and many studies have shown that under certain conditions, these species may be non-dominant or even absent in the mycobiota colonizing ericaceous hair roots (e.g., Hutton et al. 1994; Williams et al. 2004; Bougoure and Cairney 2005; Bruzone et al. 2015; Lukešová et al. 2015; Leopold et al. 2021). Indeed, the spectrum of potential ErMF is considerably larger than previously thought (Leopold 2016; Vohník 2020), hence one may ask to what extent does our current understanding of the ErM symbiosis reflect the actual diversity of the ErMF.

Early modern (~ 1970-90s) views on the diversity of the ErMF were based on morphological observations and culture-dependent approaches, with obvious advantages and disadvantages (Vohník 2020). It was believed that true ErMF were only ascomycetous and either conspecific with or at least closely related to the prominent ErMF *H. hepaticicola* (Pearson and Read 1973a). A decade later, *O. maius* has been accepted as an ErMF (Couture et al. 1983; Douglas et al. 1989). In parallel, reports started to accumulate about the “uncultivable” basidiomycetous component of the ErMF diversity, especially the clavarioid and sebacinoid fungi (Seviour et al. 1973; Bonfante-Fasolo 1980; Englander and Hull 1980; Peterson et al. 1980; Mueller et al. 1986; Allen et al. 1989). Today we recognize several confirmed and potential ErMF lineages within *Hyaloscypha* s. str. [formerly the *Hymenoscyphus ericae* aggregate, see Vrålstad et al. (2000)], namely (alphabetically) *H. bicolor* and *H. finlandica* (Hambleton and Sigler 2005), a lineage closely related to or conspecific with *H. daedaleae*/ *H. fuckelii* (Vohník and Réblová 2023), *H. gryndleri* (Vohník et al. 2022), *H. hepaticicola* (Pearson and Read 1973a), and *H. variabilis* (Hambleton and Sigler 2005) and acknowledge that ericoid mycorrhizae can be formed by both serendipitoid (Sebacinales: Serendipitaceae) (Selosse et al. 2007; Vohník et al. 2016) and non-serendipitoid (Vohník et al. 2012; Kolařík and Vohník 2018) basidiomycetes. In addition, recent re-synthesis studies (Grunewaldt-Stöcker et al. 2013; Baba and Hirose 2020) suggested that members of *Leohumicola* (Hambleton et al. 2005) might form ericoid mycorrhizae and two Australian ErMF were recently accommodated in *Cairneyella* (Midgley et al. 2016) and *Gamarada* (Midgley et al. 2018). That said, the mycorrhizal bi-directional nutrient transfer between the mycobiont and the photobiont has been demonstrated in accordance with the Koch’s postulates only for a single mycobiont, namely *H. hepaticicola* (Pearson and Read 1973b).

During ca. past two decades, studies employing culture-independent approaches (especially cloning followed by Sanger sequencing and high-throughput sequencing) have considerably broadened our understanding of the global diversity of ericaceous root mycobionts. Their major contribution was the discovery that the difficult-to-cultivate sebacinoid fungi regularly colonize hair roots of core Ericaceae, often (co-)dominating the detected mycobiont spectra (Allen et al. 2003; Selosse et al. 2007). Several other potential ErMF lineages have been revealed, especially the chaetothyrioid fungi [Lukešová et al. (2015), also see Baba and Hirose (2021)], but as yet they remain taxonomically unexplored and the mode of their functioning is not fully understood. Not least, molecular methods have revealed that the thin hair roots harbor very diverse fungal communities, perhaps the most diverse per biomass among plants (Bougoure et al. 2007), comprising not only ErMF, but also endophytes, parasites, and saprobes (Vohník 2020). That said, except the so-called dark septate endophytes (DSE) that

regularly colonize ericaceous roots, form characteristic melanized intracellular microsclerotia, and whose effects on host fitness range from positive to neutral to negative (Vohník et al. 2003, 2005; Lukešová et al. 2015), little is known about colonization patterns and eco-physiological effects of these non-ErMF mycobionts.

Core Ericaceae inhabit diverse ecosystems in all continents except Antarctica, yet most of our knowledge on the ErM symbiosis comes from heathland or heathland-like ecosystems of northern Europe and Australia. Hence, one could be curious about fungal colonization patterns and spectra in ericaceous hair roots outside these archetypal ecosystems. For example, Leake et al. (1990) stressed that ericoid mycorrhiza is intolerant of permanent waterlogging and increased water content in the substrate seems to negatively affect also the otherwise ubiquitous DSE (Hambleton and Currah 1997; Vohník and Réblová 2023), hence ErMF and DSE should be infrequent to absent in waterlogged ecosystems [but see Griffin and Kernaghan (2022)]. The presence of EcM hosts in a given ecosystem may increase the presence of EcM basidiomycetes (Bougoure et al. 2007) but decrease the presence of certain *Hyaloscypha* spp. (Kohout et al. 2011) in ericaceous hair roots.

In this study we focused on two neglected regions in Central Europe (namely Northern Bohemia) and South America (Patagonia), the latter including a waterlogged site in a Valdivian temperate rainforest. We collected root samples of local ericaceous species from a wide range of natural habitats, including treeless sites as well as sites with EcM and arbuscular mycorrhizal (AM) tree dominants, carefully selected healthy-looking hair roots and investigated their fungal colonization using light microscopy. The rest of the hair roots were surface-sterilized and their mycobiont communities characterized using 454 pyrosequencing, followed by a conservative assignment of the taxonomy and trophic modes of the detected fungi. We expected significant differences between the two geographical regions in terms of both structural and molecular diversity of their mycobionts. Since members of *Hyaloscypha* s. str. may be absent in some South American Ericaceae, possibly being substituted by local sebacinoid fungi [Bruzzone et al. (2015), also see Setaro and Kron (2011)], we expected that the differences in molecular diversity might be to a large extent due to these two mycobiont guilds. In addition, we were curious whether the present culture-independent study would yield results similar to our previous culture-dependent investigations from the same or nearby locations [N. Bohemia: Vohník and Réblová (2023), also see Lukešová et al. (2015) who employed 454 pyrosequencing; Patagonia: Bruzzone et al. (2015, 2017)].

Materials And Methods

Sampling

Representatives of ericaceous plant species were sampled at nine sites in southern Argentina along the Cordillera de los Andes and at three sites in N. Bohemia, Czechia (Fig. 1). The Argentine sites phytogeographically belong to the Subantarctic Province (Cabrera and Willink 1980) and are distributed along a latitudinal gradient from 39.593°S to 54.831°S, with altitudes between 291 meters above sea level (Ushuaia) and 1722 m a. s. l. (Volcán Lanín). One of the sites is located in Parque Municipal Llao-Llao, San Carlos de Bariloche (Bruzzone et al. 2015), the remaining ones are located in four national parks: three in Parque Nacional (PN) Nahuel Huapi (Bruzzone et al. 2017), two in PN Lanín, two in PN Los Glaciares and one in PN Tierra del Fuego (Fig. 1). These sites represent four very different native Patagonian environments: the native humid broadleaf forest dominated by deciduous or perennial *Nothofagus* species (*N. pumilio* and *N. dombeyi*), the mesic conifer forest dominated by *Austrocedrus chilensis*, the patchy scree vegetation of high Andes mainly characterized by perennial herbs, and the waterlogged peat bog located in the Valdivian rainforest dominated by native conifers (*Pilgerodendron uviferum*, *Fitzroya cupressoides*) and *Sphagnum*. The three Czech sites are located in N. Bohemia (latitude between 50.641°N and 50.856°N, altitude between 277 and 690 m a. s. l.) and represent some of the typical ericaceous habitats of C. Europe: the temperate secondary Norway spruce (*Picea abies*) forest (Vohník and Réblová 2023), the temperate Scots pine (*Pinus sylvestris*) forest growing on sandstone (Lukešová et al. 2015) and the callunetum, i.e., mostly a treeless ecosystem dominated by *Calluna vulgaris* occasionally accompanied by other Ericaceae.

Argentine ericaceous plants belong to six genera, 18 species, and two varieties (Teillier et al. 2019). The most speciose genus is *Gaultheria*, a typical South American member of core Ericaceae, and we investigated *G. antarctica*, *G. caespitosa*, *G. mucronata*, *G. phillyreifolia* [this species was investigated in the same area by Bruzzone et al. (2015) and mistakenly assigned as *P. mucronata*], and the most common species *G. poeppigii* (sampled at seven sites in four protected areas). The Czech species belonged to *Calluna* (heather, *C. vulgaris*) and *Vaccinium* (European blueberry, *V. myrtillus* and cowberry, *V. vitis-idaea*) (Table 1).

Table 1
Sites in Northern Bohemia and Patagonia investigated in this study.

Locality	Site (GPS)	Altitude (m a. s. l.)	Species sampled	Sample code	Ecosystem	Ecosystem type	Main canopy species (mycorrhizal preference)	Main understory species (mycorrhizal preference)
Pod Ptáčincem	N50.855917 E14.618117	690	<i>Calluna vulgaris</i>	Cvu12	callunetum	Ericaceae-dominated	none	<i>Calluna vulgaris</i> (ErM)
Lužické hory PL	N50.855650 E14.617233		<i>Vaccinium myrtillus</i>	Vmy10	temperate Norway spruce forest	evergreen conifer	<i>Picea abies</i> (EcM)	perennial herbs/grasses and Ericaceae (AM + NM + ErM)
Šmí u České Lípy	N50.641017 E14.57748	277	<i>Vaccinium vitis-idaea</i>	Vvi11	temperate Scots pine forest	evergreen conifer	<i>Pinus sylvestris</i> (EcM)	perennial herbs/grasses and Ericaceae (AM + NM + ErM)
Paso Tromen PN Lanín	S39.593383 W71.444683	1229	<i>Gaultheria poeppigii</i>	Gpo14	mesic Patagonian forest	deciduous broadleaf/evergreen conifer	<i>Nothofagus pumilio</i> (EcM), <i>Araucaria araucana</i> (AM)	annual and perennial herbs and shrubs (AM)
Volcán Lanín PN Lanín	S39.614667 W71.467350	1722	<i>Gaultheria poeppigii</i>	Gpo7	high mountain scree	perennial herbs and shrubs	none	perennial herbs and shrubs (AM + NM)
Puerto Blest PN Nahuel Huapi	S41.040600 W71.806250	782	<i>Gaultheria antarctica</i> <i>Gaultheria poeppigii</i>	Gan8 Gpo9	Valdivian temperate rainforest	waterlogged peat bog	<i>Pilgerodendron uviferum</i> (AM), <i>Fitzroya cupressoides</i> (AM)	<i>Sphagnum</i> and Ericaceae (NM + ErM)
Llao Llao Parque Municipal Llao Llao	S41.074250 W71.568017	778	<i>Gaultheria phillyreifolia</i> <i>Gaultheria poeppigii</i>	Gph1 Gpo2	humid Patagonian forest	evergreen broadleaf	<i>Nothofagus dombeyi</i> (EcM)	annual and perennial herbs and shrubs (AM)
Cerro Otto PN Nahuel Huapi	S41.136967 W71.360700	960	<i>Gaultheria poeppigii</i>	Gpo3	mesic Patagonian forest	evergreen conifer	<i>Austrocedrus chilensis</i> (AM)	annual and perennial herbs and shrubs (AM)
Cerro Tronador PN Nahuel Huapi	S41.185567 W71.810917	1100	<i>Gaultheria poeppigii</i>	Gpo4	humid Patagonian forest	deciduous broadleaf	<i>N. pumilio</i> (EcM)	annual and perennial herbs and shrubs (AM)
Chaltén PN Los Glaciares	S49.282883 W72.961933	737	<i>Gaultheria caespitosa</i>	Gca13	humid Patagonian forest	deciduous broadleaf	<i>N. pumilio</i> (EcM)	perennial herbs/grasses and Ericaceae (AM + NM + ErM)
	S49.303883 W72.916500	734	<i>Gaultheria poeppigii</i>	Gpo5			<i>N. pumilio</i> (EcM)	perennial herbs and shrubs (AM + NM)
Ushuaia PN Tierra del Fuego	S54.831083 W68.488850	291	<i>Gaultheria mucronata</i>	Gmu15	humid Patagonian forest	deciduous broadleaf	<i>N. pumilio</i> (EcM)	annual and perennial herbs and shrubs (AM + NM)
Details on the 12 investigated sites which are listed geographically from the north to the south (non-shaded samples are from Patagonia, shaded samples are from N. Bohemia; for more details see Materials and Methods and Fig. 1). Abbreviations: AM = arbuscular mycorrhizal; a. s. l. = above sea level; EcM = ectomycorrhizal; ErM = ericoid mycorrhizal; NM = non-mycorrhizal; PL = protected landscape; PM = municipal park (<i>Parque Municipal</i>); PN = national park (<i>Parque Nacional</i>).								

Typically, only one ericaceous species occurred at a site, but at two sites in Patagonia (Puerto Blest and Llao Llao) two *Gaultheria* species co-occurred, resulting in 14 different host × site combinations. At every site, five adult individuals per species growing at least 5 m apart were carefully extracted from the substrate (incl. adhering soil) using a spade, sealed in plastic bags, transported to the laboratory, stored in a fridge (6–8°C) and processed within the next 24 hours. First, the adhering soil was carefully removed from the roots under running tap water and the cleaned roots were stored in 50 ml plastic tubes in 70% ethanol in a fridge. Subsequently, when all samples were ready, they were again carefully washed on a sieve with running tap water and healthy-looking turgescient hair roots firmly attached to the thick main roots were selected using a dissecting microscope and excised using scissors. The sampling in Patagonia took place during February 2013 (autumn) and October/November 2014 (spring) while the sampling in N. Bohemia took place in November 2014 (autumn).

Microscopy

A smaller part of the healthy-looking turgescient roots was subjected to light microscopy. First, they were cleared by autoclaving in 10% KOH at 121°C for 20 min, washed with tap water, briefly acidified in 3% HCl, washed with tap water and stained by autoclaving in 0.05% solution of trypan blue in lactoglycerol

(glycerol/lactic acid/water 2:1:2) at 121°C for 20 min. Excess stain was removed using a sieve, the stained roots were washed with tap water and stored in 30% EtOH. Second, a random subsample (a few cm in total length) was taken from the stained hair root samples representing individual localities and screened using an Olympus BX60 upright microscope at high magnifications (×400–1000). After characterizing individual colonization patterns, the whole stained samples were screened using an Olympus SZX12 stereomicroscope to evaluate their approximate frequency. We especially focused on intracellular hyphal coils, intracellular microsclerotia, intercellular hyphae, partial hyphal mantles formed by DSE on the root surface [see Vohník and Albrechtová (2011) and references therein], hyphal sheaths on the root surface (clamped vs. non-clamped hyphae), hyphae surrounding the hair roots (clamped, non-clamped, with and without septa), and AM (arbuscules) and EcM (Hartig net) colonization (Table 2). Photos were taken with an Olympus DP70 camera, the graphic documentation was modified for clarity in FastStone Image Viewer (FastStone Soft) as needed. Figures were assembled in Paint.NET (<https://www.getpaint.net/>).

Table 2
Fungal colonization patterns in ericaceous hair roots from Northern Bohemia and Patagonia observed using light microscopy.

Sample code ¹	Ericoid mycorrhiza (intracellular hyphal coils stained blue/brown/hyaline)	DSE microsclerotia	DSE partial mantles	DSE hyphae on the surface	hyphal mantles without clamps	hyphal mantles with clamps	clamped hyphae around	non-clamped hyphae around	AM hyphae around	blue-stained intracellular spheres	intercell hyphae
Gph1	+/+/+	+	+	+	+	-	+	+	-	+	+
Gpo2	+/+/+	-	-	+	+	-	-	+	-	+	+
Gpo3	+/+/+	+	+	+	+	-	-	-	-	-	-
Gpo4	+/+/+	-	+	+	+	-	-	+	-	-	+
Gpo5	+/+/+	+	-	+	+	-	-	-	-	+	-
Gpo7	+/+/+	-	+	+	+	-	-	+	-	+	+
Gan8	+/+/+	+	-	+	+	-	-	+	-	-	+
Gpo9	+/+/+	+	+	+	+	-	-	+	-	-	-
Vmy10	+/+/+	+	+	+	+	-	-	+	-	-	+
Vvi11	+/+/+	+	+	+	+	-	+	+	-	-	+
Cvu12	+/+/+	-	+	+	-	-	-	+	-	-	+
Gca13	+/+/+	-	+	+	+	-	-	+	-	-	-
Gpo14	+/-/+	+	+	+	+	-	-	+	+	-	-
Gmu15	+/-/+	-	-	+	+	-	-	-	-	-	-

¹ Codes as in Table 1; non-shaded samples are from Patagonia, shaded samples are from N. Bohemia. Abbreviations: **AM** = arbuscular mycorrhizal; **DSE** = da endophytes.

DNA extraction, PCR amplification and pyrosequencing

The excised hair roots were surface sterilized for 60 s in 10% household bleach containing 5% active chlorine and then serially washed with sterile water. Subsequently, 10 ml of 0.9% NaCl (w/v) were added to 100 mg (fresh weight) of surface-sterilized hair roots, the mixture was frozen in liquid nitrogen and homogenized in a sterile mortar. Total DNA was extracted from each such suspension representing a unique sample (70 samples in total) using a FastDNA Green Spin Extraction and Isolation Kit (MP Biomedicals) according to manufacturer’s instructions. The resulting DNA extracts were pooled for each host × site combination, resulting in 14 pooled samples.

In the first PCR, the entire ITS1-5.8S-ITS2 region of the nrDNA was amplified using the fungal specific primer pair ITS1F + ITS4 (White et al. 1990; Gardes and Bruns 1993). Each PCR mix consisted of 15.8 µl ddH₂O, 0.2 µl Taq polymerase, 2.5 µl 10x PCR buffer, 2 µl MgCl₂ (25 mM), 0.5 µl dNTP mixture (2 mM), 0.5 µl of each primer (10 µM), 1 µl BSA (20 mg/ml; Sigma) and 2 µl of a diluted DNA template. The parameters of the first PCR were as follows: an initial denaturation step at 95°C for 5 min followed by 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 60 s, followed by the final extension at 72°C for 10 min. Purified products from the first PCR were used as templates for the second PCR employing ITS1F and ITS4 primers containing 454 pyrosequencing adaptors and sample-specific labels (Roche Applied Science). The resulting amplicon libraries were purified using an Agencourt AMPure XP kit (Beckman Coulter) according to manufacturer’s instructions, quantified and pooled into an equimolar working solution that was subsequently sequenced using the 454 GS-FLX Titanium sequencing platform (Roche).

Bioinformatics

In total, the tag-encoded amplicon sequencing yielded 74820 raw sequences. The data were filtered and trimmed using the pipeline SEED ver. 2.0.4 (Větrovský and Baldrian 2013). All sequences with mismatches in tags were removed from the dataset. A pyrosequencing noise reduction was performed using the PyroNoise algorithm translation in Mothur ver. 1.28.0 (Schloss et al. 2009) and a total of 72081 sequences were retained after the removal of low-quality sequences (mean < 25), sequences shorter than 480 bases and potentially chimeric sequences. This dataset was trimmed to the 480 bp sequence length, the ITS1 region was extracted using ITSx ver. 1.0.11 (Bengtsson-Palme et al. 2013), and all sequences containing ambiguous bases were discarded. This yielded

63513 sequences which were clustered to operational taxonomic units (OTU) using UPARSE implementation in USEARCH ver. 8.1.1861 (Edgar 2013) with 97% similarity threshold (281 chimeric sequences were excluded during this step). The consensus sequence from each OTU was constructed from a MAFFT ver. 7.222 alignment (Katoh et al. 2009) based on the most abundant nucleotide at each position. For sample comparison and diversity analyses the dataset was randomly resampled at the same sampling depth of 2900 sequences. Diversity indices were computed in SEED ver. 2.0.4. and OTU identification was performed by BLASTn against local ITS database based on UNITE ver. 8.3 [Abarenkov et al. (2010), <https://unite.ut.ee>]. OTU with insufficient taxonomic resolution (typically only at the kingdom level) or outdated/surprising taxonomy were further manually BLASTed in GenBank at NCBI [Benson et al. (2013), <https://www.ncbi.nlm.nih.gov/genbank>]. Their phylogenetic background was further checked with Blast Tree View (<https://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi>) in three modes, i.e., without limitations, excluding environmental sequences and considering only sequences from type material. Subsequently, OTU with no hits in the databases and singletons were discarded.

Representative sequences of OTU with affinities to Hyaloscyphaceae were aligned using Clustal W (Thompson et al. 1994) implemented in BioEdit (Hall 1999) with publicly available sequences of *Hyaloscypha* s. str. type specimens, including the recently described *H. gabretae* (CBS 145341/GenBank MZ520780), *H. gryndleri* (CBS 145337/MZ520785) and *H. melinii* (CBS 143705/EF093175) (Fehrer et al. 2019; Vohník et al. 2022), *H. epiporia* (CBS 125.91/MH018929), and *Hyaloscypha* sp. B10 (OP345122, (Vohník and Réblová 2023)). A NJ analysis with *Hyphodiscus brachyconius* (CBS 700.73/GU727557), *Hy. brevicollaris* (CBS 126.74/GU727561) and *Hy. luxurians* (CBS 647.75/GU727560) serving as an outgroup was performed in TOPALi (Biomathematics & Statistics Scotland) to check their phylogenetic position, especially whether they belonged to *Hyaloscypha* s. str. The same was done with representative sequences of OTU belonging to Sebaciniales that were compared to publicly available sequences of type specimens of *Serendipita herbarumans* (DSM 27534/NR_144842) and *S. indica* (CBS 125645/NR_166023) and to Serendipitaceae sp. JPK-132 (OP863025), the only isolate that has been shown to form ericoid mycorrhiza (Vohník et al. 2016). In addition, OTU delimited here were compared with dominant OTU belonging to Helotiales, Hypocreales, Pleosporales and *Oidiodendron* that were reported from Patagonia in two recent culture-dependent studies (Bruzzone et al. 2015, 2017) and with dominant OTU belonging to Helotiales and Chaetothyriales that were recently reported from N. Bohemia in one culture-independent study (Lukešová et al. 2015) and one culture-dependent study (Vohník and Réblová 2023) using methods described above.

The ratio between ascomycetous and basidiomycetous OTU and % of total reads was calculated. Variability in the fungal community structure of the samples based on dominant OTU was visualized using non-metric multidimensional scaling (NMDS) performed in R (R Core Team 2023) using the metaMDS function from vegan package (Oksanen et al. 2018). The default Bray-Curtis dissimilarity index was used for the NMDS construction. In addition, the heatmap function in R (R Core Team 2023) was used for the same purpose, including only OTU with > 5% local abundance.

Fungal taxonomy followed MycoBank (<https://www.mycobank.org/>, accessed August-September 2022), taxonomy of *Hyaloscypha* s. str. followed Fehrer et al. (2019) and Vohník et al. (2022). Sequence data and associated metadata are available in the PlutoF repository (<https://doi.plutof.ut.ee/doi/10.15156/BIO/2483938>).

Trophic modes

Based on literature searches and own experience, the detected fungal OTU were assigned to the following trophic modes: DSE [mostly members of the *Phialocephala fortinii* s. lat. – *Acephala applanata* species complex (PAC), Grünig et al. (2008)], ErMF (*H. hepaticicola*), probable ErMF (prErM; *H. variabilis* + *O. maius*), possible ErMF/endophytic fungi (poErMF/endophytic; members of *Hyaloscypha* s. str. except *H. hepaticicola* and *H. variabilis* + all Sebaciniales), parasitic/saprobic fungi, and other root-associated fungi (RAF; fungi with uncertain or unknown symbiotic mode). These categories reflect the most likely trophic mode of given OTU in healthy ericaceous hair roots, irrespectively of other possible modes in other hosts or substrates. For example, the genus *Mycena* is typically saprobic but commonly occurs in core Ericaceae hair roots [where its functioning remains under debate, e.g., Grelet et al. (2017) and Vohník and Réblová (2023)], hence its assignment in this paper is RAF; the genera *Cortinarius*, *Piloderma* and *Tomentella* contain EcM species but their functioning in ericaceous roots is not known, hence their assignment in this paper is RAF; etc.

Results

Microscopy

Dense intracellular hyphal coils (blue-stained, hyaline, or dark brown to dark grey) were abundantly present in all screened root systems (Fig. 2A–D, Table 2). In contrast, melanized intracellular microsclerotia typical of DSE (Fig. 2E&F) were very rare and totally absent in samples from six host × site combinations. Hyaline microsclerotia (Fig. 2G–J) similar to those formed by some sebacinoid fungi (Vohník et al. 2016) and in a way resembling “spore-like bodies” reported by Brook (1952) in *G. macrostigma* (syn. *Pernettya macrostigma*) were found in three host × site combinations. All root samples except *C. vulgaris* contained hair root segments covered with blue-stained or hyaline hyphal mantles (Fig. 2K–M), either discontinuously along the hair roots or more often surrounding the hair roots' tips. The mantles differed in density and thickness from single-layered sparse nets to multilayered dense structures resembling sheathed ericoid mycorrhiza (Vohník et al. 2012), except that the hyphae were much thinner and never formed clamp connections [see Massicotte et al. (2005) and Bruzzone et al. (2015)]. Sparse parenchymatous nets (Fig. 2N) formed by melanized septate hyphae [see Vohník and Albrechtová (2011)] were present on the hair roots' surfaces in ten host × site combinations (incl. all N. Bohemian samples), often at the point of emergence of lateral roots. In contrast, individual melanized septate hyphae (Fig. 2O–Q) of different thickness, typically running parallel to the hair root's axis, either on the surface or intraradically around the stele, were present in all samples. Intercellular septate hyphae (Fig. 2R) were present in eight host × site combinations (incl. all N. Bohemian samples) and were especially frequent in *V. vitis-idaea* from N. Bohemia. Six Argentine host × site combinations comprised blue-stained intracellular spheres (Fig. 2S&T), typically one per host cell. These were observed in hair roots either free of other fungal colonization or containing intracellular hyphal coils typical of the ErM symbiosis.

In most cases there were no apparent intermediates between extracellular hyphae and intracellular hyphal coils, but in some samples, especially from the Valdivian rainforest and N. Bohemia, many rhizodermal cells contained haustoria-like structures (Fig. 2U–Y) in a way resembling those reported by Vohník and

Réblová (2023), often more than one (up to six) in a single cell. Some hair roots were surrounded by clamped or non-clamped hyphae with no clear physical connection to the root; some of the latter lacked septa and resembled AM hyphae (not shown, Table 3). Irrespective of fungal colonization, some hair roots of *G. poeppigii* from the Valdivian rainforest possessed rhizodermal cells with unusually thick cell walls (Fig. 2Z).

Table 3
Taxonomy of the fungal communities detected in ericaceous hair roots from Northern Bohemia and Patagonia using 454 pyrosequencing

Phylum	OTU (reads)	Class	OTU (reads)	Order	OTU (reads)	Most common/significant OTU (total reads)	Reads ARG	Reads CZE	Taxonomy
Ascomycota	563 (48867)	Leotiomycetes	315 (39761)	Helotiales	255 (34856)	OTU 1 (9397)	7861	1536	PAC
						OTU 2 (2942)	2941	1	Hel
						OTU 3 (2399)	2396	3	Hel
						OTU4 (2037)	2032	5	Hel
						OTU 5 (1540)	1529	11	Hel
						OTU 6 (1429)	1429**	0	Hye
						OTU 8 (1372)	48	1324	Hye hep
						OTU 32 (460)	0	460	Hye
						OTU 37 (375)	375	0	Hye hep
				Rhytismatales	6 (1155)	OTU 24 (728)	0	728*	Rhy
						OTU 41 (320)	317	3	Rhy
						OTU 86 (96)	96**	0	Rhy
				Thelebolales	4 (9)	OTU 572 (3)	0	3	The
				Phacidiales	2 (5)	OTU 557 (3)	3	0	Pha
				unknown	48 (3736)	OTU 26 (698)	698	0	Lec
						OTU 29 (633)	0	633*	Lec
						OTU 30 (577)	577**	0	Lec
		Dothideomycetes	83 (2787)	Pleosporales	53 (2441)	OTU 7 (1386)	1385	1	Ple
						OTU 40 (336)	336	0	Ple
						OTU 65 (144)	144	0	Ple
				Capnodiales	4 (27)	OTU 300 (11)	11*	0	Cap
				Venturiales	3 (7)	OTU 536 (3)	3*	0	Ver
				Mycosphaerellales	1 (2)	OTU 688 (2)	0	2*	Cer
				unknown	22 (310)	OTU 62 (152)	152	0	Dot
						OTU 25 (719)	719	0	Cha
						OTU 51 (242)	0	242	Her
		Eurotiomycetes	52 (1846)	Chaetothyriales	42 (1665)	OTU 79 (112)	112	0	Cha
						OTU 81 (106)	0	106*	Cha
				Eurotiales	6 (69)	OTU 152 (34)	3	31	Per
		Sordariomycetes	36 (727)	unknown	4 (112)	OTU 93 (86)	0	86*	Eur
				Hypocreales	9 (220)	OTU 78 (114)	113	1	Tric
						OTU 129 (46)	45	1	Ilyc
						OTU 150 (36)	36	0	Fus
				Sordariales	7 (30)	OTU 288 (11)	11	0	Sor

* detected in a single host × site combination

** detected only in the Valdivian temperate rainforest in Patagonia, Argentina

Abbreviations: ARG = samples from Patagonia, Argentina; CZE = samples from N. Bohemia, Czechia; DSE = dark septate endophytes; ErM = ericoid mycorrhizal; poErM = possibly ericoid mycorrhizal; OTU = operational taxonomic unit; PAC = dark septate endophytes from the *Phialocephala fortinii* s. lat. – *Acephala* spp. associated fungus (incl. all possible symbiotic modes). For easier orientation, the ErM, prErM and poErM categories are shaded. Fungal taxonomy follows MycoBank (accessed August-September 2022).

Phylum	OTU (reads)	Class	OTU (reads)	Order	OTU (reads)	Most common/significant OTU (total reads)	Reads ARG	Reads CZE	Tax
				Coniochaetales	5 (86)	OTU 149 (36)	36*	0	Con
				Xylariales	5 (74)	OTU 145 (37)	37*	0	Xyli
				Chaetosphaeriales	2 (28)	OTU 201 (24)	20	4	Cha
				unknown	8 (289)	OTU 71 (128)	0	128	Sor
		Lecanoromycetes	5 (34)	Lecanorales	1 (2)	OTU 684 (2)	2	0	Lec
				Ostropales	1 (5)	OTU 439 (5)	5	0	Ost
				unknown	3 (27)	OTU 274 (13)	0	13	Lec
		Archaeorhizomycetes	2 (79)	Archaeorhizomycetales	2 (79)	OTU 99 (76)	0	76*	Ar
		Pezizomycetes	2 (13)	Pezizales	1 (2)	OTU 678 (2)	2*	0	Mo
				unknown	1 (11)	OTU 295 (11)	0	11	Pez
		Arthoniomycetes	1 (6)	Lichenostigmatales	1 (6)	OTU 385 (6)	6**	0	Pha
		Geoglossomycetes	1 (6)	Geoglossomycetes	1 (6)	OTU 394 (6)	0	6*	Gec
		Saccharomycetes	1 (6)	Saccharomycetales	1 (6)	OTU 372 (6)	1	5	Car
		unknown	65 (3602)			OTU 13 (1159)	2	1157	Asc
						OTU 15 (868)	824	44	Pez
						OTU 43 (309)	0	309	Oid
Basidiomycota	119 (10698)	Agaricomycetes	101 (10355)	Sebacinales	42 (8263)	OTU 9 (1341)	1	1340	Seb
						OTU 10 (1270)	1270	0	Ser
						OTU 11 (1242)	0	1242	Ser
						OTU 12 (1163)	1163	0	Ser
						OTU 14 (1151)	1151	0	Ser
						OTU 28 (652)	652	0	Ser
						OTU 31 (558)	558	0	Ser
						OTU 39 (354)	354**	0	Ser
				Agaricales	28 (1386)	OTU 66 (143)	143	0	My
						OTU 82 (105)	105	0	My
						OTU 121 (54)	54	0	My
				Auriculariales	7 (535)	OTU 49 (255)	255	0	Aur
						OTU 57 (196)	196**	0	Aur
				Thelephorales	4 (30)	OTU 233 (18)	18*	0	Tor
				Atheliales	2 (14)	OTU 305 (11)	0	11	Pilc sph
				Cantharellales	2 (6)	OTU 582 (3)	3*	0	Car
				Trechisporales	1 (4)	OTU 456 (4)	0	4*	Tre
				Russulales	1 (2)	OTU 645 (2)	2	0	Ste
				unknown	14 (115)	OTU 185 (27)	27**	0	Age

* detected in a single host × site combination

** detected only in the Valdivian temperate rainforest in Patagonia, Argentina

Abbreviations: **ARG** = samples from Patagonia, Argentina; **CZE** = samples from N. Bohemia, Czechia; **DSE** = dark septate endophytes; **ErM** = ericoid mycorrhizal; **poErM** = possibly ericoid mycorrhizal; **OTU** = operational taxonomic unit; **PAC** = dark septate endophytes from the *Phialocephala fortinii* s. lat. – *Acephala* spp. associated fungus (incl. all possible symbiotic modes). For easier orientation, the ErM, prErM and poErM categories are shaded. Fungal taxonomy follows My accessed August-September 2022).

Phylum	OTU (reads)	Class	OTU (reads)	Order	OTU (reads)	Most common/significant OTU (total reads)	Reads ARG	Reads CZE	Tax
		Tremellomycetes	8 (60)	Tremellales	4 (11)	OTU 487 (4)	0	4	<i>Vis. vici</i>
				Trichosporonales	1 (25)	OTU 196 (25)	15	10	<i>Api</i>
				Cystofilobasidiales	1 (18)	OTU 235 (18)	18	0	<i>Tau</i>
				Holtermanniales	1 (4)	OTU 492 (4)	0	4*	<i>Hol wai</i>
				Filobasidiales	1 (2)	OTU 636 (2)	1	1	<i>Filc</i>
		Exobasidiomycetes	1 (8)	Exobasidiales	1 (8)	OTU 332 (8)	8*	0	<i>Exc</i>
		Malasseziomycetes	1 (5)	Malasseziales	1 (5)	OTU 430 (5)	5	0	<i>Ma</i>
		Geminibasidiomycetes	1 (3)	Geminibasidiales	1 (3)	OTU 535 (3)	3	0	<i>Ger</i>
		Pucciniomycetes	1 (2)	Platyglloeales	1 (2)	OTU 779 (2)	2**	0	<i>Pla</i>
		unknown	6 (265)			OTU 73 (127)	127	0	<i>Bas</i>
						OTU 76 (123)	123**	0	<i>Bas</i>
Cryptomycota	7 (61)					OTU 138 (41)	0	41	<i>Cry</i>
Mortierellomycota	5 (15)	Mortierellomycetes	5 (15)	Mortierellales	5 (15)	OTU 398 (6)	0	6	<i>Poc</i>
Olpidiomycota	1 (55)	Olpidiomycetes		unknown	1 (55)	OTU 117 (55)	55	0	<i>Olp</i>
Glomeromycota	1 (2)	Glomeromycetes	1 (2)	Glomerales	1 (2)	OTU 603 (2)	2*	0	<i>Rhi</i>
unknown	15 (423)					OTU 48 (273)	273	0	<i>Fur</i>
						OTU 115 (56)	56**	0	<i>Fur</i>
* detected in a single host × site combination									
** detected only in the Valdivian temperate rainforest in Patagonia, Argentina									
Abbreviations: ARG = samples from Patagonia, Argentina; CZE = samples from N. Bohemia, Czechia; DSE = dark septate endophytes; ErM = ericoid mycorrhizal; poErM = possibly ericoid mycorrhizal; OTU = operational taxonomic unit; PAC = dark septate endophytes from the <i>Phialocephala fortinii</i> s. lat. – <i>Acephala ap</i> associated fungus (incl. all possible symbiotic modes). For easier orientation, the ErM, prErM and poErM categories are shaded. Fungal taxonomy follows My accessed August-September 2022).									

Molecular diversity

Excluding singletons, we detected 711 fungal OTU comprising 60121 reads. On average, there were 107 OTU (max. 148, min. 66) and 4294 reads (max. 5377, min. 2859) per host × site combination. The highest number of OTU was detected in the Patagonian *G. poeppigii* from Cerro Otto (Gpo3, for codes see Table 1) while the lowest in *C. vulgaris* (Supplementary Table 1). Ascomycota dominated with 563 OTU/48867 reads (79.2%/81.3% of total), followed by Basidiomycota (119/10698, 16.7%/17.8%). Other phyla were found in much lower abundance: Cryptomycota (7/61, 0.98%/0.1%), Mortierellomycota (5/15, 0.70%/0.02%), Glomeromycota (1/2, 0.14%/0.003%) and Olpidiomycota (1/55, 0.14%/0.09%). The remaining 15 OTU/423 reads (2.1%/0.7%) could not be assigned below the kingdom level (Table 3).

Ascomycetous OTU comprised ten classes with dominant Leotiomyces (315/39761, 44.3%/66.1%), followed by Dothideomycetes (83/2787, 11.7%/4.6%), Eurotiomycetes (52/1846, 7.3%/3.1%), and Sordariomycetes (36/727, 5.1%/1.2%). Other ascomycetous fungal groups were also present, but not abundantly [Lecanoromycetes (5/34, 0.7%/0.06%), Archaeorhizomycetes (2/79, 0.3%/0.1%), Pezizomycetes (2/13, 0.3%/0.02%), Arthoniomycetes (1/6, 0.1%/0.01%), Geoglossomycetes (1/6), and Saccharomycetes (1/6)]. The remaining 65 ascomycetous OTU/3602 reads (9.1%/6.0%) could not be assigned below the phylum level. The most abundant ascomycetous order were Helotiales (255/34856, 35.9%/58.0%, Leotiomyces), followed by Pleosporales (53/2441, 7.5%/4.1%, Dothideomycetes), Chaetothyriales (42/1665, 5.9%/2.8%, Eurotiomycetes) and other 19 assigned orders.

Basidiomycetous OTU comprised six classes with the largely dominant Agaricomycetes (101/10355, 14.2%/17.2%), followed by a few Tremellomycetes (8/60, 1.1%/0.1%), Exobasidiomycetes (1/8, 0.1%/0.01%), Malasseziomycetes (1/5, 0.1%/0.01%), Geminibasidiomycetes (1/3, 0.1%/0.004%) and Pucciniomycetes (1/2, 0.1%/0.003%). The most abundant basidiomycetous order were Sebaciales (42/8263, 5.9%/13.7%, Agaricomycetes), followed by Agaricales (28/1386, 3.9%/2.3%, Agaricomycetes), Auriculariales (7/535, 1.0%/0.89%, Agaricomycetes) and other 17 assigned orders.

The ratio between ascomycetous and basidiomycetous OTU ranged from 14 (*C. vulgaris*) to 3.2 (*V. vitis-idaea*) and averaged 6.5 (7.7 for the samples from N. Bohemia and 6.1 for the samples from Patagonia). On average, ascomycetous and basidiomycetous reads represented 99% of all reads (max. 99.9% in *G. poeppigii* Gpo14, min. 96.3% in *G. caespitosa*) and the average ratio between % of ascomycetous and basidiomycetous reads was 34.1 (max. 337.2 in *C. vulgaris*, min. 0.8 in *G. caespitosa*) (Table 4).

Table 4

Ascomycetous vs. basidiomycetous OTU detected in ericaceous hair roots from Northern Bohemia and Patagonia using 454 pyrosequencing.

Sample code ¹	number of OTU / % of reads				Ascomycota : Basidiomycota
	total	Ascomycota + Basidiomycota	Ascomycota	Basidiomycota	OTU / % of reads
Gph1	133	130 / 99.5	110 / 93.8	20 / 5.7	5.5 / 16.6
Gpo2	138	135 / 99.3	107 / 74.3	28 / 25.0	3.8 / 3.0
Gpo3	148	145 / 99.3	129 / 73.4	16 / 25.9	8.1 / 2.8
Gpo4	113	107 / 99.0	82 / 70.7	25 / 28.3	3.3 / 2.5
Gpo5	123	118 / 99.5	107 / 95.0	11 / 4.5	9.7 / 20.8
Gpo7	92	91 / 99.8	72 / 79.2	19 / 20.6	3.8 / 3.8
Gan8	90	87 / 99.8	77 / 97.0	10 / 2.8	7.7 / 34.8
Gpo9	72	70 / 98.4	60 / 81.3	10 / 17.1	6.0 / 4.7
Vmy10	111	105 / 99.3	90 / 71.3	15 / 28.0	6.0 / 2.5
Vvi11	95	92 / 99.6	70 / 63.2	22 / 36.4	3.2 / 1.7
Cvu12	66	60 / 98.9	56 / 98.6	4 / 0.3	14.0 / 337.2
Gca13	78	76 / 96.2	63 / 44.1	13 / 52.1	4.8 / 0.8
Gpo14	103	102 / 99.9	93 / 97.6	9 / 2.3	10.3 / 41.9
Gmu15	131	127 / 97.1	104 / 77.8	23 / 19.3	4.5 / 4.0

¹ Codes as in Table 1. Non-shaded samples are from Patagonia, shaded samples are from N. Bohemia. Maximal and minimal values for the whole dataset are in bold. For details on individual OTU see Results and Supplementary Table 1.

Differences between Patagonia and Northern Bohemia

On average, there were 111 OTU (max. 148, min. 72) and 4350 reads (max. 5377, min. 2859) per host × site combination in Patagonia, while there were on average 91 OTU (max. 111, min. 66) and 4089 reads (max. 4512, min. 3763) per host × site combination in N. Bohemia. In Patagonia, the highest number of OTU was detected in *G. poeppigii* Gpo3 and the lowest in *G. poeppigii* from the Valdivian rainforest (Gpo9). In N. Bohemia, the highest number of OTU was detected in *V. myrtillus* and the lowest in *C. vulgaris*. Out of all 711 fungal OTU, 516 (23271 reads) were detected only in Patagonia whereas 133 (5898 reads) only in N. Bohemia. Within Ascomycota, 408 OTU with 14857 reads were detected only in Patagonia whereas 98 OTU with 4441 reads only in N. Bohemia. Within Basidiomycota, 89 OTU with 7948 reads were detected only in Patagonia whereas 25 OTU with 1367 reads only in N. Bohemia.

On average, the Shannon-Wiener diversity index was higher for the samples from Patagonia ($H = 2.959$, min. 2.393, max. 3.493) than N. Bohemia ($H = 2.537$, min. 2.362, max. 2.679). When the samples from the Valdivian rainforest in Puerto Blest were considered separately ($H = 2.581$, min. 2.476, max. 2.6864), the difference between N. Bohemia and the rest of Patagonia ($H = 3.043$, min. 2.393, max. 3.493) changed only slightly. All computed diversity indices are available in Table 5.

Table 5

Alpha diversity indices of the fungal communities detected in ericaceous hair roots from Northern Bohemia and Patagonia using 454 pyrosequencing.

	Gph1	Gpo2	Gpo3	Gpo4	Gpo5	Gpo7	Gan8	Gpo9	Vmy10	Vvi11	Cvu12	Gca13	Gpo14	Gmu15
Shannon-Wiener Diversity Index	3.2065	3.3897	3.4932	3.0108	2.3926	3.014	2.6864	2.476	2.6793	2.5686	2.3623	3.0618	2.4036	3.4144
Shannon Entropy	4.626	4.8903	5.0396	4.3437	3.4518	4.3482	3.8756	3.5721	3.8654	3.7057	3.4081	4.4173	3.4677	4.9259
Species Richness (S)	150	155	158	138	124	120	102	103	129	116	92	111	110	152
Simpson Diversity Index	0.0902	0.0625	0.0629	0.1111	0.2647	0.1199	0.1726	0.1765	0.1459	0.1532	0.1725	0.092	0.2257	0.066
Evenness	0.6399	0.6721	0.69	0.6111	0.4964	0.6296	0.5808	0.5342	0.5513	0.5403	0.5224	0.6501	0.5114	0.6796
Species Richness – 80% diversity	15	16	23	12	10	16	10	8	9	8	6	14	9	19
Chao-1	293	247.13	245	231	199	186	137.2	159.89	197.14	169.45	142.65	178.56	157.05	211.91
For sample comparison and diversity analyses the dataset was randomly resampled at the same sampling depth of 2900 sequences. Diversity indices were computed in SEED ver. 2.0.4. (Větrovský and Baldrian 2013). Locality codes follow Table 1, non-shaded samples are from Patagonia, shaded samples are from N. Bohemia. Maximal and minimal values for the whole dataset are in bold.														

There was a clear biogeographic pattern for the archetypal ErMF *H. hepaticicola*: while OTU 8 was dominantly detected in two N. Bohemian hosts, OTU 37 was detected only in Patagonia. The prErMF *H. variabilis* and *O. maius* were found only in N. Bohemia and while *H. variabilis* was detected in all hosts, *O. maius* was detected in *V. myrtillus* and *V. vitis-idaea* but not in *C. vulgaris* (which was, unlike the two *Vaccinium* spp., dominated by *H. hepaticicola*). Clear biogeographic differences were observed also for the OTU belonging to several unnamed *Hyaloscypha* s. str. (possibly representing different new species), as 13 out of the total 14 occurred only in Patagonia. Similarly, in terms of sequence reads, the second to the seventh most abundant OTU (namely four Helotiales spp., *Hyaloscypha* sp. and Pleosporales sp.), totaling 11733 reads, were predominantly detected in Patagonia. Some major sebacinoid OTU were detected only in N. Bohemia, namely OTU 9 (1341) dominating *V. myrtillus* and OTU 11 (1242) dominating *V. vitis-idaea*. On the other hand, seven sebacinoid OTU with > 100 reads (totaling 5269 reads) occurred only in Patagonia. Interestingly, no sebacinoid OTU was detected in the *H. hepaticicola*-dominated *C. vulgaris* and they were also very rare in the samples from the Valdivian rainforest (*G. antarctica*: 2 OTU/1 + 2 reads, *G. poeppigii*: 2/1 + 352).

The NMDS graph (Fig. 3) and the heatmap (Fig. 4) distinguished three sample groups: N. Bohemia, the Valdivian rainforest in Patagonia, and the rest of Patagonia.

Individual OTU

In terms of the number of sequence reads, by far the most numerous OTU 1 (9397 reads) was assigned to PAC whose members are widespread DSE that cannot be distinguished using the ITS nrDNA gene (Grünig et al. 2008). OTU 1 was especially abundant in two Patagonian samples, namely *G. poeppigii* Gpo5 (2603 reads) and *G. poeppigii* Gpo14 (2237), while it was relatively rare in some other Patagonian samples, namely *G. poeppigii* Gpo4 (49), *G. antarctica* (51) and *G. poeppigii* Gpo9 (13) (all from humid forests). It was the only OTU detected in all investigated hosts and at all sampling sites. Other probable PAC members comprised OTU 46 (286) and OTU 130 (46) found only in *G. antarctica*, in addition to OTU 166 (31), OTU 460 (4), OTU 531 (3), OTU 606 (2), OTU 691 (2) and OTU 775 (2) detected in several hosts.

In contrast to OTU 1, the second most abundant OTU 2 (2942) was practically limited to the Valdivian rainforest (*G. antarctica* with 2066 reads and *G. poeppigii* Gpo9 with 875 reads), with a single other read obtained from *V. myrtillus*. In GenBank, OTU 2 shared 100/99.4% sequence coverage/similarity with an uncultured fungus from an ectomycorrhizal root tip of *N. cunninghamii* from Tasmania [FN298705, Tedersoo et al. (2009)] and 100/97% with the *Hyaloscypha* sp. isolate ISO.88 from a root of *V. calycinum* from Hawaii [MT321782, Leopold and Fukami (2021)]. When searches were limited to sequences from type specimens, it shared only 100/89.9% with *Leptodontidium boreale* (Leotiomyces: Leptodontidiaceae) CBS 682.76 from a pine wood pole from Sweden (MH861025) and 100/87% with *Davidhawksworthia quintinae* (Helotiales: Dermateaceae) CBS 146963 from *Quintinia sieberi* from New South Wales (NR_171759). Since Blast Tree View nested OTU 2 between *Dactylaria dimorphospora* (Helotiales) and *L. boreale* and near *Arboricolonus simplex* (Helotiales), *Lareunionomyces eucalypti* (Helotiales) and *Phialocephala virens* (Helotiales), our taxonomic assignment of this OTU was a Helotiales sp. Similarly, the next three OTU (in total 5976 reads) were assigned only as Helotiales sp. and were mostly detected in the Patagonian hosts.

OTU 6 (1429 reads) was detected only in the Valdivian rainforest and after the first screening, it seemed to represent a common mycobiont of ericaceous roots, the prErMF *H. variabilis*. However, comparisons with sequences in GenBank revealed only 100/93.79% with *H. variabilis* UAMH 8861/NR_121313, i.e., the type of *H. variabilis* and also other sequences from reliable *H. variabilis* cultures displayed < 97% similarity. The NJ analysis placed OTU 6 in *Hyaloscypha* s. str. and indicated that, together with OTU 367 (6), 506 (3) and 600 (3), it represents a new root-symbiotic species.

OTU 7 belonged to Pleosporales and represented the most abundant non-helotialean OTU (1386 reads); except a single read in *V. vitis-idaea*, it was detected only in the Patagonian hosts, by far being the most abundant in *P. poeppigii* Gpo5 (616 reads). In UNITE, it shared 100/92.4% with SH1645905.08FU (*Uzbekistanica rosae-hissaricae*, Pleosporales: Melanommataceae) while in GenBank, 100/91.7% with *U. rosae-hissaricae* MFLUCC 17-0819/NR_157549 and *U. yakutkhanika* strain MFLUCC 17-0809/MG828977 (both from Uzbekistan) and 100/90.1% with *Alpinaria rhododendri* WU 36914/NR_147686 (Pleosporales: Melanommataceae) from Austria.

OTU 8 represented the archetypal ErMF *H. hepaticicola* (1372 reads). It dominated the mycobiota of *C. vulgaris* (1198) and was also present in *V. myrtilus* (121), while it was absent at five and relatively rare at six Patagonian sites and *V. vitis-idaea*. In addition, *H. hepaticicola* comprised OTU 37, but in contrast to OTU 8, all its reads (375) originated from *G. poeppigii* from three sites in northern Patagonia. Congruently, its closest hit in GenBank (KX611520, 100/99.38%) represented the *H. hepaticicola* isolate C21.8 obtained from *G. caespitosa* from the same area by Bruzone et al. (2017) (also see below). Some other OTU were at first indicated as *H. hepaticicola* but manually reduced to *Hyloscypha* sp. (OTU 105 and OTU 110) or Helotiales sp. (OTU 84 and OTU 92).

OTU 9 represented the most abundant non-ascomycetous OTU with 1341 reads and it belonged to the order Sebaciales (Basidiomycota), the closest sequences in GenBank (100%/100%) originating from *G. hispidula* from Canada [MT027941 and MT027942, Griffin and Kernaghan (2022)]. It was predominantly detected in *V. myrtilus* (1156 reads) and *V. vitis-idaea* (184) with only one other read detected in *G. poeppigii* Gpo9 from the Valdivian rainforest. The next most abundant OTU 10 (1270) also belonged to Sebaciales but in contrast to OTU 9, it was found only in Patagonia. Three of the next four OTU belonged to the same order and in total, there were 42 sebacinoid OTU comprising 8263 reads. Non-sebacinoid basidiomycetous fungi comprised OTU 18 (811, Agaricales sp.), OTU 49 (255, Auriculariales sp.), OTU 57 (196, Auriculariales sp.), OTU 66 (143, *Mycena* sp.), OTU 73 (127, Basidiomycetes sp.), OTU 76 (123, Basidiomycetes sp.), OTU 82 (105, *Mycena* sp.), OTU 121 (54, *Mycena* sp.) and other 69 OTU with < 50 reads.

OTU 13 comprised 1159 reads and the great majority (1131) was detected in *V. myrtilus*, co-dominating its detected root mycobiota with OTU 9 (Sebaciales sp.). The remaining 26 reads were detected in *V. vitis-idaea* and two reads in *G. poeppigii* Gpo4 and Gpo9. In UNITE, it shared 95.2/99.4% with SH2747050.08FU (Leotiomyces sp. detected in Estonia) while in GenBank, 98/93.9% with Helotiales sp. KO-group B (AB986445) isolated from a *Cenococcum geophilum* sclerotium from USA (Obase et al. 2014). When only sequences from type material were considered, OTU 13 displayed 87/88.4% with *Cochlearomyces eucalypti* CBS 142622/NR_156382 (Helotiales: Cochlearomycetaceae) and 87/87.7% with *Variabilispora flava* CBS 144845/NR_165906 (Leotiales: Tympanidaceae), but Blast Tree View placed OTU 13 as a sister clade to *Minutiella tardicola* CBS 121757/MH863154 in Eurotiomycetes. Hence, our conservative assignment of this OTU was Ascomycota sp.

From other significant OTU with < 1000 reads, OTU 32 (460) represented the prErMF *H. variabilis* and in the NJ analysis, it grouped together with OTU 216 (20), OTU 444 (5) and OTU 681 (2), all being detected solely in the N. Bohemian hosts. OTU 43 (309) represented the prErMF *O. maius* and it was detected only in the two *Vaccinium*. Several other OTU belonged to *Oidiodendron*, but could not be reliably assigned to *O. maius*, namely OTU 227 (19 reads, solely in *V. vitis-idaea*), OTU 566 (3, solely in *V. vitis-idaea*), OTU 712 (2, solely in *V. myrtilus*), OTU 749 (2, solely in *V. vitis-idaea*) and OTU 765 (2, solely in *V. vitis-idaea*).

The NJ analyses suggested that none of the delimited OTU belonged to the EcM and poErMF *H. bicolor*, *H. finlandica* and *H. vraolstadiae*. Similarly, none of the delimited OTU belonged to the *Hyaloscypha* spp. recently described from C. Europe, namely the root-symbiotic *H. gabretae* and *H. melinii* and the prErMF *H. gryndleri*. Nevertheless, OTU 89 (in total 95 reads, majority from Patagonia) and OTU 762 (2 reads from Patagonia) showed affinities to *H. bicolor*/*H. finlandica* and OTU 110 (61 reads from Patagonia) showed affinities to *H. gryndleri*. None of the sebacinoid OTU clustered with the Serendipitaceae sp. JPK-132 originating from Mid-Norway (Vohník et al. 2012; Vohník and Réblová 2023), i.e., the only isolate shown to form ericoid mycorrhiza *in vitro* (Vohník et al. 2016).

The comparison with the four previous studies revealed only a few overlaps with OTU detected in this study and only with those with total abundance < 5% (not shown). For example, the chaetothyrialean OTU 4 with total abundance 10.7% detected in *Vaccinium* sp. from N. Bohemia by Lukešová et al. (2015) clustered with the chaetothyrialean OTU 81 and OTU 611 detected here (both detected only in *V. vitis-idaea*), but their total abundances were only 0.18% and 0.003%, respectively. The situation only slightly changed when instead focusing on local abundances. For example, these were 2.7% for OTU 81 and 0.05% for OTU 611. The only exception was OTU 37 that displayed 100%/99.38% sequence coverage/similarity with *H. hepaticicola* isolate C21.8 (GenBank KX611520) obtained from *G. caespitosa* from a treeless alpine site at Cerro Tronador by Bruzone et al. (2017). However, while OTU 37 was in this study detected at three Patagonian sites (reaching up to 11% abundance at a treeless site at Volcán Lanín), it was not detected in *G. poeppigii* from a humid Patagonian forest at Cerro Tronador.

Interestingly, when comparing our consensus sequences with those available in GenBank, a few showed > 99% (up to 100%) coverage/similarity with sequences of isolates from ericaceous roots obtained worldwide. For example, OTU 92 (Helotiales sp. detected both in N. Bohemia and Patagonia) matched 100%/100% a *Calluna vulgaris* root associated fungus from Bavaria, Germany (FM172867, unpublished), OTU 135 (Helotiales sp. from a single site in Patagonia) matched 100%/100% an *Epacris microphylla* root associated fungus #26 from Australia [AY268210, Williams et al. (2004)], and OTU 261 (Ascomycota sp. from a single site in Patagonia) matched 100%/99% an *E. microphylla* root associated fungus #21 from Australia [AY268205, Williams et al. (2004)].

Trophic modes

Most of the detected OTU, specifically 622 OTU with 37302 reads, could not be reasonably assigned to other trophic mode than RAF. Only two OTU could be reliably assigned to the prominent ErMF *H. hepaticicola*, namely OTU 8 (1372) and OTU 37 (375). The guild of prErMF comprised *H. variabilis* (4 OTU/487 reads) and *O. maius* (1/309), while poErMF were represented by sebacinoid fungi (42/8263) and some members of *Hyaloscypha* s. str. (14/1720). Interestingly, we did not detect any helotialean ErMF/prErMF/poErMF in *G. phillyreifolia*, which on the other hand contained five sebacinoid/serendipitoid OTU totaling 136 reads (2.7% of reads detected in this host species). Nine OTU with 9773 reads represented DSE from PAC. OTU 1 was dominating with 9397

reads, being present in all samples. The second most abundant DSE, OTU 46 (286), was mostly confined to Patagonia and only 4 reads were found in *V. myrtillos*. Finally, 17 OTU (520) represented probable saprobes/parasites. These seemed to be randomly scattered in low read numbers over all samples, except OTU 59 where 188 out of the total 195 reads were detected in *C. vulgaris*.

Only a few OTU with very low read numbers could be attributed to typical EcM genera/orders, namely OTU 437 (5, *Tomentella* sp.), OTU 470 (4, *Tomentella* sp.), OTU 530 (3, *Cantharellales* sp.), OTU 551 (3, *Thelephoraceae* sp.), OTU 571 (3, *Cenococcum geophilum*), OTU 582 (3, *Cantharellales* sp.), OTU 587 (3, *Piloderma* sp.), and OTU 720 (2, *Cortinarius* sp.). Only a single OTU could be attributed to AM fungi, namely OTU 603 (2) *Rhizophagus* sp. In this study, all were considered as RAF (see Materials and Methods).

Discussion

The primary goal of this study was to describe and compare the structural and molecular diversity of the fungi associated with ericaceous hair roots from N. Bohemia and Patagonia. In addition to the expected differences between the two geographic regions, we discovered a unique fungal community associated with the two *Gaultheria* spp. from the Valdivian temperate rainforest in Patagonia. The rich spectrum of mycobionts detected here suggests that rather than being dominated by a single ErMF, ericaceous hair roots host a plethora of taxonomically and probably also functionally diverse fungi. Our results challenge some of the cornerstones of the ErM research, especially the significance of the archetypal ericaceous mycobionts *H. hepaticicola*, *H. variabilis*, and *O. maius*, because in many samples they were low in abundance or even absent.

Structural diversity

The typical ErM colonization pattern, namely the intracellular hyphal coils in the host rhizodermis, was detected in all host × site combinations, including the waterlogged site in the Valdivian rainforest. A bit surprising was the high incidence of hyphal mantles that are supposed to be common in ericaceous species belonging to the Andean clade of Ericaceae (typically epiphytic) but not in those outside this clade (terrestrial) (Setaro et al. 2006a). On the other hand, the same mantles/sheaths were already reported in terrestrial *Gaultheria* spp. from Patagonia by Bruzone et al. (2015). At first glance, the hair roots with hyphal mantles observed here resemble sheathed ericoid mycorrhiza (Vohník et al. 2012) and cavendishoid ectendomycorrhiza (Setaro et al. 2006b). However, the mantles were composed of non-clamped hyphae (ruling out the former) and we were unable to unequivocally detect the Hartig net (ruling out the latter). Even more surprising was the presence of morphologically the same hyphal mantles in *Vaccinium* spp. from N. Bohemia. On the other hand, “large quantities of hyphae on rootlets” were already reported in some boreal peatland ericaceous species from Canada by Thormann et al. (1999) and similar mantles were reported in *V. myrtillos* from N. Bohemia by Vohník and Réblová (2023). Interestingly, the mantles were absent in *C. vulgaris* that lacked sebacinoid mycobionts but present in *G. phillyreifolia* that lacked known helotialean ErMF but contained some sebacinoid mycobionts. Hence, we assume that the mantles were formed by sebacinoid mycobionts, similarly to the mantles in cavendishoid ectendomycorrhiza. Given their common presence in the hair roots investigated in this study as well as in the reports listed above, and the fact that they were always accompanied by intracellular hyphal coils in the underlying rhizodermis, we suggest that hyphal mantles are regarded as an integral morphological feature of the ErM symbiosis.

Despite that DSE were present in all host × site combinations, the incidence of microsclerotia typical of DSE was very low. On the other hand, DSE might be present in the form of sparse superficial parenchymatous nets, individual surface and intraradical hyphae, etc. Frequently, there was an apparent continuum between typical ErM and DSE structures, as already reported by Vohník and Albrechtová (2011), often permitting no sharp morphological differentiation of these two root-symbiotic types.

Some hair roots (especially in *V. vitis-idaea*) contained abundant intercellular hyphae, often lightly melanized, that sometimes resembled a rudimentary Hartig net, giving an impression of arbutoid mycorrhiza, but without thick hyphal mantles (no typical arbutoid mycorrhizal hosts were present at any investigated site). Some samples from Patagonia, but none from N. Bohemia, comprised intracellular spheres interpreted here as chlamydospores of serendipitoid fungi (Vohník et al. 2016). Hair roots of *G. poeppigii* from the Valdivian rainforest often contained relatively thick melanized superficial parenchymatous nets that were connected with intracellular hyphal coils in the underlying rhizodermis through the hitherto unreported haustoria-like structures. Similar yet not identical structures were detected in the sympatric *C. antarctica*, but those rather resembled the intracellular haustorium-like structures recently reported in *Vaccinium* spp. from Mid-Norway by Vohník and Réblová (2023). It is unclear why these structures are formed in the rhizodermis of the ericaceous hair roots because they seem to represent only intermediates between extracellular hyphae and intraradical hyphal coils typical of ericoid mycorrhiza. All these morphological features undoubtedly deserve further investigations that were, however, outside the dimensions of this study.

Some hair roots of *G. poeppigii* from the Valdivian rainforest possessed rhizodermal cells with unusually thick cell walls, resembling those reported in cultivated *Rhododendron ponticum* by Duddridge and Read (1982), in *Dracophyllum secundum* from Australia by Allen et al. (1989), in *Lysinema ciliatum* from Australia by Ashford et al. (1996), in *Kalmia angustifolia* and *R. groenlandicum* from Canada by Massicotte et al. (2005), and in *G. hispidula* and *K. angustifolia* from Canada by Griffin and Kernaghan (2022). Ashford et al. (1996) reported that the thick-walled rhizodermal cells were preferentially colonized by ErM fungi and remained intact for longer than the thin-walled cells, hence they suggested that the thick-walled cells might prolong the ErM association and protect it in conditions when the soil is drying out. In addition, these cells may act as infective propagules (Ashford et al. 1996), may prevent the entry of microorganisms and to some extent restrict the passage of potentially toxic materials (Duddridge and Read 1982).

In contrast to some other studies, we did not detect intraradical structures typical of the AM and EcM symbioses. For example, Urcelay (2002) reported ErM (dense hyphal coils), DSE (microsclerotia) and AM structures (arbuscules and vesicles) in *G. poeppigii* from Central Argentina. On the other hand, whereas the ErM structures were detected in hair roots (60–120 µm in diam.), the AM structures were observed in coarser roots (80–250 µm). Such a distributional pattern has already been observed in some Hawaiian ericaceous species by Gemma and Koske (1990) and Koske et al. (1990). However, in this study we focused only on hair roots.

Variability in the mycobiont community structure

Perhaps the most surprising result of our study was the separation of the Valdivian mycobiont community from the rest of Patagonia, while their distance in the NMDS graph was practically the same as the distance between the rest of Patagonia and N. Bohemia. The N. Bohemian samples originated from *Calluna* and *Vaccinium*, i.e., the hosts whose mycobiont communities served as the backbone of the early modern ErM research (Pearson and Read 1973a). However, when looking closely at the N. Bohemian communities, only *C. vulgaris* corresponds to the original view about the dominance of ascomycetes, and especially *H. hepaticicola*, in ericaceous hair roots (also see below). Nevertheless, also *C. vulgaris* contained mycobionts with unclear trophic status and/or taxonomy, e.g., OTU 24 (ca. 19% of reads) and OTU 29 (ca. 17%). While the mycobiome of *C. vulgaris* was dominated by *H. hepaticicola* (ca. 32%) and comprised a member of PAC (ca. 5%) and *H. variabilis* (ca. 3%), very little can be said about the significance of the remaining 60%. For example, the closest SH in UNITE for OTU 24 is SH0994854.09FU/*Colpoma* sp. A mycobiont with affinities to *Colpoma* has already been detected in *C. vulgaris* [GenBank DQ309240, Bougoure et al. (2007)], but since this genus harbors weak parasites and lignicolous saprobes (Darker 1967), its functioning in ericaceous roots remains obscure. Since the archetypal ErMF *H. hepaticicola* was only recently transferred to the genus *Hyaloscypha* that contains many lignicolous saprobes (Fehrer et al. 2019), OTU 24 might turn out to be a novel ErMF. However, this can only be verified experimentally. The mycobiome of *V. myrtillus* was dominated by the sebacinoid OTU 9 (ca. 26%) and OTU 13 (ca. 25%) that displayed affinities to Tympanidaceae (SH1271393.09FU). This family harbors eight genera of weak parasites and saprobes (<https://fungalgenera.org>, accessed January 2023) and similarly to OTU 24, its trophic mode in *V. myrtillus* remains unknown. Finally, the mycobiome of *V. vitis-idaea* was dominated by sebacinoid fungi (7 OTU, ca. 35%) and comprised *O. maius* (ca. 7%), *H. variabilis* (2 OTU, ca. 1.5%), and *H. hepaticicola* (1 OTU, ca. 0.1%). On the other hand, it also comprised a member of PAC (ca. 21%) and three OTU with affinities to *Mollisia* (Helotiales, Mollisiaceae) (ca. 16%). *Mollisia* contains common saprobes typically colonizing decaying wood and graminoid culms and leaves, but also endophytes of leaves, twigs, and roots in diverse hosts (Tanney and Seifert 2020). The mycobiome of *V. vitis-idaea* was thus composed of both ascomycetous and basidiomycetous ErMF and prErMF accompanied by diverse endophytes and fungi with unknown trophic modes, whose possible interactions are probably beyond any reasonable experimental design.

The two hosts from the Valdivian rainforest harbored a unique mycobiont community with some abundant OTU endemic to this peculiar ecosystem. Examples include OTU 2, the second most abundant in the whole dataset (ca. 4.9%), the sixth most abundant OTU 6 (ca. 2.4%), and OTU 30 (ca. 1%). Despite that OTU 2 and OTU 6 dominated the mycobiomes of *G. antarctica* and *G. poeppigii* from the Valdivian rainforest, respectively, very little can be said about their precise taxonomy and practically nothing about their trophic modes (see below). Perhaps one of them is responsible for the unusual colonization pattern comprising the haustoria-like intracellular structures found in *G. poeppigii*, which can only be tested experimentally, provided that axenic isolates of these mycobionts are available. Interestingly, *G. antarctica* was practically free of sebacinoid mycobionts and the incidence of *H. hepaticicola* was very low (ca. 0.6%), while *G. poeppigii* harbored one major sebacinoid OTU (ca. 9.6%) and no *H. hepaticicola*.

The mycobiomes of the rest of the Patagonian host × site combinations were too diverse to be addressed individually, with an intriguing exception of *G. phillyreifolia* whose mycobiome lacked fungi that could be unambiguously attributed to ErMF and prErMF (see below). In general, one *H. hepaticicola* OTU occurred only in the rest of Patagonia, an observation already made by Bruzone et al. (2017), while the other was very rare in the Valdivian rainforest, even rarer in the rest of Patagonia but relatively abundant in N. Bohemia (especially in *C. vulgaris*). A similar trend could be seen for the sebacinoid fungi because those detected in Patagonia never occurred in N. Bohemian [cf. Setaro and Kron (2011)]. This might be mirrored in the occurrence of the intracellular chlamydospore-like spheres that were unique to several host × site combinations from the rest of Patagonia. There were many abundant OTU endemic to the rest of Patagonia, examples including OTU 3 (Helotiales sp.) dominating *G. phillyreifolia* (ca. 24%) and occurring in seven of the eight host × site combinations, OTU 7 (Pleosporales sp.) co-dominating *G. poeppigii* from Chaltén (ca. 12%), OTU 18 (Agaricales sp.) dominating *G. caespitosa* (ca. 24%), and OTU 25 (Chaetothyriales sp.) co-dominating *G. poeppigii* from Cerro Otto (ca. 14%). While *Hyaloscypha* s. str. belongs to Helotiales, by far not all helotialean fungi are ericoid mycorrhizal. Next to nothing can be said about functioning of members of Agaricales, Chaetothyriales, and Pleosporales in ericaceous hair roots.

Former studies from the same areas

The diversity of mycobionts associating with hair roots of *Gaultheria* spp. from Patagonia was first reported by Bruzone et al. (2015). Surprisingly, the detected cultivable spectra of *G. phillyreifolia* (misidentified as *G. mucronata* in that paper) and *G. poeppigii* lacked *H. hepaticicola* and *O. maius* and the authors hypothesized that these prominent ericaceous mycobionts were substituted by the difficult-to-cultivate sebacinoid fungi. The same host × site combinations were investigated in this study (sample codes Gph1 and Gpo2) and our results confirm the absence of *O. maius* in both hosts (also see below) as well as the absence and near absence of *H. hepaticicola* in *G. phillyreifolia* and *G. poeppigii* from Llao Llao, respectively (only 10 reads could be assigned to *H. hepaticicola* in the latter species). On the other hand, while the sebacinoid mycobionts formed ca. one fifth of the mycobiome of *G. poeppigii*, it was only 2.7% of *G. phillyreifolia*. The investigated *G. phillyreifolia* hair roots possessed ericoid mycorrhizae but the identity of the respective mycobionts remains unclear, because except the rare sebacinoid fungi, we could not identify any known ErMF and prErMF. Since the mycobiome of *G. phillyreifolia* was dominated by unnamed helotialean fungi (44 OTU, ca. 63%), we suppose that some of them might represent novel ErMF awaiting discovery.

In a follow-up study, Bruzone et al. (2017) reported spectra of cultivable mycobionts of *G. caespitosa* and *G. pumila* from a treeless alpine site at the foothills of Cerro Tronador that were dominated by *H. hepaticicola* (ca. 37% of the obtained isolates). One of the sites investigated here was at the foothills of Cerro Tronador, but at a lower altitude (1635 vs. 1100 m a. s. l.), in a humid Patagonian forest dominated by the EcM host *N. pumilio*, and we investigated *G. poeppigii* (sample code Gpo4). In contrast to Bruzone et al. (2017), the incidence of *H. hepaticicola* was negligible (4 reads), illustrating how fungal communities in ericaceous roots may dramatically differ at sites a few hundreds of meters apart, depending on the interplay between the host, the local environmental conditions (including presence/absence of EcM hosts), and the availability of the fungal inoculum.

The first report on ericaceous hair root mycobionts from C. Europe is the study by Lukešová et al. (2015), whose dataset was dominated by unnamed helotialean and chaetothyrialean species. Interestingly, at the three sites in N. Bohemia investigated by Lukešová et al. (2015), the prominent ericaceous mycobionts *H. hepaticicola*, *H. variabilis*, and *O. maius* were either absent or very low in abundance. The overlap between our dataset and the dataset of Lukešová et al. (2015) is negligible. For example, one of the dominant OTU in Lukešová et al. (2015) was the chaetothyrialean OTU 4 (total abundance 10.7%) that clustered with two of the OTU detected here, but their total abundances were only 0.18% and 0.003%.

The only other report from C. Europe is the recent study by Vohník and Réblová (2023) who investigated the mycobiota of *V. myrtilus* growing on decomposing wood at a locality identical to one of the sites investigated here (sample code Vmy10; this sample, however, did not originate from plants growing on decomposing wood). The authors revealed a novel lineage in *Hyaloscypha* s. str. that forms ericoid mycorrhizae (represented by *Hyaloscypha* sp. CBS 149189), but this lineage was missing in our dataset, illustrating how local edaphic factors (at the scale of meters) profoundly affect the composition of the mycobiota of ericaceous hair roots.

Ascomycetes vs. basidiomycetes in ericaceous hair roots

In general, our results confirm the dominance of ascomycetes in the mycobiome of ericaceous hair roots as well as the fact that ascomycetes together with basidiomycetes represent an overwhelming majority of ericaceous hair root mycobionts (here in average 99% of all reads). However, there were significant differences between individual host × site combinations, the extremes being *C. vulgaris* from N. Bohemia totally dominated by ascomycetes (98.6%) and *G. caespitosa* from Patagonia actually dominated by basidiomycetes (52.1%). On the one hand, these observations question the general significance of *C. vulgaris* as a model ErM host (Rayner 1915; Read 1974; Pearson and Read 1975; Leake and Read 1991). On the other hand, since the most abundant OTU of *G. caespitosa* displayed affinities to clavarioid fungi, they give credit to the earlier observations on the peculiar association of clavarioid fruitbodies with certain ericaceous plants (Seviour et al. 1973; Englander and Hull 1980; Mueller et al. 1986) that are now practically forgotten. Apparently, more studies focusing on both sebacinoid and non-sebacinoid basidiomycetous mycobionts are needed to fully understand the functioning of the ErM symbiosis.

Trophic modes

One of the surprising (and indeed frustrating) results of our study is the absolute dominance of mycobionts with uncertain symbiotic status (“root-associated fungi”). While many represented only a very minor part of the detected local communities, some (co-)dominated individual host × site combinations or were relatively common in many host × site combinations. Examples include OTU 2 (*Helotiales* sp.) dominating *G. antarctica* from the Valdivian rainforest (ca. 38%), OTU 6 (*Hyaloscypha* sp.) dominating *G. poeppigii* from the Valdivian rainforest (ca. 33%), and OTU 3 (*Helotiales* sp.) dominating *G. phillyreifolia* from Llao Llao (ca. 24%). At the same time, these mycobionts do not necessarily represent species limited to ericaceous hosts from Patagonia – for example, the closest relatives of OTU 2 were a mycobiont from an ectomycorrhiza of *N. cunninghamii* from Tasmania and a mycobiont from a root of *V. calycinum* from Hawaii. Certainly, it would be beneficial to isolate these mycobionts into pure culture, test their symbiotic potential in vitro, and ideally provide their formal taxonomic treatment (Vohník 2020).

Another unexpected result is the relatively low abundance of ErMF and prErMF, namely *H. hepaticicola*, *H. variabilis*, and *O. maius*, in our dataset. While it would be tempting to assign all sebacinoid OTU as ErMF, to date only a single serendipitoid isolate has been shown to form ericoid mycorrhizae in vitro (Vohník et al. 2016) and no close relatives of this mycobiont were detected in our dataset. Indeed, many serendipitoid mycobionts have a potential to colonize ericaceous hair roots but the mode of their functioning is probably endophytic rather than ericoid mycorrhizal (Vohník et al. 2016).

Contrary to our expectations but in agreement with our microscopic observations, only a relatively small proportion of the detected OTU and sequence reads could be assigned to the DSE from PAC. On the one hand, this contrasts with the results of many microscopic [e.g., Vohník and Albrechtová (2011)] and culture-dependent [e.g., Bruzone et al. (2015)] studies. On the other hand, a taxonomically wide range of fungi produce dark septate hyphae (Vohník 2022) and culture-dependent studies favor PAC mycobionts because of their fast growth.

Typical saprobes/parasites were very low in abundance, probably because we focused on healthy-looking hair roots. However, it is likely that at least some were hidden among the many OTU with unclear symbiotic status (also see above).

Missing fungi

As a prominent ErMF of core Ericaceae, *H. hepaticicola* was believed to be restricted to the Northern Hemisphere [see Bruzone et al. (2015) and references therein]. However, this was to a large extent due to limited sampling efforts and recent studies discovered this species in the roots of ericaceous plants from Patagonia (Bruzone et al. 2017), southern Africa (Kohout and Tedersoo 2017), and Australia (Midgley et al. 2017). While in this study *H. hepaticicola* was detected in all N. Bohemian and most Patagonian ericaceous species (although often in very low to negligible abundances), the Patagonian samples lacked the two other prominent ericaceous mycobionts common in the N. Hemisphere, namely *H. variabilis* and *O. maius*.

The absence of *H. variabilis* in Patagonia was surprising, especially because of the presence of *H. hepaticicola* and other *Hyaloscypha* spp. in most of the sampled *Gaultheria* spp. While *H. variabilis* does not have known sexual state and to our knowledge its cultures have never been reported to form conidia [but see the recently reported conidiogenesis of *H. bicolor* in Fehrer et al. (2019)], Grelet et al. (2010) found genetically indistinguishable populations of *H. variabilis* some 20 km apart, the mode(s) of dispersal over such a distance remaining unclear. Although *H. variabilis* is a frequent root mycobiont of both ericaceous and non-ericaceous plants in the N. Hemisphere [e.g., Hambleton and Sigler (2005); Grelet et al. (2010); Vohník et al. (2013)], for unknown reasons it indeed seems to be absent in the Southern Hemisphere [see the distribution of SH1080697.09FU and SH1277360.09FU in UNITE, also see Kohout (2017)].

Unlike *H. variabilis*, *O. maius* regularly forms abundant conidia and its probable sexual state belongs to *Myxotrichum* (Myxotrichaceae) (Rice and Currah 2002), hence its absence in Patagonia was even more surprising. Bruzone et al. (2015) obtained five isolates with affinities to *O. maius* from *G. poeppigii* from Cerro Otto and Llao Llao, but upon closer inspection, they do not seem to be conspecific with *O. maius*, displaying only ca. 97% ITS sequence similarity with

the NCBI Reference Sequence NR_111034 from the type specimen *O. maius* UAMH 1540. An *Oidiodendron* from a soil sample collected close to Lago Mascaradi (NP Nahuel Huapi, ca. 30 km from Cerro Otto and Llao Llao, in UNITE as UDB02323453) displays a similar relationship to *O. maius* UAMH 1540 and seems to be conspecific (100%/99.57% ITS rDNA sequence coverage/similarity) with the *Oidiodendron* isolated by Bruzone et al. (2015). They probably represent a new species sister to *O. maius* that awaits a formal taxonomic treatment [also see Kohout (2017)].

Other common associates of ericaceous roots that were not present in our dataset comprise *Lachnum*, *Leohumicola*, and the two recently described genera that seem to be specific to the S. Hemisphere, namely *Cairneyella* and *Gamarada*. The absence of the latter two genera in Patagonia is puzzling, because some Patagonian OTU were identical with fungi isolated from Australian *E. microphylla* (Williams et al. 2004). In agreement with the microscopic screening, we did not detect *K. argillacea*, the mycobiont forming sheathed ericoid mycorrhiza. On the other hand, it is difficult or even impossible to detect *K. argillacea* using common “universal” PCR primers, incl. those employed here (Vohník et al. 2012).

The presence of AM fungi in our dataset was negligible (a single glomeromycotan OTU with 2 reads) and only a few OTU with very low read numbers could be ascribed to genera/families/orders harboring typical EcM fungi, such as Cantharellales, *Cenococcum geophilum*, *Cortinarius*, *Piloderma*, and *Tomentella*. Similarly, we did not detect the *Hyaloscypha* spp. known to form ectomycorrhizae and at the same time supposed to form ericoid mycorrhizae, namely *H. bicolor*, *H. finlandica*, and *H. vraolstadiae*. Surprisingly, neither we detected the root-symbiotic *H. gabretae*, *H. gryndleri*, and *H. melinii*, all recently described from C. Europe (Fehrer et al. 2019; Vohník et al. 2022).

Conclusions

Fungal symbionts may constitute up to 80% of the biomass of the rhizodermal cells of the ericaceous hair roots (Read and Kerley 1999) and our study illustrates their rich structural and molecular diversity. The detected mycobiont spectra were dominated by unnamed fungi with unknown trophic statuses and most likely contain many novel ErM lineages. Many host × site combinations lacked the archetypal ericaceous mycobionts and one can only wonder what fungi took over their functioning. Our results confirm that sebacinoid mycobionts are common associates of ericaceous hair roots (Selosse et al. 2007), yet it is not clear whether all of them represent ErMF. It is unknown why some ericaceous root mycobionts have a cosmopolitan distribution (e.g., the prominent ErMF *H. hepaticicola*) while others seem to be restricted to the N. Hemisphere (*H. variabilis* and *O. maius*) or the S. Hemisphere (*C. variabilis* and *G. debralockiae*). Studies of the factors affecting the distribution of ErMF, incl. investigations of their dispersal modes [that are relatively well-known for other guilds of mycorrhizal fungi, e.g., Horton (2017)], are needed to fully understand biogeography of the ErMF.

Declarations

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Figures

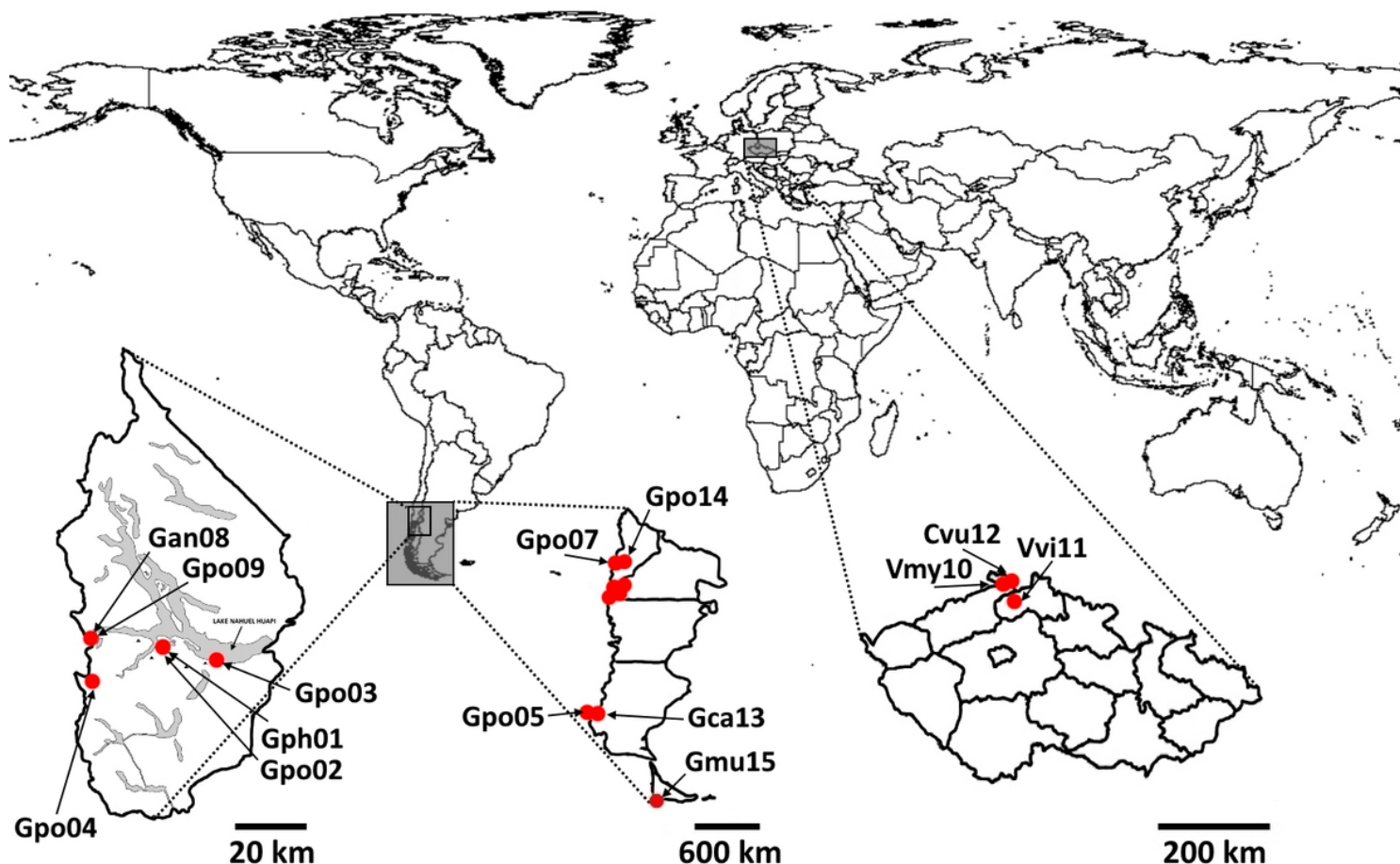


Figure 1

Sampling sites in Northern Bohemia and Patagonia. In total, 12 sites were investigated in this study (red dots), three in N. Bohemia (Czechia) and nine in Argentine Patagonia. At two Argentine localities, two ericaceous species were sampled, resulting in 14 different host × site combinations (codes Gph1–Gmu15). Abbreviations: **Cvu** = *Calluna vulgaris*; **Gan** = *Gaultheria antarctica*; **Gca** = *G. caespitosa*; **Gmu** = *G. mucronata*; **Gph** = *G. phillyreifolia*; **Gpo** = *G. poeppigii*; **Vmy** = *Vaccinium myrtillus*; **Vvi** = *V. vitis-idaea*.

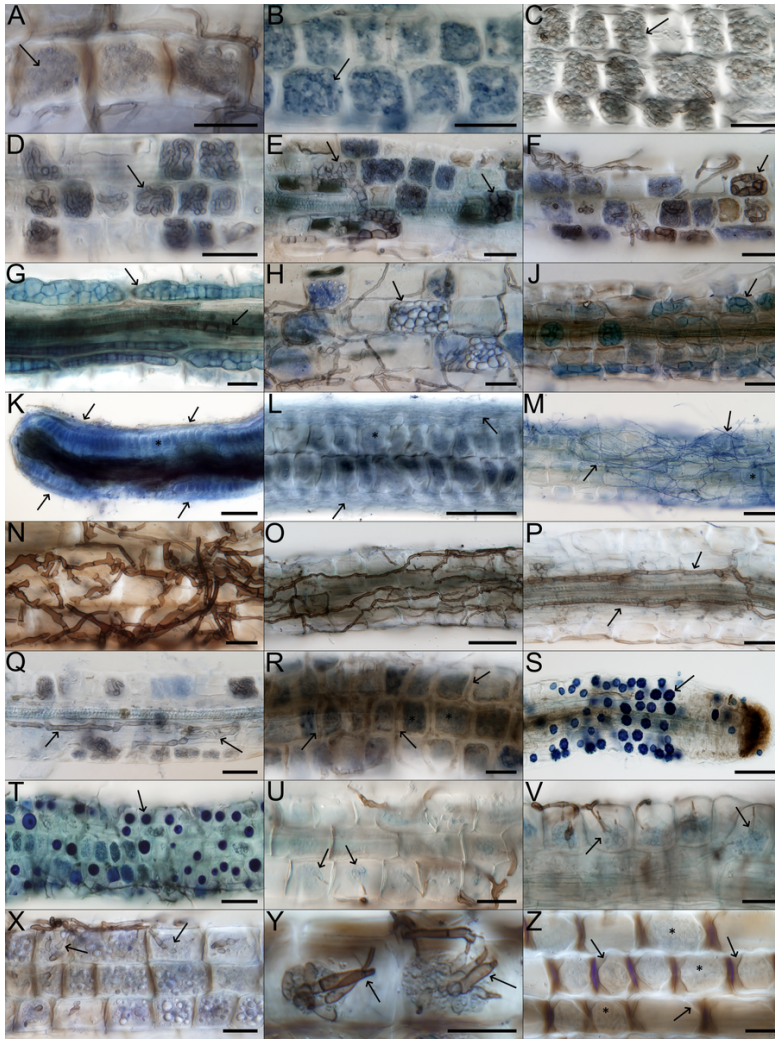


Figure 2

Morphological features of fungal colonization in ericaceous hair roots investigated in this study. **A–D:** intracellular hyphal coils typical for ericoid mycorrhiza (some examples indicated with arrows). **E&F:** Melanized intracellular microsclerotia typical for the dark septate endophytes (arrows). **G–J:** Hyaline microsclerotia, here stained with trypan blue (some examples indicated with arrows), resembling those formed by some sebacinoid fungi. **K–M:** Hyphal mantles (arrows) on the surface of hair roots whose rhizodermal cells contain hyphal coils (some examples indicated with asterisks) typical for ericoid mycorrhiza. **N:** A sparse parenchymatous net formed by melanized septate hyphae. **O:** Dark septate hyphae on the surface of an ericaceous hair root. **P&Q:** Intraradical dark septate hyphae (arrows) running parallel to vascular elements. **R:** Intercellular melanized septate hyphae (some examples indicated with arrows) accompanied by intracellular hyphal coils typical for ericoid mycorrhiza (some examples indicated with asterisks). **S&T:** Blue-stained intracellular spheres (some examples indicated with arrows), typically one per host cell, interpreted here as chlamydospores of serendipitoid fungi. They were observed in hair roots either free of other fungal colonization (S) or containing intracellular hyphal coils typical for ericoid mycorrhiza (T). **U–Y:** Intracellular haustoria-like structures (some examples indicated with arrows). **Z:** Thickened cell walls of rhizodermal cells (some examples indicated with arrows), some of them containing hyphal coils typical for ericoid mycorrhiza (some of them indicated with asterisks). Hair root samples of *Gaultheria antarctica* (U), *G. caespitosa* (A), *G. phillyreifolia* (C, G and J), *G. poeppigii* (K–N, O, P, S, T, V and X–Z), *Vaccinium myrtillus* (B, D, E, H and Q) and *V. vitis-idaea* (F and R). Scale bars 20 μm (A–J, N, P–R and U–Z) and 50 μm (K–M, O, S and T).

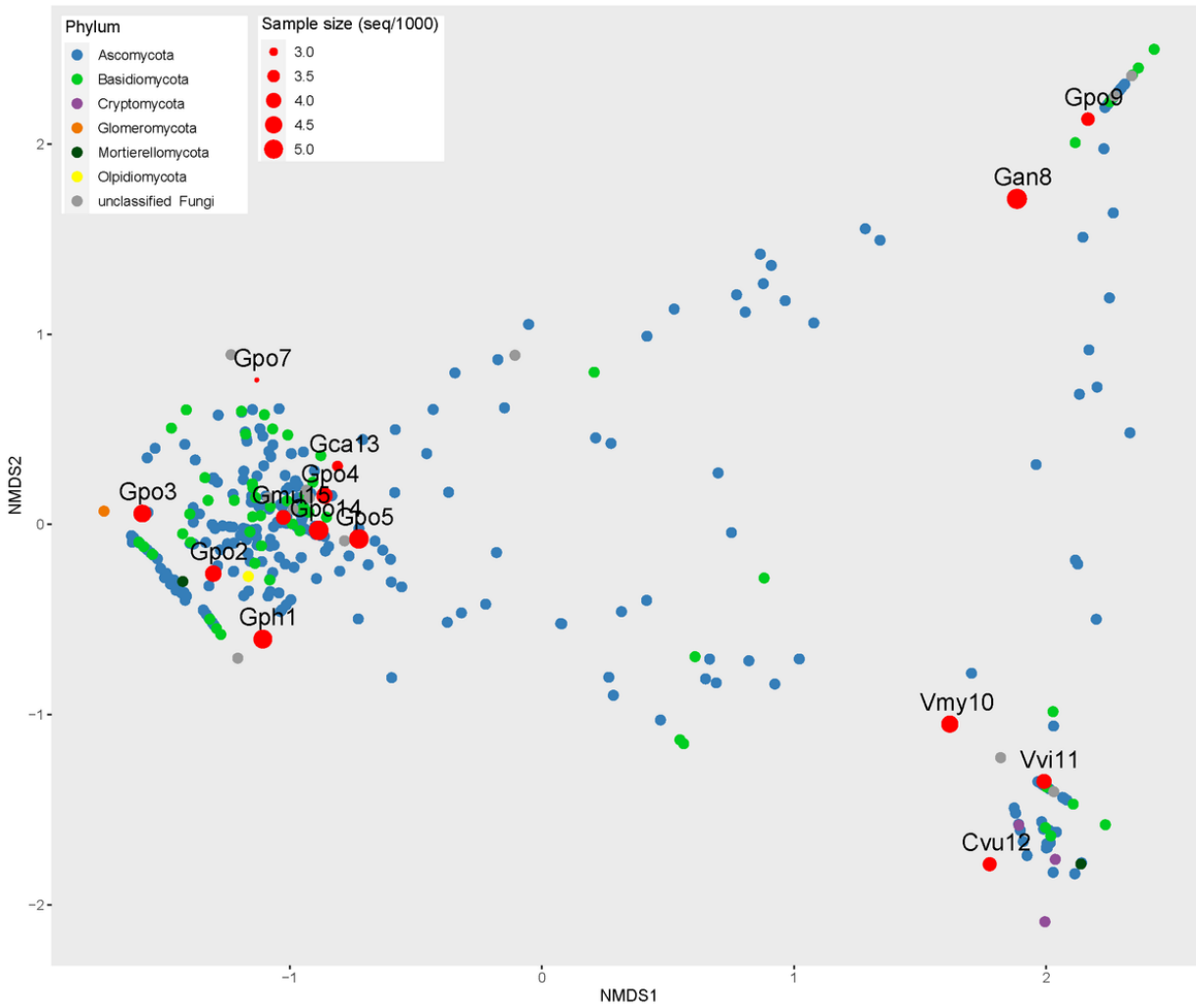


Figure 3

Non-metric multidimensional scaling (NMDS) ordination of the 14 host × site combinations by their mycobiont compositions. Abbreviations: **Cvu** = *Calluna vulgaris* (N. Bohemia); **Gan** = *Gaultheria antarctica* (Patagonia); **Gca** = *G. caespitosa* (Patagonia); **Gmu** = *G. mucronata* (Patagonia); **Gph** = *G. phillyreifolia* (Patagonia); **Gpo** = *G. poeppigii* (Patagonia); **Vmy** = *Vaccinium myrtillus* (Czechia); **Vvi** = *V. vitis-idaea* (Czechia).

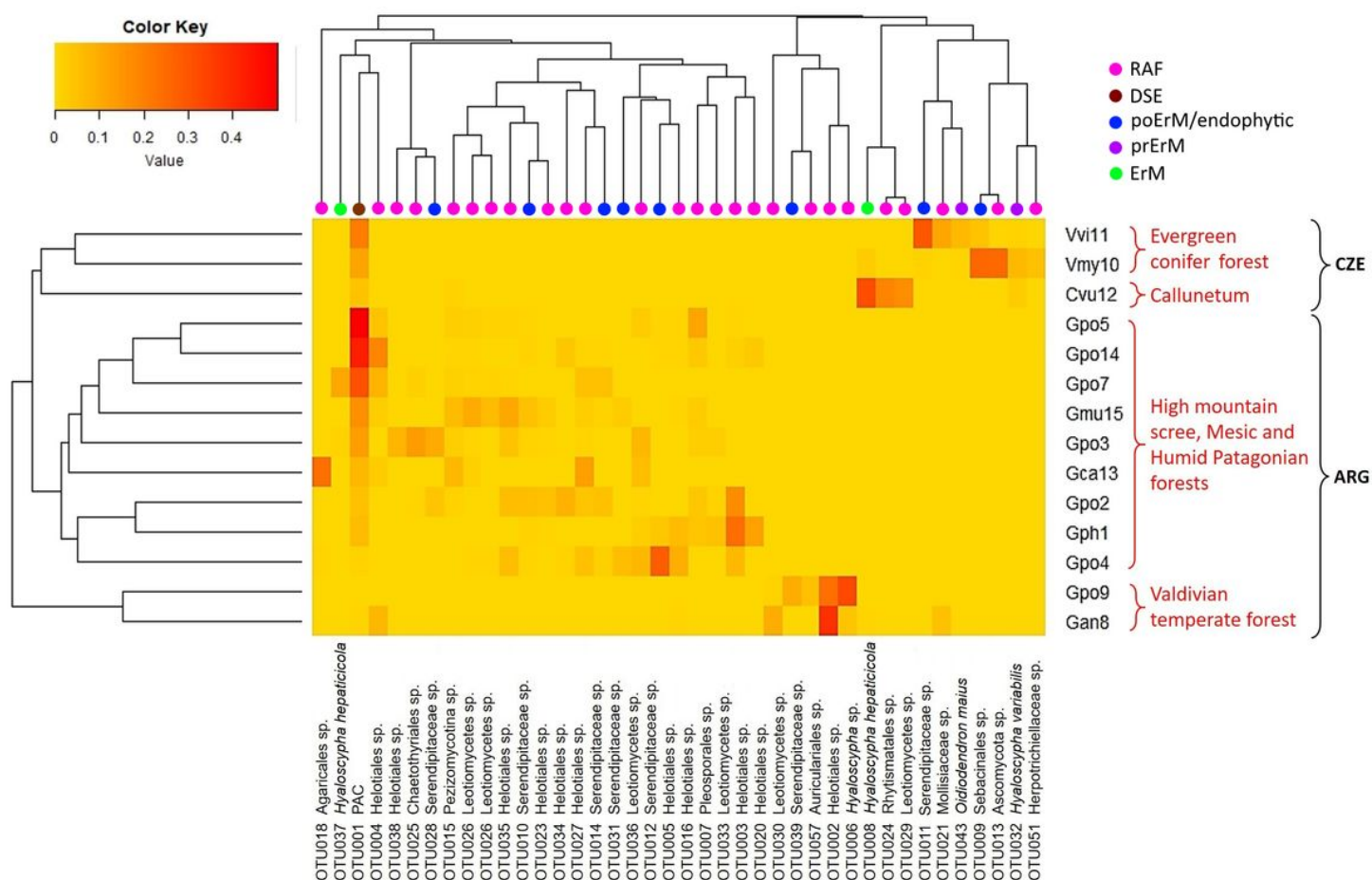


Figure 4

Heatmap displaying composition of fungal communities in ericaceous roots from Northern Bohemia and Patagonia. Due to visualization limitations, operational taxonomic units with less than 5 % as their maximum local abundance are not shown. Abbreviations host plants: **Cvu**= *Calluna vulgaris*; **Gan**= *Gaultheria antarctica*; **Gca**= *G. caespitosa*; **Gmu** = *G. mucronata*; **Gph** = *G. phillyreifolia*; **Gpo** = *G. poeppigii*; **Vmy** = *Vaccinium myrtillus*; **Vvi** = *V. vitis-idaea*. Other abbreviations: **ARG** = Argentina (Patagonia); **CZE** = Czechia (Northern Bohemia); **DSE** = dark septate endophytes; **ErM** = ericoid mycorrhizal; **poErM** = possibly ericoid mycorrhizal; **prErM** = probably ericoid mycorrhizal; **RAF** = root-associated fungi.

Supplementary Files

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- [Supplementarytable1final.xlsx](#)