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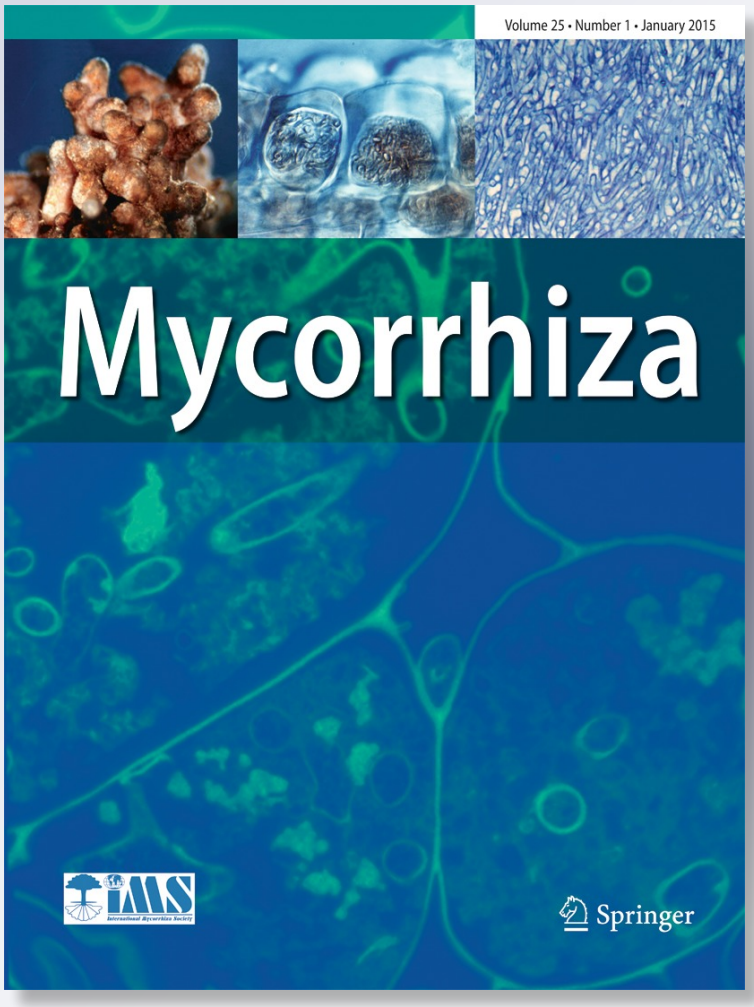

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ORIGINAL PAPER

Is the prominent ericoid mycorrhizal fungus *Rhizoscyphus ericae* absent in the Southern Hemisphere's Ericaceae? A case study on the diversity of root mycobionts in *Gaultheria* spp. from northwest Patagonia, Argentina

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Abstract Ericaceae diversity hotspots are in the mountains of the Neotropics and Papua New Guinea, South Africa's fynbos and Southeast Asia but majority of references to their root mycobionts come from the Northern Hemisphere. Here, typical cultivable ericoid mycorrhizal (ErM) fungi comprise *Rhizoscyphus ericae*, *Meliniomyces variabilis*, and *Oidiodendron maius*. It is however unclear whether this is true also for the Southern Hemisphere. Our study focused on cultivable mycobionts from hair roots of *Gaultheria mucronata* and *Gaultheria poeppigii* (Ericaceae) from two natural forests in NW Patagonia, Argentina, differing in mycorrhizal preferences of their tree dominants. We detected 62 well-defined OTUs mostly belonging to Helotiales and Hypocreales; the most frequent were *Phialocephala fortinii* s. l., *Pochonia suchlasporia*, and *Ilyonectria radicicola*. Only one out of 257 isolates showed ITS nrDNA similarity to members of the *R. ericae* aggregate (REA) but was not conspecific with *R. ericae*, and only five isolates were conspecific with *O. maius*. Microscopic observations showed that the screened roots were frequently colonized in a manner differing from the pattern typically produced by *R. ericae* and *O. maius*. A re-synthesis experiment with selected isolates showed that only *O. maius* formed colonization resembling ericoid mycorrhiza. Amplification of root fungal DNA with REA-specific and Sebacinaceae-specific primers showed that REA mycobionts were present in some of the screened samples while Sebacinaceae were present in all samples. These results suggest that *Gaultheria* spp. from NW Patagonia form ericoid mycorrhizae predominantly with the difficult-to-cultivate Sebacinaceae while the incidence of REA is relatively low and may be masked by other most likely non-mycorrhizal cultivable mycobionts.

Keywords Ericoid mycorrhizae · Root endophytes · Global fungal distribution · *Phialocephala fortinii* · Sebacinaceae · *Rhizoscyphus ericae*-specific primers

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Introduction

At present, there is reliable information about mycorrhizal status of some 336 plant families representing 99 % of all flowering plants and it was calculated that arbuscular mycorrhizae (AM), orchid mycorrhizae, ectomycorrhizae, and ericoid mycorrhizae (ErM) are present in roots of 74, 9, 2, and 1 % of angiosperm species, respectively (Brundrett 2009). The least frequent ErM symbiosis has been reported in about 30 genera (Wang and Qiu 2006) belonging to Cassiopoideae, Ericoideae, Harrimanelloideae, Styphelioideae, and Vaccinioideae subfamilies of the Ericaceae. Ericaceae also comprise groups forming arbutoid, cavendishioid and monotropoid ectendomycorrhizae, and arbuscular mycorrhizae (Selosse et al. 2007; Setaro et al. 2006). These mycorrhizal types, and especially ErM, are viewed as key adaptations enabling Ericaceae to survive and dominate in nutrient poor soils of waste areas in both Northern and Southern Hemispheres (Read et al. 2004).

The ability to form ErM has been experimentally confirmed for several ascomycetes, especially those from the

Rhizoscyphus ericae aggregate (REA) (Pearson and Read 1973; Vrålstad et al. 2002; Grelet et al. 2009), and one hitherto undescribed basidiomycete with affinities to Trechisporales (Vohník et al. 2012). There is also complex non-experimental evidence that ericoid mycorrhiza is commonly formed by the ubiquitous basidiomycetous root symbionts belonging to Sebacinaceae (Selosse et al. 2007). However, the spectrum of mycobionts detected in Ericaceae hair roots is considerably larger (e.g., Bougoure and Cairney 2005a; Bougoure and Cairney 2005b; Walker et al. 2011; Gorzelak et al. 2012) suggesting that further re-synthesis experiments may expand the list of confirmed ErM fungi.

Although Ericaceae diversity hotspots are in the mountains of the Neotropics and Papua New Guinea (Luteyn 2002), South Africa's fynbos (Oliver 2000) and Southeast Asia (Luteyn 2002), the majority of references to their root-associated fungi come from the Northern Hemisphere, especially Europe and North America (Wang and Qiu 2006). In these areas, ericaceous root symbiont communities seem to be dominated by dark septate endophytes (DSE) (Ishida and Nordin 2010; Walker et al. 2011; Vohník and Albrechtová 2011; Gorzelak et al. 2012), members of REA, especially *R. ericae* and *Meliniomyces variabilis* (Grelet et al. 2010; Kjøller et al. 2010), *Oidiodendron maius* (Usuki et al. 2003) and/or Sebacinales (Allen et al. 2003). In contrast, the few reports from other regions (especially Australia) suggest that Southern Hemisphere Ericaceae may host different root mycobiont spectra which possibly lack the prominent ericoid mycorrhizal fungus *R. ericae* sensu stricto (Williams et al. 2004; Bougoure and Cairney 2005a; Curlevski et al. 2009). For example, Bougoure and Cairney (2005b) investigated mycobionts of *Rhododendron lochiae* in an Australian tropical cloud forest and obtained only one isolate out of 331 (RFLP type 19, GenBank AY699657) which belonged to REA but was not conspecific with *R. ericae*. Additionally, the authors obtained one DGGE band amplified from the DNA extracted directly from *R. lochiae* roots which belonged to REA but had closest ITS sequence similarity (94 %) to *Meliniomyces vraolstadiae*. On the other hand, there seem to be no reports from other large and Ericaceae-rich Southern Hemisphere areas (e.g., South Africa, South America) which must be investigated before any solid conclusion on the global distribution of *R. ericae* is made.

The moderate knowledge of root-fungus symbioses of South America's Ericaceae is mostly derived from microscopic observations of cleared and stained hair roots. By this way, the presence of ErM has been documented in *Gaultheria caespitosa* (syn. *Pernettya minima*), *Gaultheria mucronata*, *Gaultheria phillyreifolia*, and *Gaultheria pumila* in southern Chile (Godoy et al. 1994), *Gaultheria poeppigii* in central Argentina (Urcelay 2002), *G. mucronata* in northwest Patagonia (Fontenla et al. 2001), and *Cavendishia melastomoides*, *Disterigma humboldtii*, and *Gaultheria erecta* in Costa Rica (Rains et al. 2003). Additionally, a morphologically distinct mycorrhizal type formed by a sebacinaceous mycobiont named cavendishioid ectendomycorrhiza was discovered in *Cavendishia nobilis* var. *capitata* in southern Ecuador (Setaro et al. 2006). Although it is plausible to assume that all South American members of Ericoideae and Vaccinioideae form ericoid mycorrhizae (or cavendishioid ectendomycorrhizae), there is no information available on the diversity of their root mycobionts.

Gaultheria is one of the five largest genera in Ericaceae comprising some 115 to 180 species (Luteyn 2002) commonly occurring throughout the continental areas and islands bordering the Pacific Rim (Lu et al. 2010). About 16 *Gaultheria* species are endemic to Andean páramo and approximately 60 to Southeast Asia (Luteyn 2002). *G. mucronata* and *G. poeppigii* commonly occur in South America while *Gaultheria shallon* is widespread along the North America's Pacific coast from Alaska to California (Fraser et al. 1993). To our knowledge, the diversity of fungi colonizing roots of the two former species in South America has not yet been examined; on the other hand, *G. shallon* is a species threatening regeneration of local mostly ectomycorrhizal conifers after clear-cutting and slash burning (Mallik 2003) and as ErM was expected to be one of the significant factors in this process; its root mycobionts obtained relatively large attention (Xiao and Berch 1996; Berch et al. 2002; Allen et al. 2003).

The objectives of our study therefore were the following: (1) to determine the diversity of cultivable fungal root symbionts of *G. mucronata* and *G. poeppigii* growing in two forests, one dominated by arbuscular mycorrhizal *Austrocedrus* and the other by ectomycorrhizal *Nothofagus*, in NW Patagonia, Argentina; (2) to test the potential for the obtained cultivable fungal symbionts to form ErM structures in *Gaultheria* roots in a re-inoculation experiment; and (3) to use specific primers on total root DNA extracts to detect REA mycobionts as well as sebacinoid fungi, the ubiquitous inhabitants of Ericaceae roots which are notoriously difficult to cultivate (Selosse et al. 2007). Our hypotheses were that (1) the screened *Gaultheria* spp. roots will harbor fungal symbionts differing from those commonly detected in roots of the Northern Hemisphere Ericaceae, (2) the cultivable spectra of fungal root symbionts will comprise ErM fungi not common in the Northern Hemisphere Ericaceae, and (3) we will not detect any member of the *R. ericae* aggregate but the roots will contain abundant sebacinoid fungi.

Materials and methods

Collection of *G. mucronata* and *G. poeppigii* samples

Root samples were collected at the end of the NW Patagonia vegetation season (June 2011) in two forests in the vicinity of

San Carlos de Bariloche, Río Negro, Argentina. The first was a humid *Nothofagus* forest located in Bahía Lopez of the Nahuel Huapi Lake (Llao Llao area); the sampling site (S 41° 04.455′ W 71° 34.081′; 778 m above sea level) had ca. 2-cm-thick continuous layer of leaf litter with high percentage of soil coverage; the tree vegetation was dominated by *Nothofagus dombeyi*, an evergreen broad-leaved tree that forms ectomycorrhiza (Fontenla et al. 1998) while the ground vegetation consisted mainly of shrubs and perennial herbs such as *Alstroemeria aurea*, *Aristotelia chilensis*, *Azara microphylla*, *Chusquea culeou*, *Luma appiculata*, *Maytenus boaria* and *Vicia nigricans*, all of them forming AM, and *G. mucronata* and *G. poeppigii* which forms ErM (Fontenla et al. 1998). In total, there are some 34 genera of shrubs and small trees registered in this area (Amico and Aizen 2005). The second sampling site (S 41° 08.218′ W 71° 21.642′; 960 m a. s. l.) was at a steep slope below Cerro Otto; it had nearly no plant litter and only sparse vegetation cover; the tree vegetation was dominated by *Austrocedrus chilensis*, an evergreen tree that forms AM (Fontenla et al. 1998) while the ground vegetation consisted mainly of ericoid mycorrhizal *G. poeppigii* and AM hosts *Lomatia hirsuta*, *M. boaria*, *Schinus patagonica*, and perennial herbs. The sampling sites were located within the municipal area of S. C. de Bariloche which is situated inside the Parque Nacional Nahuel Huapi (http://www.nahuelhuapi.gov.ar/).

Two *Gaultheria* species were sampled: *G. poeppigii* in both *Nothofagus* (Llao Llao) and *Austrocedrus* (Cerro Otto) forests and *G. mucronata* only in the *Nothofagus* forest (this species was absent at Cerro Otto). These are endemic species of Argentina and Chile; both of them have been described for the Patagonian Andean forests; *G. poeppigii* is also present in mountains from Córdoba and San Luis (Argentina) (Zuloaga et al. 2008). There were no other ericaceous species present within and in the immediate surrounding of both sampling sites. At both sites, five individuals of each species were sampled; each individual was carefully excavated with roots and the adhering soil using a spade and sealed in a plastic bag. Samples were transported to the laboratory of Microbiología Aplicada y Biotecnología (MABB) at Centro Regional Universitario Bariloche, placed in a fridge, and then processed as follows. The soil bulks with individual *Gaultheria* plants (approx. volume 500 ml each) were carefully washed on a sieve under running tap water and hair roots attached to the plants were separated using scissors. Hair roots from each individual were divided into three subsamples: one for microscopy, one for mycobiont isolation, and one for DNA analysis using specific primers.

Along with the roots samples, we additionally took separate soil samples for chemical analyses. The soil samples were pooled for each site and immediately dried at room temperature and sieved either through a 2-mm sieve for analysis of phosphorus content and pH measurement or through a 0.5-mm sieve for analysis of carbon and nitrogen content. They were analyzed according to Sparks et al. (1996) and Burt (2004): pH in water (1:2.5 soil to water ratio); total carbon and total nitrogen with an elemental analyzer (Thermo Electron Corporation Flash EA 1112); P extracted in 0.5 M NaHCO3 (1:20, soil to solution ratio) and determined by the molybdate ascorbic acid method (Olsen-P). These analyses were performed in the Laboratory of the Soil Group at Centro Regional Universitario Bariloche. Ripe fruits were collected from both *Gaultheria* species for a re-synthesis experiment. Fruits were stored in the fridge until used.

Microscopic observations

One third of root samples were cleared with 10 % KOH (20 min at 121 °C), washed in tap water, acidified with 3 % HCl (30 s), washed in tap water, and stained with trypan blue (0.05 %) in lactoglycerol (glycerol to lactic acid to water in the ratio of 2:1:2) overnight. Roots were further de-stained in lactoglycerol and observed at ×400 and ×1000 magnification using an Olympus BX-60 microscope equipped with DIC. Pictures were taken with an Olympus DP70 camera; graphic documentation was modified for clarity in Paint.NET (Brewster, Jackson and contributors+Microsoft Corporation) as needed.

Isolation of mycobionts

Healthy-looking lightly pigmented turgescent hair roots were selected using a dissecting microscope for mycobiont isolation; the isolation procedure followed methods described in Vohník et al. (2012). We used modified Melin-Norkrans agar (MMN) supplemented with novobiocin (50 mg/l) to prevent growth of bacteria (M-medium); half of the media was additionally supplemented with benomyl (4 mg/l) to reduce growth of ascomycetes (B-medium). The media were poured in four-compartment Petri dishes (diam. 9 cm) and five surface-sterilized root pieces (length ca. 3 mm each) were placed into each compartment. There were three dishes (i.e., 60 root pieces) per each plant-site-medium combination. The dishes were sealed with an air-permeable film and placed in a cultivation chamber in the dark at room temperature and periodically checked for fungal growth. Sporulating mycelia (based on microscopy observations) were discarded as contaminants while all non-sporulating mycelia were transferred to new dishes with MMN. Isolations were terminated after 12 weeks when there were no new colonies formed within two consequent weeks.

Mycobiont DNA extraction and amplification, sequence analyses

DNA was extracted from all transferred cultures using Extract-N-Amp Plant Kits (Sigma-Aldrich, Germany) following

manufacturer's instructions. The nuclear ITS1-5.8S-ITS2 rDNA region was amplified using the ITS1+ITS4 and ITS1F+ITS4 primer pairs (White et al. 1990; Gardes and Bruns 1993). For PCR parameters and electrophoresis, see Vohník et al. (2012). PCR products were purified and sequenced by Macrogen Europe Laboratory (Macrogen Inc., South Korea) using ITS1F and ITS1 forward primers, respectively.

All sequences were screened in Finch TV v1.4.0 (geospiza.com/finchtv), and those of high quality were checked for possible machine errors and edited when needed. They were subsequently aligned in BioEdit v7.0.5.3 (Hall 1999), and the alignment was used for delimitation of operational taxonomic units (OTUs) in TOPALi v2.5 (topali.org) at 99 % sequence similarity (NJ, default F84-G model). Sequences within separate OTUs were further aligned to screen their heterogeneity, and the most divergent were subjected to BLAST searches (megablast/blastn algorithms) in GenBank (Altschul et al. 1997). The most frequent *Phialocephala fortinii* s. l. isolates belonging to OTU1 (see "Results") were further separated into clusters according to 99.9 % sequence similarity. Isolates/sequences were taxonomically assigned to orders (based on 95 % sequence similarity to 2–3 reliably identified GenBank sequences derived preferably from isolates with deposited vouchers), genera (97 %), and species (100 %), and their taxonomic position was further checked with Blast Tree View (NJ, max. seq. difference 0.75). By this way, several ascomycetous OTUs could not be assigned even at the order level; these were typically similar to unnamed sequences derived from isolates of endophytic origin and presumably belonged to Helotiales. We did not proceed with more complex phylogenetic analyses as the ITS region is not suitable for resolution of deeper phylogenetic relationships within Helotiales (Wang et al. 2006). Sequences representing all available plant/site combinations within respective OTUs were deposited in GenBank under acc. numbers KC180658-KC18180755.

Biodiversity analyses

To characterize the cultivable fungal community obtained with the use of two media for each plant species at each site, the Shannon–Weaver diversity index (*H*) was calculated. To evaluate differences between the samples, the Hutchinson *t* test ($p=0.05$) was performed according to Moreno (2001). Similarity among all plant/site combinations was analyzed using the classical and modified Jaccard's indexes. All indexes were calculated using EstimateS v. 8.20. To estimate the efficiency of the isolation procedure rarefaction curves were calculated using the same software. Rarefaction curves were calculated with the same software using 50 randomizations, sampling without replacement and default settings for upper incidence limit for infrequent species (Colwell 2006). Observed richness values Sobs (Mao Tau) were fitted using Origin 6.1 software with a first-order exponential decay model y=y0+A1*e(-x/t1). The curve asymptote was used to estimate the number of samples that would have been necessary to reach the maximum yield of fungal species present in each host plant species (Moreno 2001).

Extraction of total root DNA and its amplification with specific primers

Hair roots for DNA extraction were stored in 50 % ethanol in a fridge until used. Prior to DNA extraction, they were surface sterilized by shaking for 30 s in a 10 % solution of SAVO (household bleach, 4.5 % available chlorine) and rinsed two times in autoclaved de-ionized water. Root DNA was extracted using MO-BIO Power Plant Pro DNA isolation kit (MO BIO Laboratories, USA) or Extract-N-Amp Plant Kit (Sigma-Aldrich, Germany) following manufacturer's instructions. Concentrations of the extracted DNAs were measured using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Extracted DNA was further amplified using the fungal-specific primer pair ITS1F+ITS4 followed by amplification with a primer pair specific for Sebacinaceae (ITS3Seb+TW13; Selosse et al. 2007) and two primer pairs specific for the members of the *R. ericae* aggregate (HericaeITS1+ITS4; Vrålstad et al. 2000) and HYM1+HYM2 (Urban et al. 2008). PCR parameters followed Vohník et al. (2012) with Tm modified according to the respective primer pairs. We used Combi PPP Master Mix (Top-Bio, Czech Republic) with 4 μl of non-diluted template DNA. DNAs of *Sebacina* sp. and *M. variabilis* were used as positive controls. The length, quality, and quantity of the PCR products were checked by gel electrophoresis (1.5 % agarose, 50 V, 45 min).

In vitro re-synthesis

For the re-synthesis trial, we chose several isolates of mycobionts which were most frequent in the investigated *Gaultheria* roots (i.e., they were isolated with the highest frequencies) and at the same time represented groups which are less common/absent in the Northern Hemisphere Ericaceae or occur in the Northern Hemisphere but their mycorrhizal status remains dubious. By this way, we omitted the most frequent consortium of the *Phialocephala*-related isolates (OTU 1 and 2; see "Results") and a frequent group of isolates related to *Ilyonectria radicicola* which seems to be an Ericaceae root saprobe/pathogen (OTU 26). We also omitted isolates which could be plausibly classified as contaminants, i.e., those related to *Umbelopsis* sp. (OTU 56) and *Mortierella* sp. (OTU 57). For inoculation, we therefore selected an isolate of *Tetracladium* cf. *breve* (OUT 4) as a

representant of the *Tetracladium*-related mycobionts, one isolate of *Cadophora olivo-luteacea* (OUT 9), and one isolate of *Pochonia bulbillosa* (OTU 25) representing the *Pochonia*-related mycobionts. We also included an isolate of *O.* cf. *maius* (OTU 45) as a representative of typical ErM fungi. Further replications within these OTUs were not possible due to the low number of seedlings available for inoculation.

G. poeppigii seeds were extracted from ripe fruits (see above), washed in tap water, surface sterilized with 10 % chlorine (55 g/l) for 45 s, and then serially washed in sterile water. The seeds were further transferred to Petri dishes with MMN with reduced carbon sources (1/10 concentration of glucose, i.e., 10 g/l, no malt extract) and kept in the dark at 21 °C until germination occurred. Young seedlings were transferred to new dishes with the same medium and placed in a growth chamber (16/8 h light/dark regime, average temperature 21 °C) to let them develop and to eliminate possible contaminations. Two-month-old seedlings were transferred to split Petri dishes (Vohník et al. 2007) with the same medium and inoculated with 2–3 agar plugs covered by the mycelium of the selected fungi. There were five seedlings per each inoculated isolate and non-inoculated control. Dishes sealed with an air-permeable film were maintained in the growth chamber and harvested 10 weeks after inoculation. Their shoots and roots were separated, shoots were dried at 70 °C and weighed, and roots were treated as above (see "Microscopic observations").

Results

Soil analysis

Total extractable phosphorus content was 5.9 μg/g in the soil from Cerro Otto and 5.8 μg/g in the soil from Llao Llao. The content of carbon and nitrogen and pH was 6.1 %, 0.4 % and 6.08 for the Cerro Otto soil and 6.5 %, 0.3 % and 5.23 for the Llao Llao soil, respectively.

Microscopic observations

Ericoid mycorrhizal colonization patterns found in most of the screened hair roots are depicted in Fig. 1a–d. They were typically represented by dense intracellular coils formed by pigmented septate hyphae. Also, dense intracellular coils formed by relatively thin hyaline hyphae and corresponding to typical ErM were observed but less frequently (Fig. 1c, d). Hyphal content in some cells seemed to be partially digested with hyphae contours barely visible (Fig. 1d). Some ErM hair roots were embedded in sheaths formed by hyaline narrow hyphae without clamp connections (Fig. 1e) while others were covered with parenchymatous nets formed by dark septate hyphae (Fig. 1f). Three types of colonization patterns were less frequent than those already described. Firstly, some rhizodermal cells were completely filled with dark microsclerotia typically formed by DSE (Fig. 1g). Secondly, some rhizodermal cells contained vesicules similar to those formed in AM (Fig. 1h); these were sometimes formed in cells already containing ErM hyphal coils (Fig. 1j). Lastly, a few rhizodermal cells were filled with loose hyphal loops possessing clamp connections typical for basidiomycetes (Fig. 1k).

Isolation of mycobionts

In total, 364 mycelia were transferred from original four-compartment dishes to new media and their ITS nrDNA was subsequently sequenced. After discarding low-quality and *Penicillium*-like sequences, we further processed 257 isolates/sequences. One hundred sixteen originated from B-medium whereas 141 from M-medium. One hundred thirteen isolates were derived form *G. poeppigii*/Cerro Otto, 77 from *G. poeppigii*/Llao Llao, and 67 from *G. mucronata*/Llao Llao (Table 1).

Sequence analyses, identity of isolates

Sixty-two OTUs were delimited within our sequence dataset, and OTU 1 (*P. fortinii* s. l.) was for better resolution further subdivided into four clusters (Table 1). Most frequent were ascomycetes (225 isolates) followed by zygomycetes (27) and basidiomycetes (5). Ascomycetes were dominated by Helotiales (23 OTUs/97 isolates) followed by Hypocreales (7/71), Pleosporales (7/19), Diaporthales (1/10), Myxotrichaceae (1/5), Eurotiales (1/4), Sordariales (2/2), Xylariales (1/2), Capnodiales (1/1), and Coniochaetales (1/1). Ten ascomycete OTUs with 14 isolates in total remained unidentified. Zygomycetes were represented by Mucorales (1/16) and Mortierellales (1/11), and the least frequent basidiomycetes by Polyporales (3/3), Tremellales (1/1), and Russulales (1/1). The most frequent OTUs were OTU 1 (*P. fortinii* s. l., Helotiales, 43 isolates), OTU 24 (*Pochonia suchlasporia*, Hypocreales, 21 isolates) and OTU 26 (*I. radicicola*, Hypocreales, 21 isolates). The most frequent genera were *Phialocephala* (two OTUs, 44 isolates), *Pochonia* (2/30), and *Tetracladium* (6/16). There was no apparent isolation medium preference except that the *P. fortinii* s. l. isolates were all except one derived from M-medium, and *Tetracladium* and the basidiomycetous isolates were obtained only from B-medium (Table 1). Out of 65 fungal groups representing the 62 OTUs (see subdivision of OTU 1 to four clusters), 42 groups (i.e., 64.6 %) were unique for one of the investigated plant/site combinations: 21 for *G. poeppigii* from Cerro Otto, 10 for *G. poeppigii* from Llao Llao, and 11 for *G. mucronata* from Llao Llao (Fig. 2).

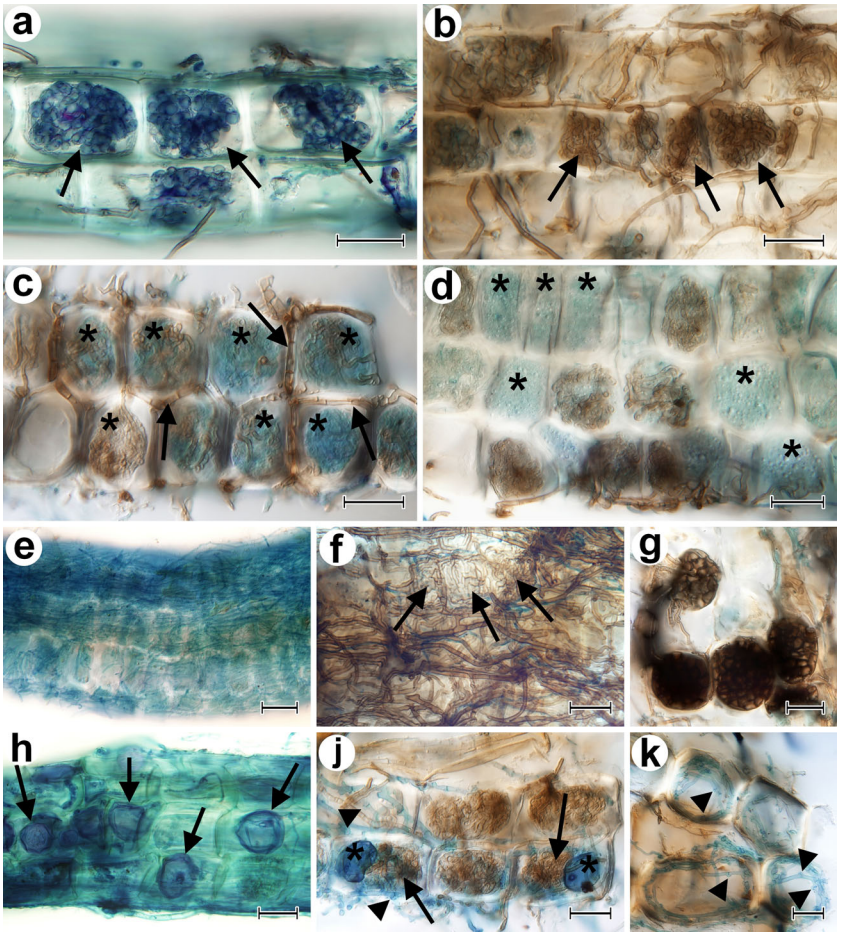

Fig. 1 Colonization patterns found in hair roots of *Gaultheria mucronata* and *G. poeppigii* in two natural forests in NW Patagonia, Argentina. **a** Dense intracellular coils formed by hyaline thick hyphae which turn blue after staining (*arrows*). **b** As in 1**a**, but hyphae remain light brown even after staining (*arrows*). **c** Intercellular (*arrows*) and intracellular (*asterisks*) phases of ericoid mycorrhizal colonization. Intercellular dark septate hyphae may turn hyaline when entering rhizodermal cells. **d** Hyphal content in some cells seemed to be partially digested with hyphae contours barely visible (*asterisks*). **e** A hyphal sheath formed by narrow hyaline hyphae which turn blue after staining. **f** Some roots were embedded in loose nets formed by dark septate hyphae. These often formed finger-like parenchymatous tissue right on the surface of the colonized hair roots (*arrows*). **g** Intracellular microsclerotia formed by thick dark brown septate hyphae. **h** Intracellular vesicules resembling those formed in arbuscular mycorrhiza (*arrows*). **j** Vesicules as in 1**h** (*asterisks*) were sometimes formed in rhizodermal cells alongside hyphal coils typical for ericoid mycorrhiza (*arrows*). Note basidiomycetous hyphae bearing clamp connections (*arrowheads*). **k** Loose intracellular loops formed by basidiomycetous hyphae possessing clamp connections (*arrowheads*). All samples stained with trypan blue and observed with an upright microscope equipped with DIC (see "Materials and methods"). All *bars* represent 20 μm

However, most of these unique groups were represented by very few isolates (often only one). At Llao Llao, 15 groups were shared between *G. mucronata* and *G. poeppigii* but most of them (10 groups) were ubiquitous fungi found in all three plant/site combinations. These ubiquitous mycobionts represented only 15.4 % of the diversity within separate fungal groups, but were represented by 144 isolates (56 %) (Fig. 2). Interestingly, all Pleosporales isolates originated from *G. poeppigii* from Cerro Otto.

In general, most of the closest sequence relatives in GenBank were derived from vascular plants (belonging to 12 different families) but also from Nematoda eggs, Hymenoptera and Isoptera nests, and as associates of other fungi. They came from 21 different countries from America, Asia, Australia, and Europe (Table 1).

Biodiversity analysis

The Shannon–Weaver diversity index (*H*) indicated that *G. poeppigii* from Llao Llao had the highest mycobiont diversity (H=3.52) which was significantly different from the other two plant/site combinations (p=0.00418 and 0.02942 in comparison with *G. poeppigii* from Cerro Otto (H=3.07) and *G. mucronata* from Llao Llao (H=3.39), respectively). There was however no significant difference between the two latter plant/site combinations (p=0.28477).

When analyzing similarity between plant/site combinations using only presence and absence of fungal species (classic Jaccard's index *J*), the community compositions seemed to be quite different, the two more similar being *G. mucronata* and *G. poeppigii* from Llao Llao (J=0.33) followed by

Table 1 Identity of the isolates obtained in this study

OTU #[a]	Identity	GenBank #	Host	Site	B[c]	M[d]	Sequence length	Coverag/ similarity (%)	Closest match[e]	Match identity[f]	Match source	Match origin	Match reference
OTU 1[b]	*P. fortinii* s.l. cluster A	KC180658	GPO	Cerro Otto	2	11	502	99/99	EU888624	*P. fortinii* s.l.	*Rhododrendron fortunei* (Ericaceae) root	China	Zhang et al. (2009)
		KC180659	GPO	Llao Llao	0	15	524	100/100	EU888624	*P. fortinii* s.l.	*Rhododrendron fortunei* (Ericaceae) root	China	Zhang et al. (2009)
		KC180660	GMU	Llao Llao	0	7	490	100/100	EU888624	*P. fortinii* s.l.	*Rhododrendron fortunei* (Ericaceae) root	China	Zhang et al. (2009)
	P. fortinii s.l. cluster B	KC180661	GPO	Cerro Otto	0	1	524	100/100	JQ711965	*P. fortinii* s.l.	*Pinus contorta* (Pinaceae) ectomycorrhiza	Canada	Jones et al. (2012)
		KC180662	GMU	Llao Llao	0	2	500	100/100	JQ711965	*P. fortinii* s.l.	*Pinus contorta* (Pinaceae) ectomycorrhiza	Canada	Jones et al. (2012)
	P. fortinii s.l. cluster C	KC180663	GMU	Llao Llao	0	1	498	99/99	EU888624	*P. fortinii* s.l.	*R. fortunei* root	China	Zhang et al. (2009)
	P. fortinii s.l. cluster D	KC180664	GPO	Cerro Otto	0	1	523	100/99	EU888624	*P. fortinii* s.l.	*R. fortunei* root	China	Zhang et al. (2009)
		KC180665	GMU	Llao Llao	0	3	493	100/99	EU888624	*P. fortinii* s.l.	*R. fortunei* root	China	Zhang et al. (2009)
OTU 2	*P. fortinii* s.l.	KC180666	GPO	Llao Llao	0	1	541	96/96	EU888624	*P. fortinii* s.l.	*R. fortunei* root	China	Zhang et al. (2009)
OTU 3	*Tetracladium furcatum*	KC180667	GPO	Cerro Otto	7	0	511	100/100	FJ000375	*T. furcatum*	Spore	Czech Republic	Letourneau et al. (2010)
		KC180668	GMU	Llao Llao	1	0	516	100/100	FJ000375	*T. furcatum*	Spore	Czech Republic	Letourneau et al. (2010)
OTU 4	*Tetracladium* cf. *breve*	KC180669	GMU	Llao Llao	1	0	475	100/99	EU883431	*T. breve*	Spore	Portugal	Letourneau et al. (2010)
OTU 5	*Tetracladium* sp.	KC180670	GPO	Llao Llao	1	0	517	100/97	EU883430	*T. maxilliforme*	Spore	Czech Republic	Letourneau et al. (2010)
		KC180671	GMU	Llao Llao	1	0	485	100/97	FJ000374	*T. setigerum*	Spore	Czech Republic	Letourneau et al. (2010)
OTU 6	*Tetracladium* sp.	KC180672	GPO	Llao Llao	1	0	508	100/97	FJ000374	*T. setigerum*	Spore	Czech Republic	Letourneau et al. (2010)
OTU 7	*Tetracladium* sp.	KC180673	GPO	Cerro Otto	2	0	530	99/98	FJ000374	*T. setigerum*	Spore	Czech Republic	Letourneau et al. (2010)
OTU 8	*Tetracladium* sp.	KC180674	GPO	Cerro Otto	2	0	470	100/97	JX029127	*Tetracladium* sp.	NA	China	unpubl.
OTU 9	*Cadophora* cf. *olivo-luteacea*	KC180675	GPO	Cerro Otto	2	6	583	100/97	FJ486274	*C. luteo-olivacea*	Wood, Shackleton's Hut	Antarctica	Blanchette et al. (2010)
		KC180676	GPO	Llao Llao	1	1	595	100/98	FJ486274	*C. luteo-olivacea*	Wood, Shackleton's Hut	Antarctica	Blanchette et al. (2010)
		KC180677	GMU	Llao Llao	1	1	556	99/99	JN859261	*Cadophora* sp.	*Juniperus communis* (Cupressaceae) root	Hungary	Knapp et al. (2012)
OTU 10	Helotiales sp.	KC180678	GPO	Cerro Otto	0	2	542	99/96	JN859267	Helotiales sp.	*Helianthemum ovatum* (Cistaceae) root	Hungary	Knapp et al. (2012)
		KC180679	GPO	Llao Llao	2	1	547	98/97	JN859267	Helotiales sp.	*Helianthemum ovatum* (Cistaceae) root	Hungary	Knapp et al. (2012)
		KC180680	GMU	Llao Llao	0	1	519	99/97	JN859267	Helotiales sp.	*Helianthemum ovatum* (Cistaceae) root	Hungary	Knapp et al. (2012)
OTU 11	Helotiales sp.	KC180681	GPO	Cerro Otto	0	1	533	99/98	HE814201	Helotiales sp.	Root tip	China	unpubl.
		KC180682	GPO	Llao Llao	0	1	502	100/95	AF081443	Mycorrhizal sp.	*Gaultheria shallon* (Ericaceae) root	Canada	Monreal et al. (1999)
		KC180683	GMU	Llao Llao	0	1	529	90/98	HE814201	Helotiales sp.	Root tip	China	unpubl.
OTU 12	*Leptodontidium* cf. *orchidicola*	KC180684	GPO	Cerro Otto	0	1	592	100/99	GU479910	*L. orchidicola*	*Trillium tschonoskii* (Melanthiaceae)	China (NA)	unpubl.
		KC180685	GMU	Llao Llao	0	1	589	100/99	GU479910	*L. orchidicola*	*Trillium tschonoskii* (Melanthiaceae)	China (NA)	unpubl.
OTU 13	*Scytalidium lignicola*	KC180686	GPO	Cerro Otto	1	0	474	100/100	FJ914697	*S. lignicola*	NA	NA	unpubl.
		KC180687	GMU	Llao Llao	0	1	465	100/100	FJ914697	*S. lignicola*	NA	NA	unpubl.

Table 1 (continued)

OTU #[a]	Identity	GenBank #	Host	Site	B[c]	M[d]	Sequence length	Coverag/ similarity (%)	Closest match[e]	Match identity[f]	Match source	Match origin	Match reference
OTU 14	Helotiales sp.	KC180688	GPO	Llao Llao	0	1	529	96/99	AY627827	Root-associated fungus	*Epacris pulchella* root	Australia	Bougoure and Cairney (2005a)
		KC180689	GMU	Llao Llao	0	1	492	97/99	AY627827	Root-associated fungus	*Epacris pulchella* root	Australia	Bougoure and Cairney (2005a)
OTU 15	Helotiales sp.	KC180690	GPO	Cerro Otto	2	0	477	100/99	AY046402	ErM sp.	Mediterranean soil	Italy	Bergero et al. (2003)
OTU 16	*Cryptosporiopsis* cf. *ericae*	KC180691	GPO	Cerro Otto	0	1	483	99/97	HQ157903	*C. ericae*	*Abies balsamifera* (Pinaceae) root	Canada	Kernaghan and Patriquin (2011)
OTU 17	*Lachnum* sp.	KC180692	GMU	Llao Llao	1	0	473	100/98	JN655650	*Lachnum* sp.	*Pseudorchis albida* (Orchidaceae) root	Czech Republic	Kohout et al. (2013)
OTU 18	*Meliniomyces* sp.	KC180693	GMU	Llao Llao	0	1	495	97/97	EF093175	*Meliniomyces* sp.	Endophytic in *Picea abies* ectomycorhiza	Czech Republic	Vohník et al. (2013)
OTU 19	*Pseudaegerita viridis*	KC180694	GMU	Llao Llao	0	1	479	100/100	EF029235	*P. viridis*	NA	New Zealand	unpubl.
OTU 20	Helotiales sp.	KC180695	GPO	Cerro Otto	0	1	515	93/96	JQ780618	*Crocicreas* sp.	Peat	Russia	unpubl.
OTU 21	Helotiales sp.	KC180696	GMU	Llao Llao	0	1	488	100/100	JN859270	Helotiales sp.	*J. communis* root	Hungary	Knapp et al. (2012)
OTU 22	Helotiales sp.	KC180697	GPO	Cerro Otto	0	1	480	99/96	JN859279	Helotiales sp.	*J. communis* root	Hungary	Knapp et al. (2012)
OTU 23	Helotiales sp.	KC180698	GPO	Llao Llao	0	1	491	100/97	AY279189	Epacrid root endophyte	*Epacris microphylla* (Ericaceae) root	Australia	unpubl.
OTU 24	*Pochonia suchlasporia*	KC180699	GPO	Cerro Otto	5	1	590	100/100	AB214658	*P. suchlasporia*	*Heterodera avenae* (Nematoda) egg	Sweden	Watanabe et al. (2006)
		KC180700	GPO	Llao Llao	9	1	600	100/99	AB214658	*P. suchlasporia*	*Heterodera avenae* (Nematoda) egg	Sweden	Watanabe et al. (2006)
		KC180701	GMU	Llao Llao	0	3	554	99/100	AB214658	*P. suchlasporia*	*Heterodera avenae* (Nematoda) egg	Sweden	Watanabe et al. (2006)
OTU 25	*Pochonia bulbillosa*	KC180702	GPO	Cerro Otto	4	0	528	100/100	AB378551	*P. bulbillosa*	*Cordyceps coccidiicola*	Japan	Watanabe et al. (2006)
		KC180703	GPO	Llao Llao	0	3	481	100/100	AB378551	*P. bulbillosa*	*Cordyceps coccidiicola*	Japan	Watanabe et al. (2006)
		KC180704	GMU	Llao Llao	0	4	563	100/100	AB378551	*P. bulbillosa*	*Cordyceps coccidiicola*	Japan	Watanabe et al. (2006)
OTU 26	*Ilyonectria radicicola*	KC180705	GPO	Cerro Otto	5	7	493	100/100	FJ430729	*N. radicicola*	Saline acidic soil	Czech Republic	Hujslová et al. (2010)
		KC180706	GPO	Llao Llao	1	0	429	100/99	JF735278	*I. rufa*	*Pseudotsuga menziesii* (Pinaceae) root	Canada	Cabral et al. (2012)
		KC180707	GMU	Llao Llao	4	4	499	100/100	FJ430730	*N. radicicola*	Saline acidic soil	Czech Republic	Hujslová et al. (2010)
OTU 27	*Fusarium tricinctum*	KC180708	GPO	Cerro Otto	7	4	490	100/100	JQ690084	*F. tricinctum*	Soil	China	unpubl.
		KC180709	GPO	Llao Llao	2	0	521	100/100	JQ690084	*F. tricinctum*	Soil	China	unpubl.
OTU 28	*Paecilomyce carneus*	KC180710	GPO	Llao Llao	1	0	517	100/100	JF311959	*P. carneus*	Soil	Canada	unpubl.
		KC180711	GMU	Llao Llao	0	2	613	99/100	JF311959	*P. carneus*	Soil	Canada	unpubl.
OTU 29	*Eucasphaeria* cf. *capensis*	KC180712	GPO	Llao Llao	1	0	546	100/99	EU272516	*E. capensis*	*Espeletia* sp. (Asteraceae)	Colombia	unpubl.
OTU 30	Hypocreales sp.	KC180713	GPO	Cerro Otto	1	0	533	100/95	EU272516	*E. capensis*	*Espeletia* sp. (Asteraceae)	Colombia	unpubl.
OTU 31	*Embellisia astragali*	KC180714	GPO	Cerro Otto	4	0	523	99/100	FJ914716	*E. astragali*	NA	NA	unpubl.
OTU 32	*Leptosphaeria* sp.	KC180715	GPO	Cerro Otto	2	2	505	100/100	GU934536	*Leptosphaeria* sp.	*Salix* sp. root	Canada (NA)	unpubl.
OTU 33	*Paraphoma* cf. *chrysanthemicola*	KC180716	GPO	Cerro Otto	2	1	512	100/98	JN123358	*P. chrysanthemicola*	*Astragalus adsurgens* (Fabaceae) root	China	unpubl.
OTU 34	*Ulocladium chartarum*	KC180717	GPO	Cerro Otto	2	1	528	100/100	EF568098	*U. chartarum*	NA	NA	unpubl.
OTU 35	*Alternaria alternata*	KC180718	GPO	Cerro Otto	1	1	523	99/100	JX045850	*A. alternata*	NA	China (NA)	unpubl.
OTU 36	Pleosporales sp.	KC180719	GPO	Cerro Otto	0	2	464	100/95	JN859337	Pleosporales sp.	*J. communis* root	Hungary	Knapp et al. (2012)

Table 1 (continued)

OTU #[a]	Identity	GenBank #	Host	Site	B[c]	M[d]	Sequence length	Coverag/ similarity (%)	Closest match[e]	Match identity[f]	Match source	Match origin	Match reference
OTU 37	*Phoma putaminum*	KC180720	GPO	Cerro Otto	1	0	426	100/100	GU237843	*P. putaminum*	*Ulmus* sp. branch, sec. on *Ceratocystis ulmi*	Netherlands	Aveskamp et al. (2010)
OTU 38	*Phomopsis columnaris*	KC180721	GPO	Cerro Otto	0	2	495	100/100	GU934562	*P. columnaris*	*Salix* sp. (Salicaceae) root	Canada (NA)	unpubl.
		KC180722	GPO	Llao Llao	1	5	531	100/99	GU934562	*P. columnaris*	*Salix* sp. (Salicaceae) root	Canada (NA)	unpubl.
		KC180723	GMU	Llao Llao	0	2	504	99/99	GU934561	*P. columnaris*	*Salix* sp. (Salicaceae) root	Canada (NA)	unpubl.
OTU 39	Eurotiales sp.	KC180724	GPO	Llao Llao	1	3	547	99/100	JN859385	Eurotiales sp.	*Ailanthus altissima* (Simaroubaceae) root	Hungary	Knapp et al. (2012)
OTU 40	*Podospora* sp.	KC180725	GPO	Cerro Otto	0	1	388	100/100	HQ647346	*Podospora* sp.	Bamboo	China	unpubl.
OTU 41	*Trichocladium* cf. *opacum*	KC180726	GPO	Llao Llao	0	1	500	100/99	FN386299	*T. opacum*	*Holcus lanatus* (Poaceae)	Spain	Sanchez Marquez et al. (2010)
OTU 42	*Truncatella* cf. *angustata*	KC180727	GPO	Cerro Otto	0	1	511	100/98	JN038391	*T. angustata*	NA	India	unpubl.
		KC180728	GMU	Llao Llao	0	1	509	100/100	JN038391	*T. angustata*	NA	India	unpubl.
OTU 43	*Capnodium* sp.	KC180729	GPO	Cerro Otto	0	2	460	97/99	AY805548	*Capnodium* sp.	*Picea abies* (Pinaceae) wood disc	Sweden	Menkis et al. (2004)
OTU 44	*Lecythophora* cf. *mutabilis*	KC180730	GMU	Llao Llao	0	1	494	100/99	HQ637304	*L. mutabilis*	soil	China	unpubl.
OTU 45	*Oidiodendron* cf. *maius*	KC180731	GPO	Cero Otto	0	1	521	100/97	AF062798	*O. maius*	Soil (cedar bog)	China	Hambleton et al. (1998)
		KC180732	GPO	Llao Llao	2	2	474	100/97	AF062798	*O. maius*	Soil (cedar bog)	China	Hambleton et al. (1998)
OTU 46	Ascomycota sp.	KC180733	GPO	Llao Llao	0	1	492	100/92	JN831360	–	–	–	unpubl.
		KC180734	GMU	Llao Llao	1	0	443	97/93	JN831360	–	–	–	unpubl.
OTU 47	Ascomycota sp.	KC180735	GPO	Llao Llao	0	2	505	100/98	EU139250	Ascomycota sp.	*Macrotermitinae* nest (Isoptera)	France	unpubl.
OTU 48	Ascomycota sp.	KC180736	GPO	Llao Llao	1	0	563	93/98	FN394690	Fungal endophyte	*H. lanatus*	Spain	Sanchez Marquez et al. (2010)
		KC180737	GMU	Llao Llao	1	0	515	94/98	FN394690	Fungal endophyte	*H. lanatus*	Spain	Sanchez Marquez et al. (2010)
OTU 49	Ascomycota sp.	KC180738	GPO	Cerro Otto	1	0	554	96/90	GU973639	–	–	–	unpubl.
OTU 50	Ascomycota sp.	KC180739	GPO	Cerro Otto	1	1	566	100/99	AM208714	Ascomycota sp.	*Rhododrendron* sp. root	China	unpubl.
OTU 51	Ascomycota sp.	KC180740	GPO	Cerro Otto	0	1	552	95/94	JN859362	–	–	–	Knapp et al. (2012)
OTU 52	Ascomycota sp.	KC180741	GMU	Llao Llao	0	1	483	98/99	HM439547	Ascomycota sp.	*Ophiocordyceps sinensis*	China	Zhang et al. (2010)
OTU 53	Ascomycota sp.	KC180742	GPO	Cerro Otto	0	1	478	100/94	JN859274	–	–	–	Knapp et al. (2012)
OTU 54	Ascomycota sp.	KC180743	GMU	Llao Llao	0	1	485	100/98	HQ117865	Sordariomycetes sp.	*Psammisia roseiflora* (Ericaceae)	Ecuador	Unpubl.
OTU 55	Ascomycota sp.	KC180744	GMU	Llao Llao	0	1	621	100/95	EU019299	*T. betulinum*	*Betula verrucosa* (Betulaceae) dead branch	Netherlands	Crous et al. (2007)
OTU 56	*Umbelopsis* sp.	KC180745	GPO	Cerro Otto	1	0	569	100/99	HQ157958	*Umbelopsis sp.*	*Picea glauca* (Pinaceae) root	Canada	Kernaghan and Patriquin (2011)
		KC180746	GPO	Llao Llao	4	2	529	100/99	HQ157958	*Umbelopsis sp.*	*Picea glauca* (Pinaceae) root	Canada	Kernaghan and Patriquin (2011)
		KC180747	GMU	Llao Llao	7	2	569	100/99	HQ157958	*Umbelopsis sp.*	*Picea glauca* (Pinaceae) root	Canada	Kernaghan and Patriquin (2011)

Table 1 (continued)

OTU #[a]	Identity	GenBank #	Host	Site	B[c]	M[d]	Sequence length	Coverag/ similarity (%)	Closest match[e]	Match identity[f]	Match source	Match origin	Match reference
OTU 57	*Mortierella* sp.	KC180748	GPO	Cerro Otto	1	1	577	100/99	HQ608143	*Mortierella sp.*	*Trachymyrmex septentrionalis* (Hymenoptera) nest	USA	Rodrigues et al. (2011)
		KC180749	GPO	Llao Llao	5	0	581	100/100	HQ608143	*Mortierella sp.*	*Trachymyrmex septentrionalis* (Hymenoptera) nest	USA	Rodrigues et al. (2011)
		KC180750	GMU	Llao Llao	4	0	579	100/100	HQ608143	*Mortierella sp.*	*Trachymyrmex septentrionalis* (Hymenoptera) nest	USA	Rodrigues et al. (2011)
OTU 58	*Daedalea* cf. *quercina*	KC180751	GPO	Llao Llao	1	0	603	100/99	EU787455	*D. quercina*	Fruitbody	Germany (NA)	unpubl.
OTU 59	*Fomitopsis pinicola*	KC180752	GPO	Cerro Otto	1	0	616	100/100	FJ608588	*F. pinicola*	NA	Czech Republic	Homolka et al. (2010)
OTU 60	*Trametes versicolor*	KC180753	GPO	Llao Llao	1	0	559	100/100	EF524042	*T. versicolor*	NA	Germany (NA)	unpubl.
OTU 61	*Trichosporon porosum*	KC180754	GMU	Llao Llao	1	0	490	100/100	HQ005755	*T. porosum*	*Odontotaenius disjunctus* (Coleoptera)	USA	Gujjari et al. (2011)
OTU 62	*Stereum gausapatum*	KC180755	GPO	Llao Llao	1	0	590	99/100	FN539048	*S. gausapatum*	Tree sapwood	UK	Partfitt et al. (2010)

GMU G. mucronata, *GPO G. poeppigii*, *NA Not Available*

[a] Grouping according to 99 % whole alignment similarity

[b] OTU 1 subgrouping according to 99.9 % partial alignment similarity (see "Materials and methods")

[c] Number of isolates obtained on the B-medium

[d] Number of isolates obtained on the M-medium

[e] In GenBank according to BLAST

[f] According to GenBank. Details for the closest matches are given only if similarity of sequences is at least 95%

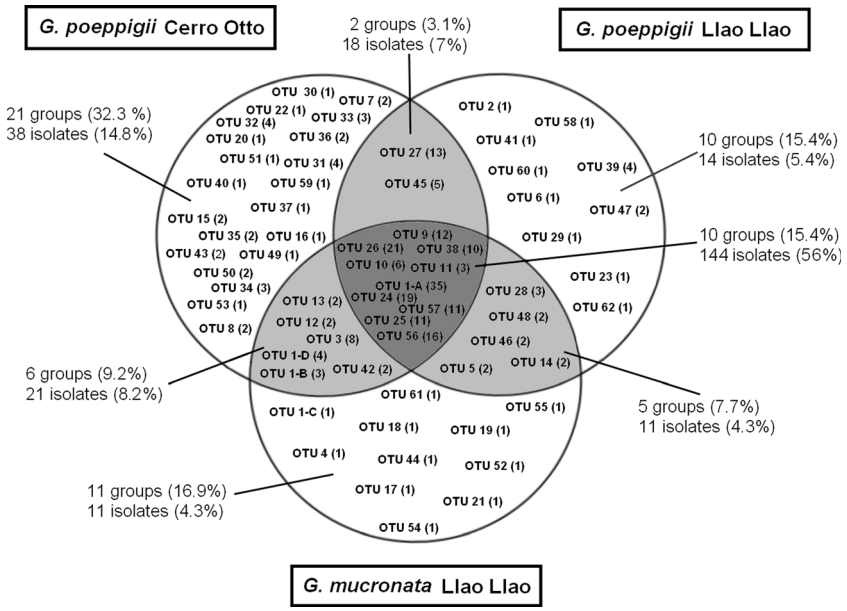

Fig. 2 Distribution of OTUs within respective plant-site combinations. *Gaultheria mucronata* and *G. poeppigii* root mycobionts were screened at two localities, Cerro Otto and Llao Llao. For taxonomic identity of respective OTUs see Table 1

G. poeppigii from Cerro Otto and *G. mucronata* from Llao Llao (J=0.29) and *G. poeppigii* from Cerro Otto and *G. poeppigii* from Llao Llao (J=0.22). However, when weight was given to the abundance of species (modified Jaccard's index Jmod.), the plant/site combinations showed greater similarity, the most similar being *G. mucronata* from Llao Llao and *G. poeppigii* from Llao Llao (Jmod.=0.56), followed by *G. poeppigii* from Cerro Otto and *G. poeppigii* from Llao Llao (Jmod.=0.48) and *G. poeppigii* from Cerro Otto and *G. mucronata* from Llao Llao (Jmod.=0.47).

Rarefaction curves showing the number of species recovered as related to the number of samples collected for each plant/site combination are shown in Fig. 3. In all cases, the number of *Gaultheria* individuals collected does not reach the stabilization phase of the curve which suggests that more extensive sampling would reveal additional fungal diversity. The estimated asymptotes (maximum number of fungal species) and their associated parameter errors showed no differences between forest types (asymptote for *G. poeppigii* from Cerro Otto and Llalo Llao=48.32±0.48 and 46.62±3.95, respectively, and for *G. mucronata* from Llao Llao=53.24±2.95).

Amplification with specific primers

Results of PCRs with the three specific primer pairs are summarized in Table 2. Interestingly, while at least a weak band appeared on a gel in all samples from *G. poeppigii* from Cerro Otto when amplified with the *R. ericae* aggregate-specific primer pairs, about half of samples from Llao Llao produced no band when amplified with HericaeITS1+ITS4 and only one Llao Llao sample produced a band when amplified with HYM1+HYM2. In contrast, all samples from all plant/site combinations produced at least a weak band when

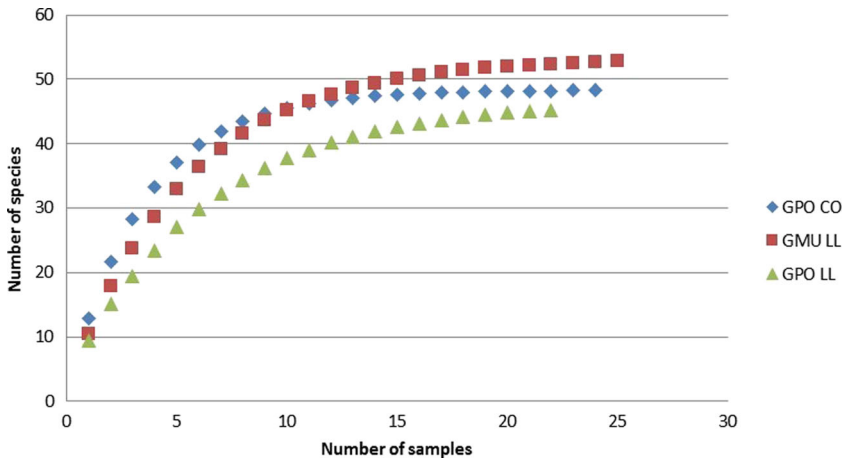

Fig. 3 Rarefaction curves. Accumulation curves of mycobiont species from *Gaultheria poeppigii* at Llao Llao (*GPO LL*), *G. mucronata* at Llao Llao (*GMU LL*), and *G. poeppigii* at Cerro Otto (*GPO CO*)

Table 2 Screening of root mycobiont spectra with specific primers

Plant/site	Sample #	HericaeITS1+ITS4[b]	HYM1+HYM2[c]	ITS3Seb+TW13[d]
G. poeppigii/Cerro Otto	1	+/–	++	+/–
	2	++	++	++
	3	+	+	++
	4	++	+/–	+
	5	+	++	++
G. mucronata/Llao Llao	7	+/–	+	++
	8	–	–	+/–
	9	–	–	+/–
	10	+	–	+/–
	11	–	–	+/–
G. poeppigii/Llao Llao[a]	12	–	–	++
	13	–	–	+/–
	14	+	–	+
	15	+	–	+

"+" corresponds to presence of a strong band ("++" for two bands), "+/–" corresponds to presence of a weak band, and "–" stands for a sample with no visible band. The forest at Cerro Otto was dominated by *Austrocedrus chilensis* which forms arbuscular mycorrhiza while the dominating tree at Llao Llao was ectomycorrhizal *Nothofagus dombeyi* (see "Materials and methods"). Both sites had comparable soil C, N, and P concentrations, and pH (see "Results")

[a] Only four DNA extractions with suitable concentration were recovered in this plant/site combination

[b] After Vrålstad et al. (2000)

[c] After Urban et al. (2008)

[d] After Selosse et al. (2007)

amplified with the *Sebacina*-specific ITS3Seb+TW13 primer pair.

In vitro re-synthesis

All tested isolates had the ability to intracellularly colonize *Gaultheria* rhizodermal cells but only the *O.* cf. *maius* isolate formed typical ErM structures (dense intracellular hyphal coils), although at very low rates (only 0.8 % of all screened rhizodermal cells). On the other hand, this isolate formed vigorous intracellular colonization which differed from typical ErM colonization pattern (in 41.3 % of all screened rhizodermal cells). Similarly, both *T.* cf. *breve* and *C.* cf. *olivo-luteacea* frequently colonized rhizodermal cells (68.8 and 40.8 %, respectively) but their colonization patterns did not resemble typical ErM. In contrast, the non-ErM intracellular colonization formed by *P. bulbillosa* was relatively low (2.4 %). We detected no fungal colonization in the non-inoculated control plants.

Discussion

Because of the complete lack of information on the diversity of Ericaceae root mycobionts in South America, our study primarily aimed at *Gaultheria* spp. from NW Patagonia. Despite the relatively high diversity of the cultivable mycobionts, we were not able to obtain any isolate belonging to the prominent ErM fungus *R. ericae*. This is congruent with studies investigating root mycobionts of Australian Ericaceae (Williams et al. 2004; Bougoure and Cairney 2005a; Bougoure and Cairney 2005b; Curlevski et al. 2009). At the same time, our microscopic observations confirmed that the screened *Gaultheria* roots possessed ericoid mycorrhizae. These facts raised two questions: (1) is *R. ericae* sensu stricto indeed absent in the screened roots and (2) if true, which mycobionts substitute its ErM functioning?

Microscopic observations

Well-developed morphologically variable fungal colonization was observed in all screened hair roots. All screened hair roots were ericoid mycorrhizal, i.e., possessed the characteristic intracellular hyphal loops and/or coils. The observed variability in the ErM colonization pattern suggests that the screened *Gaultheria* spp. were colonized by at least two morphologically different guilds of ErM fungi (cf. McLean and Lawrie 1996). The most frequent colonization pattern (Fig. 1a, b) does not fully resemble the typical pattern formed by *R. ericae* and is rather similar to structures formed in Ericaceae hair roots by isolates belonging to Sebacinaceae

(M. Vohník, unpublished in vitro studies). Congruently, no *R. ericae* isolate was obtained from the *Gaultheria* spp. hair roots, and Sebacinaceae DNA was successfully amplified from all tested root samples. This hypothesis could be further tested by transmission electron microscopy due to Sebacinaceae characteristic dolipores (Selosse et al. 2007), by methods targeting single mycorrhizal rhizodermal cells, e.g., laser capture microdissection or the maceration method after Pearson and Read (1973), or groups of cells (Allen et al. 2003), followed by amplification of fungal DNA.

The hyphal sheaths occasionally found at the surface of some roots resembled fungal mantles described by Massicotte et al. (2005). In contrast to sheathed ericoid mycorrhiza (Vohník et al. 2012), the hyphae forming these sheaths lacked clamp connections. The parenchymatous nets formed by dematiaceous septate hyphae correspond to structures described by Fernando and Currah (1996), Wurzburger and Bledsoe (2001), and Vohník and Albrechtová (2011) which are typically formed by DSE. The presence of intracellular vesicules resembling those formed in the AM symbiosis in the rhizodermal cells of Ericaceae is not frequent but has been already reported for some Australian ericoid species (McLean and Lawrie 1996), *G. poeppigii* from Central Argentina (Urcelay 2002), and for Himalayan rhododendrons (Chaurasia et al. 2005). As we did not detect any arbuscules, we conclude that the screened *Gaultheria* spp. did not form functional AM and that the vesicules were most probably formed by extraradical hyphae originating from neighboring AM host plants. Similarly to Vohník and Albrechtová (2011), we found that some rhizodermal cells were colonized by hyphae possessing clamp connections. However, this basidiomycetous colonization pattern was infrequent and seemed to be restricted to already senescing cells. Congruently, all basidiomycetes isolated in this study belong to soil saprobic/wood-decaying fungi (cf. Vohník et al. 2012).

The community of cultivable mycobionts

The analysis of similarity of the cultivable mycobiont communities in the three plant/site combinations yielded comparably low index values and the diversity indexes among the plant/site combinations were relatively high with significant differences between *G. poeppigii* from Llao Llao and the two other combinations. It seems that the differences between the respective plant/site combinations are primarily in the relatively rare species (i.e., with low number of isolates). Clearly, the most frequent species were shared among all combinations. The rarefaction curves however suggest that more samples are needed to obtain more complete diversity patterns for the screened plant/site combinations.

In agreement with many studies targeting Ericaceae mycobiont communities (e.g., Walker et al. 2011; Gorzelak et al. 2012), the majority of recovered isolates belonged to Helotiales, especially to the *P. fortinii* s. l.—*Acephala applanata* species complex (PAC; Grünig et al. 2008). Although these DSE may form intracellular hyphal loops in Ericaceae rhizodermal cells (Vohník et al. 2003), there is currently no evidence that they form functional ErM and they should be thus regarded as probably harmless ericaceous endophytes. The high frequency of PAC mycobionts among the isolates likely reflects their fast growth on the isolation media. On the other hand, in contrast to all *Tetracladium* and basidiomycetous isolates, the PAC isolates were recovered mostly on the benomyl-free media suggesting that employing of additional specific isolation media would reveal broader spectrum of cultivable root mycobionts.

Besides PAC, the screened *Gaultheria* hair roots hosted relatively diverse spectrum of mycobionts. However, most of them could not be assigned as typical ErM fungi. The second and third most frequent genera, *Pochonia* and *Tetracladium*, represent common soil saprobic/parasitic fungi and aquatic hyphomycetes also known to associate with plant roots, respectively (Vohník et al. 2011; Selosse et al. 2008). Congruently, their respective isolates failed to form ErM in the re-synthesis experiment. Similarly, *I.* (*Neonectria*) *radicicola* and *C. olivo-luteacea* are known root and trunk pathogens, respectively (Cabral et al. 2012; Agusti-Brisach et al. 2013).

Surprisingly, we recovered only five isolates of *O.* cf. *maius* and one isolate of *Meliniomyces* sp. which represent the expected ErM symbionts of the screened *Gaultheria* spp. (cf. Wurzburger et al. 2011). Moreover, the prominent ErM fungus *R. ericae* has not been isolated at all. This might be explained by their comparably slower growth on artificial isolation media especially when the fast-growing DSE dominate the root-associated fungal communities (Walker et al. 2011). On the other hand, mycobionts belonging to the *R. ericae* aggregate can dominate cultivable spectra of ericaceous root-associated fungi even when DSE are simultaneously present (Ishida and Nordin 2010). We suggest that in our case, the low recovery rate rather reflects their low incidence in the screened roots. More light could be shed on this issue using culture-independent techniques. For example, Wurzburger et al. (2011) investigated mycobionts of *Rhododendron maximum* (Ericaceae) from North America (NC, USA) and while they failed to isolate any *R. ericae* s. s. using the culture-dependent approach, its cloned sequences were relatively frequent (10.4 % in the A horizon). Similar was true for *P. fortinii*: it was not cultured but frequently detected by cloning (16.7 % in the A horizon). At the same time, the cultivable mycobiont spectrum was dominated by *O. maius* (46 % of the obtained isolates) but its recovery by cloning was low (4.7 and 3.8 % in the O and A horizons, respectively). Apparently, it is best to combine both approaches as already discussed by other authors (e.g., Allen et al. 2003, Bougoure and Cairney 2005b).

Amplification with specific primers

To our knowledge, in the Southern Hemisphere, *R. ericae* s. s. has been so far detected only in the rhizoids of liverworts from Antarctica and Australia (Chambers et al. 1999; Upson et al. 2007). Indeed, some authors already suggested that Southern Hemisphere's Ericaceae may host ErM fungi differing from those colonizing Northern Hemisphere's Ericaceae (Hutton et al. 1994; McLean et al. 1999). This study extends the known range of the *R. ericae* aggregate for NW Patagonia. However, further studies are needed to specifically detect the prominent ErM fungus *R. ericae* in South America's Ericaceae.

Using PCR-based methods, we were able to show that REA members colonized at least some of the examined *Gaultheria* roots. Reasons for the substantial difference between the plants from Cerro Otto and Llao Llao remain unknown but might include the different tree dominants with contrasting mycorrhizal preferences. Kohout et al. (2011) showed that presence of Ericaceae dwarf shrubs significantly altered ectomycorrhizal colonization of neighboring *Pinus* spp. seedlings. Most notably, Ericaceae suppressed the REA species *Meliniomyces bicolor* which formed abundant ectomycorrhizae when pine seedlings were cultivated alone, without ericaceous plants. Perhaps, ectomycorrhizal trees might have analogous effect on the community of ErM fungi in neighboring Ericaceae roots?

Similarly to Berch et al. (2002) and Allen et al. (2003), the results obtained here with the Sebacinaceae-specific primer pair support the hypothesis that the majority of the screened *Gaultheria* roots were colonized by the difficult-to-cultivate Sebacinaceae. This hypothesis is also supported by the low recovery rate of the typical ErM fungi, the failure of most of the in vitro tested isolates to form typical ErM structures and by microscopic observations (see above).

Conclusions

To conclude, it seems that the major part of the screened Gaultheria spp. roots was colonized by the mostly non-cultivable Sebacinaceae which were likely responsible for the most frequent ErM colonization pattern. The culture-based approach thus discriminated these ErM fungi in favor of root endophytes, saprobes, and/or parasites. Members of the *R. ericae* aggregate were comparably less frequent, detectable only by the direct PCR-based method, mostly at a locality dominated by arbuscular mycorrhizal *A. chilensis* trees. A follow-up study is underway to resolve the effect of tree cover on the community of *Gaultheria* root mycobionts as well as to answer the question whether *R. ericae* s. s. is indeed absent in the Ericaceae from NW Patagonia.

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