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Fungal associates of *Pyrola rotundifolia*, a mixotrophic Ericaceae, from two Estonian boreal forests

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Abstract *Pyrola rotundifolia* (Ericaceae, Pyroleae tribe) is an understorey subshrub that was recently demonstrated to receive considerable amount of carbon from its fungal mycorrhizal associates. So far, little is known of the identity of these fungi and the mycorrhizal anatomy in the Pyroleae. Using 140 mycorrhizal root fragments collected from two Estonian boreal forests already studied in the context of mixotrophic Ericaceae in sequence analysis of the ribosomal DNA internal transcribed spacer region, we recovered 71 sequences that corresponded to 45 putative species in 19 fungal genera. The identified fungi were mainly ectomycorrhizal basidiomycetes, including *Tomentella*, *Cortinarius*, *Russula*, *Hebeloma*, as well as some ectomycorrhizal and/or endophytic ascomycetes. The *P. rotundifolia* fungal communities of the two forests did not differ significantly in terms of species richness, diversity and nutritional mode. The relatively high diversity retrieved suggests that *P. rotundifolia* does not have a strict preference for any fungal taxa. Anatomical analyses showed typical arbutoid mycorrhizae, with variable mantle structures, uniseriate Hartig nets and intracellular hyphal coils in the large epidermal cells. Whenever compared, fungal ultrastructure was congruent with the molecular identification. Similarly to other mixotrophic and autotrophic pyroloids in the same forests, *P. rotundifolia* shares its mycorrhizal fungal associates with surrounding trees that are likely a carbon source for pyroloids.

Keywords Arbutoid mycorrhizae · Ericaceae · Ectomycorrhizal communities · Endophytic fungi · Mixotrophy · Mycorrhizal anatomy

Introduction

A major recent breakthrough in plant nutrition was the discovery that some green forest plants, although able to perform photosynthesis, recovered significant amount of carbon (C) from their mycorrhizal fungi. This strategy, called mixotrophy (MX), was documented in green orchids (Gebauer and Meyer 2003; Bidartondo et al. 2004; Julou et al. 2005; Abadie et al. 2006) and, more recently, in a group of forest perennial subshrubs from the Ericaceae (Tedersoo et al. 2007; Zimmer et al. 2007), namely the sub-tribe Pyroleae, here referred to as pyroloids. These two plant families also harbour fully achlorophyllous, heterotrophic species that obtain all their C from their mycorrhizal fungi, the so-called mycoheterotrophic (MH) plants (Leake 2004). Indeed, the occurrence of MX species was proposed as an evolutionary predisposition to the rise of MH taxa (Tedersoo et al. 2007). Namely, *Pyrola aphylla* is fully non-chlorophyllous and thus MH (Zimmer et al. 2007).

Two major tools contributed to advances in the study of MX (and MH) species, namely stable isotopes and

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molecular methods, to identify fungal symbionts. Organisms usually have similar abundance in ^{13}C to their food source, such as MH plants and their associated fungi (Trudell et al. 2003; Tedersoo et al. 2007). As an exception, however, fungi obtaining C from living or dead plants are richer in ^{13}C than autotrophic plants (Hobbie et al. 2003; Zeller et al. 2007). A contribution of fungal C to MX biomass can thus be detected in the ^{13}C content as a deviation from that of surrounding autotrophic plants. The comparison of the ^{13}C content of MX and fully heterotrophic MH plants and fungi allows quantification of fungal C used in MX biomass (Gebauer and Meyer 2003). With such assumptions, Tedersoo et al. (2007) found a variable level of C heterotrophy in four green pyroloid species from two Estonian sites, ranging from 0% to 67.5%. Zimmer et al. (2007) found MX in pyroloids in only one out of six and none out of three sites in Germany and California, respectively.

Fungi associated with pyroloids form arbutoid mycorrhizae, sometimes referred to as ‘ectendomycorrhizae’, i.e. mycorrhizae with a hyphal sheath, an intercellular Hartig net and coils in root cells (Robertson and Robertson 1985; Smith and Read 1997; Massicotte et al. 2008). Molecular methods demonstrated that most fungal associates of European green pyroloids were biotrophic, falling into two ecological categories (Tedersoo et al. 2007; Zimmer et al. 2007). Some species belonged to taxa often reported as plant roots endophytes (e.g. Helotiales) whose physiological roles, if any, remain unknown (Schulz and Boyle 2005). Most species belonged to taxa forming ectomycorrhizal (ECM) associations with forest trees, belonging to Asco- and mainly Basidiomycetes. This suggests that overstorey trees are the ultimate C source for MX pyroloids, via the fungi, exactly as described for MX orchids (Bidartondo et al. 2004; Julou et al. 2005; Abadie et al. 2006; Selosse et al. 2006) and MH plants from temperate forests (Leake 2004). Accordingly, Hashimoto et al. (2005) provided ex situ evidence that labelled C can move from Japanese larch (*Larix kaempferi*) to co-cultivated *Pyrola incarnata*, suggesting that fungal links to the tree are crucial for MX pyroloids.

Despite this, mycorrhizal associates of pyroloids are hitherto known from a limited number of species and sites only. Especially, the fungal associates of *P. rotundifolia*, the most heterotrophic green pyroloid reported so far (with the exception of the MH *P. aphylla*), are still unknown. Despite careful ultrastructural studies of arbutoid mycorrhizae in six North-American pyroloid species (Robertson and Robertson 1985; Massicotte et al. 2008), no morphological nor ultrastructural data are available for *P. rotundifolia* mycorrhizae. In this study, we further document fungal associates of pyroloids by investigating mycorrhizal associates of *P. rotundifolia* in two boreal forests in Estonia where other pyroloid mycorrhizal fungi were already identified (Tedersoo et al. 2007). In addition, we included molecular data from the site where a high heterotrophy level (67.5% of fungal C in the biomass) was previously detected for *P. rotundifolia* (Tedersoo et al. 2007). A combination of molecular and transmission electron microscopy (TEM) methods was used to identify the mycorrhizal fungi and to describe their interaction with host roots.

Materials and methods

Study site and sampling

Mycorrhizal sampling was performed on August 14th, 2005 at Kärla (Saaremaa Island, NW Estonia; geocode: 58°20′N, 22°18′E) and on September 10th, 2005 at Värska (SE Estonia: 57°57′N, 27°40′E). These sites, distant from approximately 350 km, were sampled and described more profoundly in Tedersoo et al. (2007). Briefly, they were selected because of their dense population of at least three pyroloid species and a canopy covering <70% of the area. The tree host community in Kärla was mainly composed of 100- to 120-year-old Scots pine (*Pinus sylvestris*) with sparse Norway spruce (*Picea abies*) undergrowth. The site of Värska comprised a 60- to 80-year-old mixed forest of Scots pine and silver birch (*Betula pendula*). Haplic podzol on limnoglacial sand dunes is the dominant soil type at both sites.

At each site, root systems of the *P. rotundifolia* were manually separated from ten 20×40 cm (depth=20 cm) soil cores, taken at least 10 m apart from each other. Plant roots were identified by tracing root systems to rhizomes and leaves. Roots covered by more or less dense wefts of hyphae were considered potentially mycorrhizal. Seven 2- to 3-mm root fragments colonised by fungi were selected from different parts of each root system. Healthier and cleaner roots were selected, as they were more likely to contain a higher proportion of living tissue of true mycorrhizal fungi rather than of saprotrophs or endophytes. These 140 root fragments (seven fragments from ten root systems in two sites) were photographed using a Carl Zeiss Stemi 2000-CS stereo microscope and Axioskop2 light microscope (Fig. 1), transferred to 100 ml of cetyltrimethylammonium bromide (CTAB) lysis buffer [100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% CTAB] and kept at −20°C before molecular investigations. Moreover, whenever mycorrhizae were long enough (more than 3 mm), a subsample of the remaining mycorrhizal length was preserved for TEM investigations by quick fixation using 2.5% (*v*/*v*) glutaraldehyde in 0.1 ml 0.1 M cacodylate buffer (pH 7.2) and stored at 4°C during 3 months.

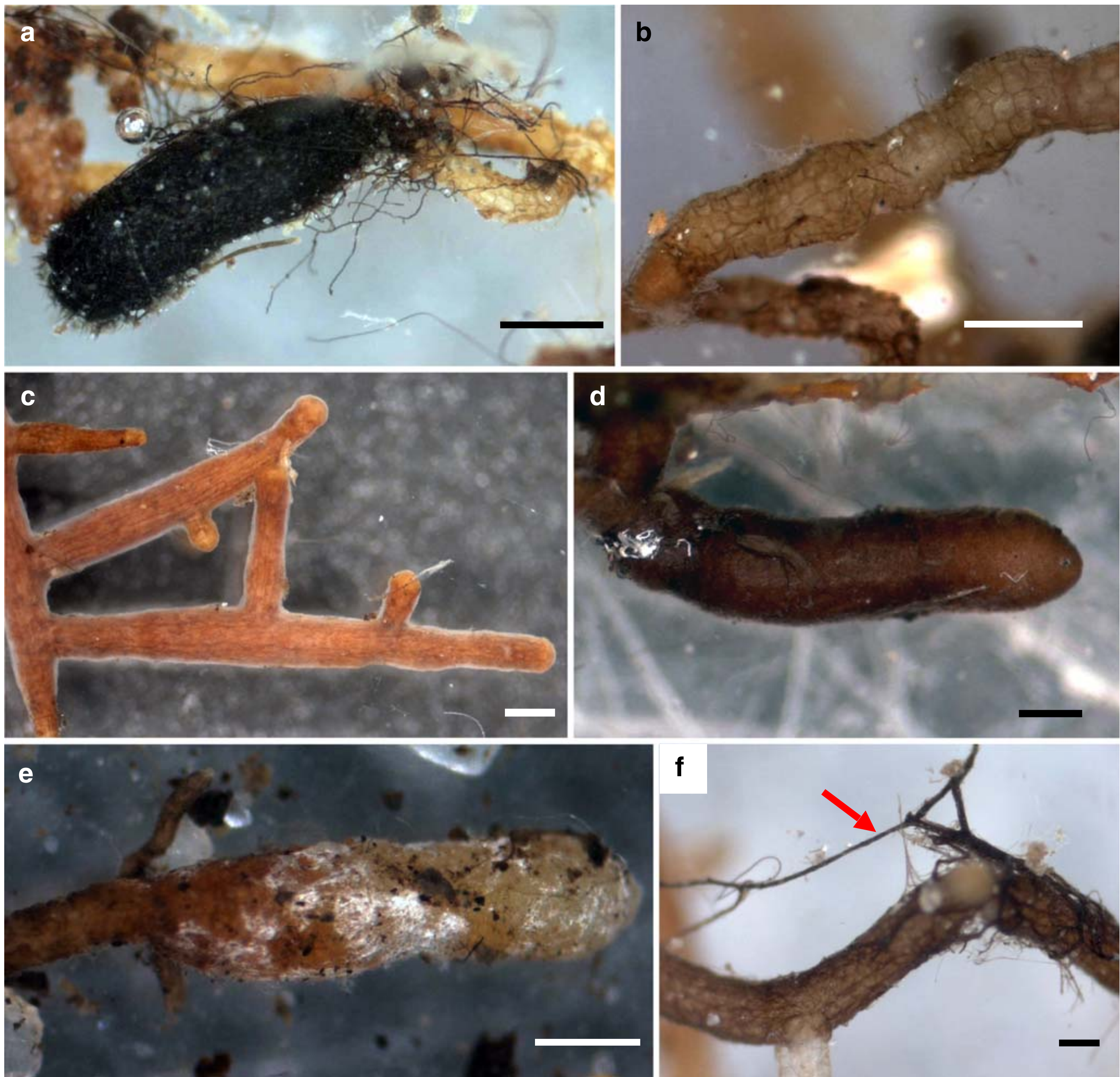

Fig. 1 External morphology of selected *P. rotundifolia* mycorrhizae identified by ITS sequencing. **a** *Tomentella* sp.1; **b** *Amphinema-Tylospora* sp.1; **c** *Inocybe* sp.1; **d** *Tomentella* sp.5; **e** *Hebeloma* sp.2; **f** *Tomentella* sp.6 with a rhizomorph (*arrowed*). *Bars* are 0.4 mm

Molecular identification of fungal partners

DNA extraction and polymerase chain reaction (PCR) amplification of fungal intergenic ribosomal DNA spacer (ITS) were performed as in Selosse et al. (2002) using the two primers sets ITS1F + ITS4 and ITS1F + ITS4B. Whenever a single fragment was amplified, it was tentatively sequenced from both strands using the two amplification primers, as in Tedersoo et al. (2007). Whenever more than one fragment was amplified, as revealed on gel after PCR or by sequence data, the PCR product was discarded from further analysis. This is because (1) we aim at documenting qualitatively the link to ECM fungi, but not the whole fungal diversity on *P. rotundifolia*, and (2) our previous analyses suggested that samples with multiple PCR products were mostly colonised by endophytic fungi in addition to an ECM fungus (Tedersoo et al. 2007). Sequences from both strands were edited using Sequencher™ 4.6 for MacOS X (Genes Codes, Ann Arbor, USA), and all sequence stretches that were ambiguous, due to the presence of additional fungal endophytes, were pruned from the edited sequence. To

identify the fungi, the sequences were queried against GenBank, European Molecular Biology Laboratory (EMBL) and the ECM fungal sequence database UNITE (Kõljalg et al. 2005) using blastN or fasta3 algorithms (all reported sequence identities are based on full-length pairwise alignments). To infer species, sequences were grouped based on >97.0% sequence identity over the whole ITS region (Tedersoo et al. 2003). Although there is no universally applicable threshold (Nilsson et al. 2008), we found 97.0% the most suitable molecular species criterion due to the low quality of some sequences. For each species, the best corrected sequence was deposited in EMBL sequence database. Putative trophic status of the detected species is that of the reported lifestyle of the closest matching taxa.

TEM investigations

Among mycorrhizae successfully typed by molecular analysis (see above), ten subsamples showing divergent morphotypes were available for TEM analysis. They were rinsed three times with the fixing buffer and then dehydrated in an ascending series of ethanol solution to 100%. They were then incubated in two changes of absolute acetone and infiltrated in Epon–Araldite resin that was polymerised for 24 h at 60°C. Embedded samples were processed for ultramicrotomy: semi-thin sections of 0.5 µm were stained with 1% toluidine blue and used to check for the pattern of fungal colonisation. Ultra-thin (70 nm) sections were counter-stained with uranyl acetate and lead citrate and used for TEM analyses under a JEM-1200 EX II electron microscope (Jeol, Tokyo, Japan) to score mycorrhizal colonisation and features corroborating molecular identification (e.g. intercellular communications, dolipores or Woronin bodies; Selosse et al. 2004; Abadie et al. 2006).

Statistics

Differences in PCR amplification and sequencing success between study sites were tested using chi-square tests. When the sample size in a distribution was too low ($n<5$), as for the comparisons of the main genus abundances, the fungal ecologies and the ratio of Ascomycota to Basidiomycota, a chi-square tests with Yates' correction were applied. As the number of successfully typed mycorrhizae differed between Kärla and Värska (respectively 50 and 20), data were rarefied as in Richard et al. (2005) to the size of the smaller sample to compare the fungal richness between sites. To compare the fungal diversity, Shannon diversity index was calculated. Because this index is sensitive to rare species, we also calculated the Fisher's alpha value for each pyroloid species using data from this and previous (Tedersoo et al. 2007) study.

Results

Identification of mycorrhizal fungi

Using the primer set ITS1F + ITS4, PCR amplifications were successful for 123 out of the 140 samples (Table 1), with a similar success rate at both sites ($\chi^2=0.603$, $df=1$, $p=0.438$). Direct sequencing of PCR products was successful for 71 root fragments (Table 1), with a higher success at Kärla: 85.0% of the PCR products were successfully sequenced at Kärla compared to only 31.7% at Värska ($\chi^2=27.463$, $df=1$, $p<0.001$). Because samples showing evidence of dual colonisation (more than one PCR fragments or mixed sequences) were not further considered, we obtained 51 sequences at Kärla and 20 sequences at Värska. Out of the seven root fragments sampled per plant, one to four produced a readable sequence at Värska compared to one to six at Kärla. All PCR amplifications that amplified with the basidiomycete-specific primer set ITS1F + ITS4B were also successful with the more general, fungal-specific ITS1F + ITS4 set. Whenever both primer pairs produced sequences ($n=31$), these were 100% identical.

In all, the sequence analysis resulted in 65 sequences (Tables 1 and 2) that were attributed to 45 species including 40 Basidiomycota. *Amphinema-Tylospora* sp.1 and *Hebeloma* sp.2 were found in four and three root samples, respectively. Eight fungal species were found in two different root samples. Several fungal genera were identified at both two sites (Table 1), including most of the dominant ones. The relative frequency of *Cortinarius* and *Tomentella* were similar at both sites ($\chi^2=5.256$, $df=2$, $p=0.072$). *Tomentella*, *Russula*, *Hebeloma*, *Cortinarius* and *Sebacina* spp. that were relatively frequent at Kärla were rare or absent from Värska (Table 1), but with no significant difference ($p>0.05$). Only two species, *Amphinema-Tylospora* sp.1 and *Tomentella* cf. *subclavigera* 1, were shared between the two sites (Table 2). The relative proportion of Asco- and Basidiomycota was similar at the two sites ($\chi^2=0.386$, $df=1$, $p=0.534$). All recovered taxa were ECM (Table 2) besides four putative endophytes and a single uncertain species (*Tulasnella* sp.1). The ecology of the associated fungi ('ECM' vs. 'endophytes and unknowns') did not significantly differ between the two sites ($\chi^2=0.279$, $df=1$, $p=0.597$).

The fungal richness for each site, as estimated by rarefaction of the data to a sample size of 11, was slightly higher at Värska than at Kärla (respectively, 7.25 and 6.9; Table 1). The Shannon diversity index were similar at both sites (2.37 at Kärla, 2.14 at Värska), whilst Fisher's alpha values, less influenced by rare species, were 10.61 and 15.65 for Kärla and Värska, respectively (Table 1), indicating that the relatively higher diversity at Värska was masked by the low typing success.

Table 1 Summary of the fungal taxa found on *P. rotundifolia* at Kärla and Värska

	Kärla	Värska	Both sites
Number of samples	70	70	140
Successful PCR[a]	60 (85.7%)	63 (90.0%)	123 (87.8%)
Successful typing[b]	51 (72.9%)	20 (28.6%)	71 (48.6%)
Number of diverging sequences	47	18	65
Number of species[c]	33	14	45
Richness after rarefaction[d]	6.93	7.25	–
Shannon diversity index	2.37	2.14	–
Fisher's alpha value	10.61	15.65	–
Genera			
Ascomycetes			
Helotiales	0	1	1
Humaria	1	0	1
Otidea	1	0	1
Phialocephala	1	1	2
Wilcoxina	1	0	1
Basidiomycetes			
Amphinema/Tylospora	2	1	2
Ceratobasidium	0	1	1
Cortinarius	3	4	7
Hebeloma	3	0	3
Inocybe	2	0	2
Piloderma	0	1	1
Russula	4	1	5
Sebacina	3	0	3
Sistotrema	0	1	1
Suillus	1	0	1
Tomentellopsis	1	0	1
Tomentella	10	2	11
Tricholoma	1	1	2
Tulasnella	1	0	1

[a] All primer sets included
[b] After successful amplification and discard of the samples showing evidence of dual colonisation
[c] All sequences showing more than 97% similarity are supposed to be from the same species—see "Materials and methods"
[d] Rarefied to a sample number of ten

Mycorrhizal morphology

Extraradical and mantle hyphal colonisation of the sampled mycorrhizae varied strongly among arbutoid mycorrhizal roots (Fig. 1). The mantle ranged from dense (Fig. 1a, d) to none (Fig. 1b), with sometimes emanating rhizomorphs (Fig. 1f), depending on fungal species. Variation in mantle thickness and anatomy was confirmed in transverse sections. In particular, plectenchymatous mantles of one to four hyphal layers occurred in *Hebeloma*, *Cortinarius* and *Tricholoma* mycorrhizae (Figs. 2a–c), whereas dense pseudoparenchymatous mantles comprising four to six hyphal layers occurred on mycorrhizae of *Russula* and *Tomentella* spp. (Figs. 2d–f and 3a, c). No organised mantle was seen in *Wilcoxina*, *Tulasnella* (Fig. 3e) and *Amphinema-Tylospora* mycorrhizae. All mycorhizae investigated by TEM (n=10; Table 2) showed a uniseriate Hartig net between the outermost cortical cells (Fig. 3d), but in some cases, the wall between some epidermal cells remained uncolonised. Fungi never invaded more deeply the root tissues. As a result of colonisation, cortical cells appeared compressed and deformed radially (Fig. 3a). Several hyphal layers were sometimes present at the junction between three or more cortical cells, as well as around the innermost part of the Hartig net, at the basis of epidermal cells (not shown). The cortical cells were large compared to the central cylinder and were filled with hyphal pelotons (Fig. 3a, c). Hyphae penetrated from the Hartig net without producing any visible plant wall reaction at the penetration site (Fig. 3d). Intracellular hyphae had similar size and cell wall thickness in Hartig net and epidermal cells (Fig. 3a, d). The colonisation often occurred on lateral roots (Fig. 1a–e), but did not always reach the root apex. Uncolonised apex showed that the large cortical cells were of epidermal origin (Fig. 3b) and were often collapsed when uncolonised. Their nucleus was laterally positioned before colonisation (Fig. 3b), but central upon fungal colonisation.

TEM analyses revealed dolipores in all ten investigated samples, further supporting their identification as members of Basidiomycota. No Woronin bodies were observed in these root fragments, indicating the paucity of ascomycetous hyphae. Unfortunately, no sample was available to investigate the possible ascomycete mycorrhizae detected by molecular tools. In mycorrhizae of *Tulasnella* sp.1 and *Sebacina* (clade A) sp.1, imperforate parenthesomes featur-

Table 2 Mycorrhizal fungi species associated with *P. rotundifolia* at Kärla and Värska

GB accession numbers	Putative species	Occurrences on sites[a]		Putative ecology[b]	Best blastN match[c]	% similarity
		Kärla	Värska			
EU668904, EU668905, EU668906	*Amphinema-Tylospora* sp. 1[d]	2 (2)	2 (3)	ECM	1—*Amphinema byssoides* AY838271	85.5
					2—Atheliaceae AM181408	93.0
EU668907	*Amphinema-Tylospora* sp.2[d]	1 (1)		ECM	*Amphinema byssoides* AY219838	83.4
					Atheliaceae AM181414	93.0
EU668908	*Ceratobasidium* sp.1		1 (1)	ECM	*Ceratobasidium* sp. AY805606	98.9
					Rhizoctonia sp. AJ419929	99.0
EU668909	*Cortinarius* cf. *anisatus* 1		1 (1)	ECM	*Cortinarius anisatus* UDB001318	98.3
					Cortinarius anisatus DQ117929	99.0
EU668910	***Cortinarius* cf. *saniosus* 1**		1 (1)	ECM	*Cortinarius saniosus* DQ102669	99.5
					Cortinarius saniosus DQ102681	99.0
EU668911	***Cortinarius* sp.1**	1 (1)		ECM	*Cortinarius hinnuleoarmillatus* UDB001460	96.5
					Cortinarius umbrinolens AY669658	96.0
EU668912	*Cortinarius* sp.2	1 (1)		ECM	*Cortinarius atrocoeruleus* UDB001011	98.6
					Cortinarius cf. *flexipes* DQ974714	98.0
EU668913	*Cortinarius* sp.3	1 (1)		ECM	*Cortinarius sertipes* UDB000068	95.8
					Cortinarius sp. EF218749	99.0
EU668914	*Cortinarius* sp.4		1 (1)	ECM	*Cortinarius sertipes* UDB000068	96.1
					Cortinarius sp. EF411087	98.0
EU668915	*Cortinarius* sp.5		1 (1)	ECM	*Cortinarius belleri* AY669685	95.8
					Cortinarius traganus DQ367900	95.0
EU668916	*Hebeloma* cf. *sinapizans* 1	1 (1)		ECM	*Hebeloma sinapizans* AY320380	99.7
					Hebeloma sinapizans AF096977	99.0
EU668917	*Hebeloma* sp.1	1 (1)		ECM	*Hebeloma leucosarx* AB211268	97.4
					Hebeloma velutipes AF430254	94.0
EU668918, EU668919	*Hebeloma* sp.2	3 (5)		ECM	*Hebeloma velutipes* UDB002445	99.8
					Hebeloma sp. EF093151	99.0
EU668920	Helotiales sp.1		1 (1)	End.	Uncultured Helotiales EF026053	98.9
					Epacris microphylla root associated fungus AY268217	99.0
EU668921, EU668922	*Humaria hemisphaerica* 1	2 (2)		ECM	*Humaria hemisphaerica* UDB000988	100.0
					Humaria hemisphaerica DQ200832	99.0
EU668923	*Inocybe* sp.1[d]	1 (1)		ECM	*Inocybe leiocephala* UDB000635	94.6
					Cortinariaceae AM181384	99.0
EU668924	*Inocybe* cf. *geophylla* 1	1 (1)		ECM	*Inocybe geophylla* UDB000632	99.3
					Inocybe geophylla var. *lilacina* AM882869	98.0
EU668925	*Otidea* cf. *tuomikoskii* 1	1 (1)		ECM	*Otidea tuomikoskii* AF072086	92.1
					Otidea tuomikoskii AF072085	99.0
EU668926	*Phialocephala* sp.1	1 (1)		End.	*Phialocephala fortinii* EU103612	92.0
					Phialocephala sp. EF101770	92.0
EU668927	*Phialocephala* sp.2		2 (2)	End.	*Phialocephala fortinii* AY394921	99.7
					Phialocephala fortinii AY394915	99.0
EU668928	*Piloderma* sp.1		1 (1)	ECM	*Piloderma sphaerosporum* UDB001750	99.8
					Piloderma sp. AY0970053	99.0
EU668929	***Russula* sp.1**		1 (2)	ECM	*Russula favrei* EF530944	96.7
					Russula pascua AY061705	96.0
EU668930	*Russula fuscorubroides* 1	1 (1)		ECM	*Russula fuscorubroides* AF418624	100.0
					Russula queletii EU248592	95.0
EU668931	***Russula* sp.2**	1 (1)		ECM	*Russula postiana* AF230898	99.3
					Russula sp. EF218809	96.0
EU668932	***Russula* sp.3**	1 (3)		ECM	*Russula acrifolia* UDB0002470	94.0
					Russula sp. EF218808	98.0
EU668933	*Sebacina* (clade A) sp.1	2 (2)		ECM	*Sebacina* sp.UDB000773	92.5
					Sebacinales EF030946	98.0
EU668934	*Sebacina* (clade B) sp.1	1 (1)		End.	*Sebacina vermifera* DQ520096	87.6
					Sebacinaceae DQ273405	98.0

Table 2 (continued)

GB accession numbers	Putative species	Occurrences on sites[a]		Putative ecology[b]	Best blastN match[c]	% similarity
		Kärla	Värska			
EU668935	*Sistotrema* cf. *alboluteum* sp. 1		1 (1)	ECM	*Sistotrema alboluteum* AJ606043	99.8
					Sistotrema alboluteum AJ606042	99.0
EU668936, EU668937	*Suillus* cf. *variegatus* 1	2 (2)		ECM	*Suillus variegatus* UDB000664	98.9
					Suillus luteus DQ658861	98.0
EU668938	***Tomentella* sp.1**	1 (1)		ECM	*Tomentella lateritia* UDB000267	94.1
					Tomentella sp. EF218839	93.0
EU668939	*Tomentella* sp.2	1 (1)		ECM	*Tomentella badia* UDB000961	96.2
					Tomentella sp. EF218830	100.0
EU668940, EU668941, EU668942	***Tomentella* sp.3**	2 (3)		ECM	*Tomentella bryophila* UDB000035	95.7
					Tomentella sp. U83482	93.0
EU668943	*Tomentella* sp.4	2 (2)		ECM	*Tomentella bryophila* UDB000035	93.2
					Tomentella sp. EF411113	96.0
EU668944	*Tomentella* sp.5	1 (1)		ECM	*Tomentella bryophila* UDB000035	91.7
					Tomentella sp. AJ534917	99.0
EU668945	*Tomentella* sp.6	1 (3)		ECM	*Tomentella lilacinogrisea* UDB000272	94.7
					Tomentella sp. EF218821	98.0
EU668946	*Tomentella* sp.7	1 (1)		ECM	*Tomentella fuscocinerea* UDB000776	96.0
					Tomentella sp. EF218826	99.0
EU668947	*Tomentella* sp.8		1 (1)	ECM	*Tomentella bryophila* UDB000035	91.5
					Tomentella sp. EF218827	98.0
EU668948	*Tomentella* sp.9	1 (1)		ECM	*Tomentella fuscocinerea* UDB00240	95.0
					Tomentella sp. EF218835	95.0
EU668949, EU668950	*Tomentella* cf. *subclavigera* 1	1 (1)	1 (3)	ECM	*Tomentella subclavigera* UDB000259	100.0
					Tomentella subclavigera AF272939	99.0
EU668951	*Tomentella* cf. *subclavigera* 2	1 (2)		ECM	*Tomentella subclavigera* AY010275	100.0
					Tomentella sp. EF218839	98.0
EU668952	*Tomentellopsis* sp.1	2 (2)		ECM	*Tomentellopsis submollis* AY641459	96.7
					Tomentellopsis sp. AJ893353	98.0
EU668953	***Tricholoma* sp.1**		1 (1)	ECM	*Tricholoma portentosum* UDB001429	99.8
					Tricholoma sp. AF349688	99.0
EU668954	*Tricholoma* sp.2	1 (1)		ECM	*Tricholoma triste* UDB001691	99.8
					Tricholoma myomyces AF377210s	99.0
EU668955	***Tulasnella* sp.1**	1 (2)		?	Orchid mycorrhizal *Tulasnella* DQ925521	99.3
					Tulasnellaceae DG925521	99.0
EU668956	*Wilcoxina* sp.1	1 (1)		ECM	*Wilcoxina rehmii* AF266708	97.8
					Wilcoxina sp. AJ893249	99.0

Species for which one mycorrhiza was investigated by TEM are in bold.
[a] Indication of the number of root tips harbouring the fungus with the number of plant individuals with such root tips (under brackets)
[b] *ECM* ectomycorrhizal fungus, *End.* endophytic fungus
[c] Best blast: (1) in Unite + EMBL data bases and (2) GenBank (only the best blast with informative taxonomy is given)
[d] Taxa already found on other pyroloids by Tedersoo et al. (2007)

ing these two taxa and a slime typical for cell walls of tulasnelloids further supported the molecular identification (Fig. 2f). Several hyphae exhibited intra-hyphal growth (Fig. 3g). Since plant cytoplasm was poorly preserved, probably due to insufficient fixation, we obtained little evidence that the cytoplasm of the host cells was alive. However, with exception of the *Tulasnella* sp.1, mycorrhizae preservation was poor, and all cells appeared to have been fixed in a turgescent state (Fig. 3a, c): Given the thin wall, the spheroid cell shape can only be explained by turgescence, which is an indirect evidence of biotrophic interaction. No evidence of collapsed or lysed hyphae in apparently healthy, turgescent cells was found; instead, non-collapsed hyphae were sometimes found in possibly senescent host cells (Fig. 3c) as well as joint senescence of hyphae and host cytoplasm (data not shown).

Discussion

The general shape of *P. rotundifolia* mycorrhizae (Figs. 1, 2 and 3) is congruent with previous report from other

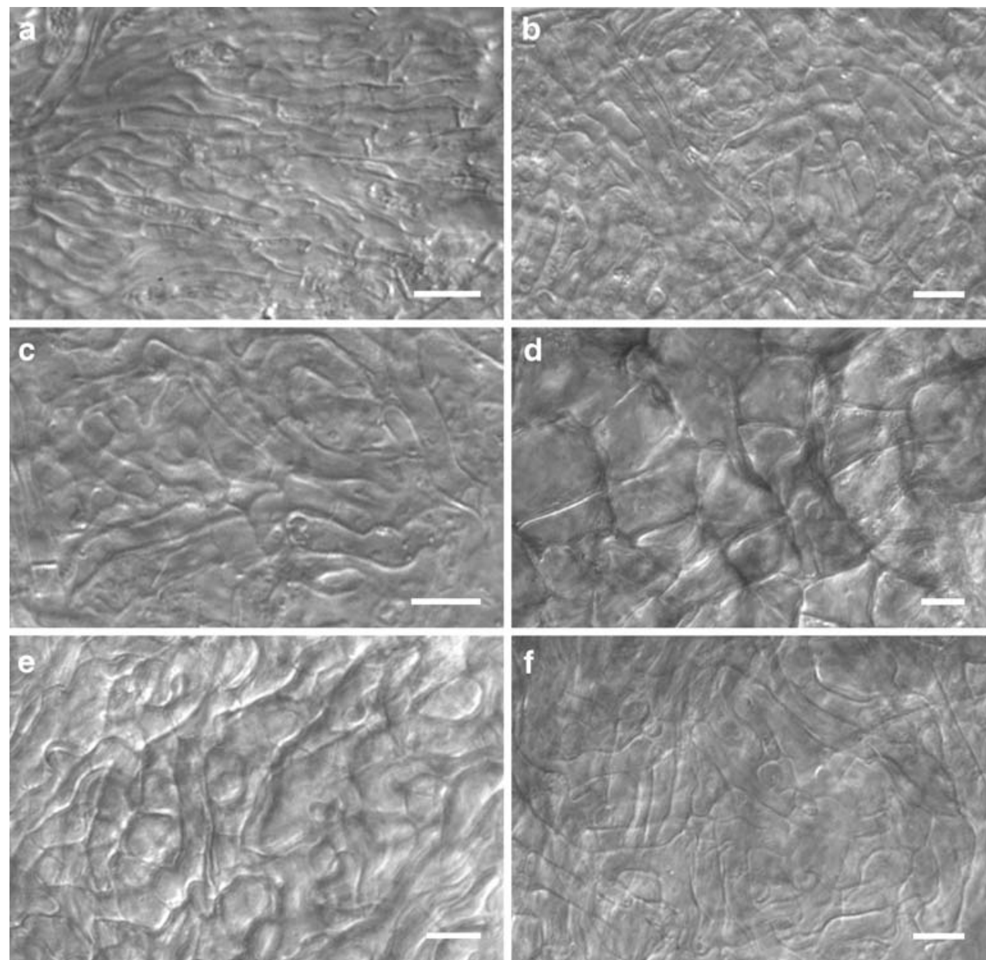

Fig. 2 Mantle anatomy of selected mycorrhizae of *P. rotundifolia* in Kärla. **a**–**c** Mycorrhizae of *Hebeloma* sp.2; **d**–**f** mycorrhizae of *Tomentella* sp.5. **a**, **d** Outer mantle layers; **b**, **e** middle mantle layers; **c**, **f** inner mantle layers. *Bar* 5 µm

pyroloids (Robertson and Robertson, 1985; Smith and Read, 1997; Massicotte et al. 2008). They show a reduced central cylinder surrounded by a single layer of large cells colonised by fungi whose origin is epidermal (Fig. 3b) as also described for ericoid mycorrhizae (Berta and Bonfante-Fasolo 1983). The apex is often not colonized, and hyphal density in the mantle and extraradical zone is variable. Massicotte et al. (2008) also reported that the mantle can be absent (e.g. in *P. chlorantha*) or dense (e.g. in mycorrhizae formed by *Thelephoraceae*-like and *Atheliaceae*-like fungi). The pattern of intracellular colonisation and the narrow uniseriate Hartig net, with several entries in cells, is also shared with other pyroloids (Robertson and Robertson 1985; Massicotte et al. 2008). A pending question concerns the mechanism of C transfer from fungi to pyroloids. In our TEM analysis, host cytoplasm senesce before the fungal hyphae and, together with Robertson and Robertson (1985), we were not able to support the claim by Lück (1941) that any intracellular digestion of hyphae occurs. Digestion may thus not to be involved in fungus-to-plant C transfer, whereas this is a commonly invoked mechanism (Trudell et al. 2003). Further microscopic investigations are needed to assess this point.

A diverse but phylogenetically similar fungal diversity was recovered from the Kärla and Värska sites, dominated by ECM fungi (Tables 1 and 2). Since no additional cloning was performed for unsequenced samples and since sampling was limited to two sites, the results of the fungal community composition are not exhaustive. Broadening the geographical scope and linking it with ecological variables would certainly reveal more fungal diversity. Only four fungal species were already found on other pyroloids from Kärla studied by Tedersoo et al. (2007), including *Amphinema-Tylospora* sp. (AM181414-15 from *Chimaphila umbellata*), *Inocybe* sp.1 (AM181384 from *Orthilia secunda*) and *Tulasnella* sp.1 (AM181390 from *C. umbellata* and *O. secunda*). The moderate values of the Shannon diversity index at Kärla and Värska (Table 1) reflect the presence of many rare fungal taxa, detected only once in the study, in part due to the limited sampling size. The fungal richness after rarefaction and the Fisher's alpha value were higher for Värska than for Kärla (Table 1), but it is difficult to speculate on the reasons for this.

The taxa found on *P. rotundifolia* encompassed those identified on other pyroloids from European and Californian sites by Zimmer et al. (2007) and from Canada by Massicotte et al. (2008). This suggests that pyroloids

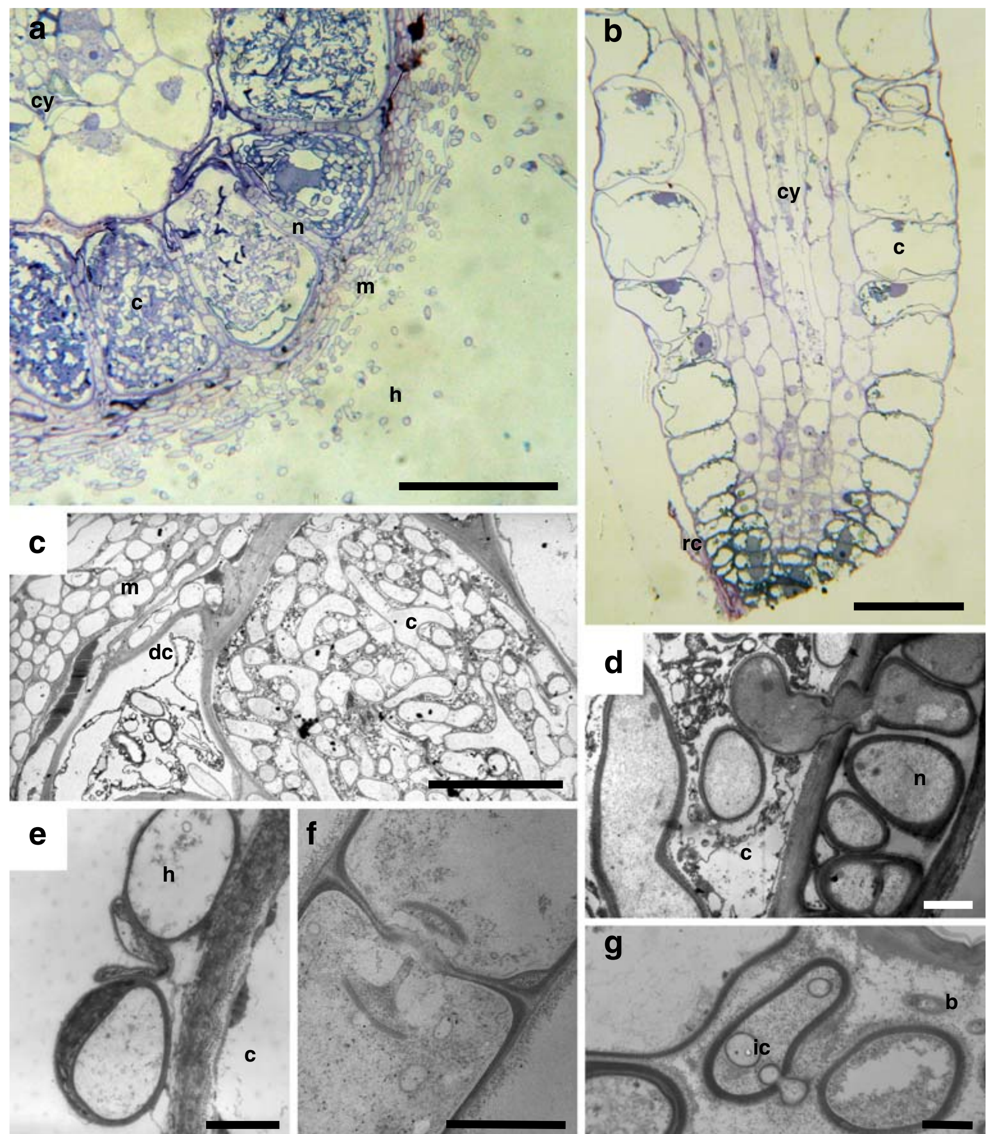

Fig. 3 Anatomy of *P. rotundifolia* mycorrhizae identified by ITS sequencing. **a** Transverse section of mycorrhizae formed by *Tomentella* sp.1, showing the large cortical cells (*c*) colonised by hyphae, the uncolonised central cylinder (*cy*), the Hartig net (*n*) and a dense hyphal mantle (*m*) as well as external hyphae (*h*). **b** Longitudinal section of an uncolonised, broken apex flanking a *Tomentella* sp.5 mycorrhiza with remains of root cap (*rc*), central cylinder (*cy*) and cortical cells (*c*). Note that the oldest uncolonised cortical cells have laterally positioned nuclei and tend to plasmolyse. **a**, **b** Light microscopy, *bars*= 100 µm. **c** Interface between a dense hyphal mantle (*m*) and cortical cells (*c*) in TEM of a *Russula* sp.3 mycorrhiza. Note non-collapsed, living hyphae in a dead host cell (*dc*); *bar*= 50 µm. **d** Detail of a hypha connecting the uniseriate Hartig net (*n*) and hyphal coils in cortical cells (*c*) from a *Russula* sp.1 mycorrhiza. **e** Detail of superficial hyphae (*h*) on a cortical cell (*c*) of a *Tulasnella* sp.1 mycorrhiza. **f** A tulasnelloid dolipore in a *Tulasnella* sp.1 mycorrhiza. **g** Bacteria (*b*) and intra-hyphal fungal colonisation (*ic*) in loose mantle hyphae of a *Hebeloma* sp.2 mycorrhiza. **d**–**g** TEM with *bars*=5 µm

species tend to associate with similar fungal taxa. The diversity indices calculated from Tedersoo et al. (2007) at Kärla for other MX pyroloids correspond to the values found here (Table 1). Namely, Fisher's alpha values of the fungal community were 14.1 for *Orthilia secunda*, 10.9 for *Pyrola chlorantha* and 8.3 for *Chimaphila umbellata*, i.e. well in the range of the present study. In our previous analysis, we suggested that pyroloids possibly had a preference for *Tricholoma* spp., but Zimmer et al. (2007) challenged this. The present study rather supports the absence of apparent fungal preference in pyroloids, and few *Tricholoma* spp. were found (Table 1). A striking difference with our previous work at Kärla is that the relative abundance of ECM fungi versus simple endophytes is higher for *P. rotundifolia* than for other pyroloids. Cloning efforts in Tedersoo et al. (2007) most often revealed endophytic fungi in addition to ECM fungi, so that endophytes and ECM fungi were about equally frequent in this previous study. The methodological option of the present study is sufficient to confirm a potential link to surrounding trees, but does not allow conclusions as to whether *P. rotundifolia* harbours more or less frequently ECM fungi than other pyroloids. Although direct in situ evidence is still lacking, the finding of a diverse ECM fungal community in the root of the strongly MX *P. rotundifolia* is in agreement with the idea that MX plants derive C from surrounding trees by way of shared mycorrhizal fungi (Selosse et al. 2006; Tedersoo et al. 2007; Zimmer et al. 2007).

The contrasting typing success in the two sites can be explained by time of sampling, differential preservation of samples or site effects affecting fungal colonization patterns. Similarly, Zimmer et al. (2007) reported strong differences in PCR success between sites. Given our standardised procedures, different preservation of the samples between the two sites is unlikely to explain the

more frequent multiple fungal colonisations at Värska. Since roots were sampled 1 month later at this site, it may be that colonisation by endophytes progressively increases during the growing season due to a continuous recruitment from soil. Multiple fungal colonisations do occur on both sites in *P. rotundifolia* roots, as suggested (1) by samples for which direct sequencing of PCR product failed, (2) by some sequences whose chromatogram showed faint contamination by at least one other fungal ITS and (3) by the intra-hyphal growth (Fig. 3g) that may represent colonisation by other fungi. Indeed, a few fungi usually considered as root endophytes were found (Table 2), but in absence of anatomical data for these, we ignore whether they behave as true mycorrhizal fungi or as simple endophytes on *P. rotundifolia*. Poor physiological conditions of roots at time of sampling may also explain the abundance of endophytic fungi. Nevertheless, the success of direct sequencing was twice higher than that for *Chimaphila umbellata* root sampled at the same time on both sites (Selosse and Tedersoo, unpublished data). The fact that ITS of putative endophytes was directly amplified (Table 2) does not mean that they were sole root colonisers, since the mycorrhiza-forming fungus can remain hidden due to our primers sets. This applies to *Tulasnella* sp.1 whose ecology is unknown, although some *Tulasnella* spp. are ECM (Bidartondo et al. 2003; Tedersoo et al. 2008a, b). In this study, we were only able to document a loose interaction (Fig. 3f) in which TEM failed to demonstrate biotrophy due to poor tissue preservation. Similarly, to our knowledge, only one interaction between pyroloids and ascomycetes is reported at anatomical level (Robertson and Robertson 1985). The interactions between pyroloids and putatively endophytic fungi and ECM ascomycetes deserve further analyses. Selosse et al. (2007) hypothesised that endophytic ascomycetes (Helotiales) and basidiomycetes (sebacinoids) had been recruited as predominant mycorrhizal partners when ericoid mycorrhizae emerged in the other Ericaceae (Ericoideae and Vaccinioideae tribes) at time of their divergence from pyroloids and related species. In our study, *Helotiales* sp.1 and *Sebacina* (clade B) sp.1 belong to taxa that commonly form ericoid mycorrhizae (Smith and Read 1997; Selosse et al. 2007). Morphological analysis of their interaction with pyroloid roots, compared with their interaction with ericoid mycorrhizae, could allow reconstructing the features of the ancestral mycorrhizal association in Ericaceae.

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