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Ectomycorrhizal fungi at tree line in the Canadian Rockies

II. Identification of ectomycorrhizae by anatomy and PCR

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Abstract Ectomycorrhizae of *Picea*, *Abies*, *Dryas* and *Salix* were collected at two tree-line sites at an altitude of 2,000–2,500 m in the Front Range of the Canadian Rockies. Six mycobionts were identified to species by direct comparison of PCR-amplified ribosomal DNA with that from locally collected sporocarps. Four of these (*Cortinarius calochrous*, *Hydnellum caeruleum*, *Laccaria montana* and *Russula integra*) are newly described symbioses. Twelve other ectomycorrhizae had no conspecific RFLP match with the sporocarps analyzed, but were identified to species, genus or family by anatomical comparison with sporocarps and literature descriptions or by phenetic clustering based on the presence or absence of restriction fragments. The majority of species identified have northern and/or montane distributions. Mycorrhizae are described on the basis of both anatomical and molecular characters.

Keywords Mycorrhizae · Alpine · Subalpine · Restriction fragment length polymorphism · DNA

Introduction

At tree line on the eastern slopes of the Canadian Rockies, the subalpine forest merges with alpine vegetation into a mosaic of the two vegetation types. This habitat is relatively diverse because of the unique combination of subalpine and alpine plant species and includes the ectotrophic plant genera *Picea*, *Abies*, *Dryas* and *Salix*. A previous survey of sporocarps in this habitat showed it to be also rich in both arcto-alpine and northern/montane ectomycorrhizal fungi (Kernaghan and Currah 1998). The species composition and relative abundance of ectomycorrhizae in montane habitats are still poorly understood (Gardes and Dahlberg 1996). Only recently have efforts been made to identify and describe ectomycorrhizae from subalpine forests and adjacent alpine zones (Debaud et al. 1981; Debaud 1987; Treu 1990; Graf and Brunner 1996; Kernaghan et al. 1997).

Studies such as these have used a variety of methods for mycobiont identification: tracing hyphal connections between sporocarps and mycorrhizae (Agerer 1991a), comparing field-collected mycorrhizae to mycorrhizae synthesized in-vitro (Fortin et al. 1980; Molina and Palmer 1982), comparing cultures obtained from sporocarps to those from mycorrhizae (Chu-Chou 1979; Danielson 1982; Chu-Chou and Grace 1983; Hutchison 1991), referring to anatomical descriptions such as by Agerer (1987–1998), Gronbach (1988), Brand (1991), Ingleby et al. (1990) and Goodman et al. (1996–1997) and, more recently, comparing PCR-amplified DNA from mycorrhizae to that of sporocarps (Gardes and Bruns 1993, 1996a).

Identification by PCR has become the method of choice (Erland et al. 1994; Gardes and Bruns 1996b; Dahlberg et al. 1997; Kårén and Nylund 1997; Kernaghan et al. 1997; Pritsch et al. 1997) because of its speed, reproducibility and potential for comparisons among mycorrhizae and sporocarps collected at different times and locations. Restriction fragment length polymorphism (RFLP) data from PCR-amplified internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) generally vary above the species level (Bruns et al. 1991; Egger 1995) and are, therefore, well-suited for demonstrating conspecificity between root-colonizing tissue and sporocarps. Although RFLP patterns from ectomycorrhizae do not always correspond to those of local sporocarps (Gardes and Bruns 1996b; Dahlberg et al. 1997; Kårén and Nylund 1997; Pritsch et al. 1997), closely related fungi can be grouped together on the basis of the presence or absence of ITS restriction frag-

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ments (Henrion et al. 1992; Kårén et al. 1997; Farmer and Silvia 1998; Dresler-Nurmi et al. 1999).

The objective of the present study was to use RFLP data from previously collected sporocarps, in combination with anatomical characters, to identify and describe the most common ectomycorrhizae of angiosperms and gymnosperms growing at tree line in the southern Canadian Rockies.

Materials and methods

Site description and collection

Sites were located at 2,100 m asl on the south-east slope of Mt. Tripoli, in the Nikanassin Range, Alberta (52° 52′ N, 117° 17′ W), and at 2,300 m asl on the south-west slope of Mt. Rae, Peter Lougheed Provincial Park, Alberta (50° 36′ N, 114° 59′ W). *Picea* and *Abies* occur as erect trees or as stunted multi-stemmed "krummholz", separated by *Salix, Dryas*, *Phyllodoce* and *Cassiope*. Soils are Dystric and Eutric Brunisols and Orthic and Orthic Humic Regosols (Trottier 1972; Mortimer 1978). During the growing season (June–September) mean daily temperatures are 6–10°C and mean monthly precipitation is 23–110 mm (Environment Canada, Archive of Climatological Data). More detailed site descriptions are given in Kernaghan and Currah (1998).

Ectomycorrhizae were collected each month during the growing season of 1994 by removing 3.5-cm-diameter cores of the organic horizon at 1-m intervals along short transects through three habitats: (1) the upper subalpine forest, supporting erect conifers, (2) the lower alpine zone, supporting *Salix* and *Dryas* as ectomycorrhizal hosts, and (3) the intervening alpine/subalpine ecotone, supporting both conifers (as stunted "krummholz") and alpine plants. Selected locations along the transects were resampled in 1995–1997. More detail on sampling design is given in Kernaghan and Harper (2001).

Ectomycorrhizae were separated from soil by washing samples through an 850-µm soil sieve placed over a 600-µm sieve. The overall morphologies of the ectomycorrhizae were described after examination with a dissecting photomicroscope with fiber-optic lighting. Ectomycorrhizae from within each core were then separated into morphological groups, which were further divided into two parts. One part was frozen in water for subsequent anatomical description and the other lyophilized for DNA extraction. Methods used for light and scanning electron microscopy are described in Kernaghan et al. (1997). Descriptive terminology is based on Agerer (1987–1998), Ingleby et al. (1990), Goodman et al. (1996–1997) and Kernaghan et al. (1997). Ectomycorrhizae formed by species of *Lactarius* and *Russula* [other than *R. integra* (Vittad.) Fr.] on coniferous hosts from the same sites are described in Kernaghan et al. (1997).

Ectomycorrhizae described in the present study are placed into three general abundance categories: (1) "very common", present in >40% of soil cores taken in a particular habitat; (2) "common", present in 10–40% of cores taken within a habitat; and (3) "localized", abundant in a few neighboring soil cores. Rare ectomycorrhizal types (i.e. those found in only one soil core) were not fully analyzed. For quantitative community analyses, see Kernaghan and Harper (2001).

When possible, host plant identity was determined either by tracing the root system from the mycorrhizae to the tree or by analysis of cross-field pitting in attached secondary root tissue (Core et al. 1979). For mycobionts identified from multiple host plant species, the anatomical characters described are common to ectomycorrhizae on all hosts.

DNA amplification and characterization

Forty-two representative taxa, collected as sporocarps, were used to construct an RFLP data base for comparison with ectomycorrhizae. The majority of sporocarps were collected on the sites used for the present study (Kernaghan and Currah 1998), although pertinent taxa from other sites were also included (Table 1). Sporocarp DNA was extracted using a modification of the protocol outlined by Gardes and Bruns (1993). Up to 10 mg dried tissue was ground in liquid nitrogen in a ceramic mortar and incubated for 1 h at 65°C in 500 µl CTAB extraction buffer. Chloroform (500 µl) was then added and the mixture centrifuged at 16,000 *g* for 15 min. The supernatant was then mixed with isopropanol and centrifuged at 16,000 *g* for 15 min. The resulting pellet was washed with 80% ethanol, dried, resuspended in 60 µl water and cleaned with the Glass MAX DNA isolation spin cartridge system (Life Technologies, Gaithersburg, Md.) prior to amplification. For mycorrhizae, DNA was extracted from up to 5 mg ground, lyophilized tissue using a DNeasy Plant Mini Kit (Quiagen, Hilden, Germany). Protocols for PCR amplification with the primers ITS1-F and ITS-4 and RFLP analyses of the resulting products with the restriction enzymes *Alu*I, *Hha*I, *Hin*fI and *Rsa*I were as described in Kernaghan et al. (1997).

In some cases, PCR amplification from ectomycorrhizae resulted in secondary products, possibly because of the presence of a second fungus within the root tissue, or intragenomic heterogeneity of the ITS region (Gardes and Bruns 1996b). In these cases, RFLP matching was accomplished by identifying the sporocarp profile within the dual profile obtained from the mycorrhizae. In cases where ectomycorrhizae without conspecific sporocarps produced two PCR products, RFLP fragments resulting from the restriction of the dominant product were scored. In the case of the ascomycetous fungi *Geopora arenosa* (Fuckel) Ahmad and "alpine ascomycete", two products of similar intensity were amplified and the sizes of both products and their restriction fragments are reported.

Phenetic clustering analysis

In cases where RFLP data obtained from mycorrhizae did not correspond to that of the sporocarps analyzed, and anatomical characters suggested affinities with the Tricholomataceae or the Cortinariaceae, taxa were clustered on the basis of presence or absence of restriction fragments. Restriction fragments produced by each enzyme, as well as non-digested ITS products, were binned into categories in which fragment sizes varied by no more than 2%. In the case of *Hin*fI, fragment sizes were essentially continuous between 277 and 355 bp and were binned into 20-bp categories. The resulting categories were then used to construct a species-character matrix for members of the Cortinariaceae, Tricholomataceae and unidentified mycorrhizae. Non-digested ITS sizes were given twice the weight of restriction fragment sizes, to reflect the (sub)generic level of variation in this character. Phenograms were then generated by the neighbor-joining method of Saitou and Nei (1987) using Potemkin software (Brzustowski 1998).

Results

Ectomycorrhizae formed by 19 fungal taxa were analyzed and are described below. Six were identified to species by RFLP matching and four (*Cortinarius calochrous*, *Hydnellum caeruleum*, *Laccaria montana* and *Russula integra*) are newly described symbioses. Twelve ectomycorrhizae gave RFLP patterns that did not match any sporocarps analyzed, but were identified to the species, genus or family level on the basis of anatomical characters and phenetic clustering of RFLP data.

Table 1 Collection data, restriction fragment sizes and non-digested ITS region sizes of ectomycorrhizae and sporocarps analyzed. Values in bold are common between sporocarps and mycorrhizae. Names in quotations refer to mycorrhizae only. Accession numbers refer to sporocarp material in the University of Alberta Cryptogamic Herbarium (*ALTA*) or cultures in the University of Alberta Microfungus Collection and Herbarium (*UAMH*). Mycorrhizae identified to species are deposited in ALTA under the same accession numbers as the associated sporocarps

Taxon	Location	Sporocarp accession #	Restriction enzyme				ITS region
			*Alu*I	*Hha*I	*Hin*fI	*Rsa*I	
Amphinema byssoides (Pers.:Fr.) J. Erikss.	Mt. Tripoli, AB	ALTA 10351	**491** **120**	**343** **154** **122**	**322** **296**	**614**	**614**
A. byssoides	Bonnyville, AB	UAMH 9577	“	“	“	“	“
Catathelasma imperiale (Fr.) Sing.	Mt. Rae, AB	ALTA 10367	435 126 98	382 280	355 301	662	650
C. brunneus Fr.	Mt. Tripoli, AB	ALTA 10146	474 104	350 292	320 299	642	642
C. calochrous (Pers.: Fr.) Fr.	Mt. Rae, AB	ALTA 10185	**512** **88**	**415** **286**	**400** **305**	**710**	**715**
C. delibutus Fr.	Mt. Rae, AB	ALTA 10199	452 115	632	336 279	304	632
C. evernius (Fr.:Fr.) Fr.	Mt. Rae, AB	ALTA 10154	457 104	350 292	302 283	620	632
C. favrei M. M. Moser ex Henderson	Mt. Tripoli, AB	ALTA 10209	537 113	416 304	390 330	725	715
C. glaucopus Fr.	Mt. Tripoli, AB	ALTA 10183	541 113	416 304	393 330	725	715
C. hinnuleus (Sowerby: Fr.) Fr.	Mt. Rae, AB	ALTA 10158	461 103	350 292	302 289	626	632
C. multiformis (Fr.) Fr.	Mt. Tripoli, AB	ALTA 10192	478 178	310 246	306 188	514 201	715
C. muscigenus Peck	Mt. Rae, AB	ALTA 10212	541 112	416 304	390 330	720	715
C. percomis Fr.	Mt. Rae, AB	ALTA 10196	471 115	413 304	393 330	719	715
C. triformis Fr.	Mt. Tripoli, AB	ALTA 10173	466 115	632	342 279	324 306	632
C. uraceus Fr.	Mt. Tripoli, AB	ALTA 10177	470 113	347 286	316 299	632	632
Dermocybe crocea (Schaeff.:Fr.) M. M. Moser	Mt. Rae, AB	ALTA 10229	624 105	738	410 295	383 320	715
Geopora arenosa (Fuckel) Ahmad	Wilcox Pass, AB	ALTA 12254	408 230	410 372 288	336 285 220	475 380 230	615 557
Hebeloma crustuliniforme(Bull.) Quél.	Robb, AB	ALTA 10400	322 193	402 202 118	392 259	745	745
Hydnellum caeruleum (Hornem.) P. Karst.	Mt. Rae, AB	ALTA 10272	**457** **176** **127**	**347** **285** **127**	**365** **168** **133** **86**	**754**	**738**
Hygrophorus chrysodon (Fr.) Fr.	Mt. Tripoli, AB	ALTA 10330	263 215 107	368 301	345 321	657	658
H. korhonenii Harmaja	Mt. Rae, AB	ALTA 10337	368 104	347 292	315	625	633
H. pudorinus (Fr.) Fr.	Mt. Tripoli, AB	ALTA 10343	355 102	348 297	326 317	636	636
Hysterangium separabile Zeller	Mt. Rae, AB	ALTA 10198	284 245 157	376 308	343 206 132	682	682

Table 1 (continued)

Taxon	Location	Sporocarp accession #	Restriction enzyme				ITS region
			*Alu*I	*Hha*I	*Hin*fI	*Rsa*I	
Inocybe dulcamara (Alb.& Schwein.) Kumm.	Mt. Tripoli, AB	ALTA 10311	550 252	330 315	327 220 189	435 349	800
I. geophylla (Sow.:Fr.) Kumm.	Jasper Nat. Park, AB	ALTA 10402	593	212	313 277	430 286	745
I. lacera (Fr.:Fr.) Kumm.	Mt. Tripoli, AB	ALTA 10320	537 224	441 333	201 170 143 128	430 327	765
I. lanuginosa (Bull.:Fr.) Kumm.	Jasper Nat. Park, AB	ALTA 10403	499 204	409 307	384 324	504 202	700
I. rimosa (Bull.:Fr.) Kumm.	Mt. Tripoli, AB	ALTA 10323	503 185	413 298	380 309	547 151	699
I. whitei (Berk. & Broome) Sacc.	Mt. Tripoli, AB	ALTA 10325	581 136	283 235 191	304 285 129	315 290 110	700
Laccaria bicolor (Maire) Orton	Madely Lake, BC	UAMH 9438	382 123	405 307	338 209	515 215	730
L. bicolor	Abitibi, PQ	UAMH 8232	“	“	“	“	“
Laccaria montana Singer	North Cascades Nat. Park, WA	ALTA 12253	**210** **111**	**410** **338**	**395** **355**	**730**	**738**
Piloderma fallax (Lib.) Stalpers	Brown-LoweryProv. Park, AB	ALTA 10144	**361** **201**	**371** **130**	**357** **151**	**663**	**744**
Russula integra (Vittad.) Fr.	Mt. Tripoli, AB	ALTA 9880	**441** **218**	**360** **355**	**350** **340**	**680**	**720**
Sarcodon imbricatus (Fr.) P. Karst.	Bragg Creek, AB	ALTA12255	520 171	419 327	397 345	770	770
S. scabrosus (Fr.) P. Karst	Breton, AB	ALTA 12252	528 220	410 173 112	328 328 117	720	770
S. cf. versipellis (Fr.) Quel.	Mt. Tripoli, AB	ALTA 10395	389 210 147	377 270 130	372 248 137	523 184	770
Thaxterogaster pingue Singer & A. H. Sm.	Mt. Rae, AB	ALTA 10349	528	416 304	393 330	719	716
Thelephora caryophyllea Fr.	Mt. Tripoli, AB	ALTA 10392	256 124 108	377 286	350 325	480 204	679
T. americana Lloyd	Edmonton, AB	ALTA 10399	597 80	377 309	346 231 110	231 114	680
T. americana	Smoky Lake, AB	UAMH 9578	“	“	“	“	“
Tomentella umbrinospora M. J. Larsen	Edmonton, AB	ALTA 10398	457 198	390 169 151	350	699	696
Tricholoma saponaceum (Fr.) Staude	Mt. Rae, AB	ALTA 10295	496 96 86	302 215	390 318	620 102	730
T. virgatum (Fr.) Kumm.	Mt. Rae, AB	ALTA 10305	298 260 133	431 310	406 330	731	730
Tuber rufum Pico	Notikewin Prov. Park., AB	ALTA 9086	454 283	523 308	297 213 123 78	712 123	828
“*Cortinarius* sp. 1”	Mt. Rae, AB	Unaccessioned	472 97	336	368	640	640
“*Cortinarius* sp. 2”	Mt. Tripoli, AB	Unaccessioned	504 222	352 250	372	653	715

Table 1 (continued)

Taxon	Location	Sporocarp accession #	Restriction enzyme				ITS region
			*Alu*I	*Hha*I	*Hin*fI	*Rsa*I	
"*Cortinarius* sp. 3"	Mt. Tripoli, AB	Unaccessioned	466 117	346 295	315	642	635
"*Cortinarius* sp. 4"	Mt. Rae, AB	Unaccessioned	483 117	344 172 126	319	328 215	632
"*Inocybe* sp."	Mt. Rae, AB	Unaccessioned	668	400 201	382 319	705	738
"*cf. Hygrophorus*"	Mt. Rae, AB	Unaccessioned	422 125	678	336	370 301	660
"*cf. Hebeloma*"	Mt. Rae, AB	Unaccessioned	510 190	349 252 124	410 320	738	745
"Alpine ascomycete"	Mt. Rae, AB	Unaccessioned	529 404	440 380 320	400 325 325 315	630 540 183	715 630
"*cf. Tubulicrinis*"	Mt. Tripoli, AB	Unaccessioned	512 152	362 255 130	365	653	738
"Thelephoraceae 3"	Mt. Tripoli, AB	Unaccessioned	329 198 141	415 231	399 261	730	730

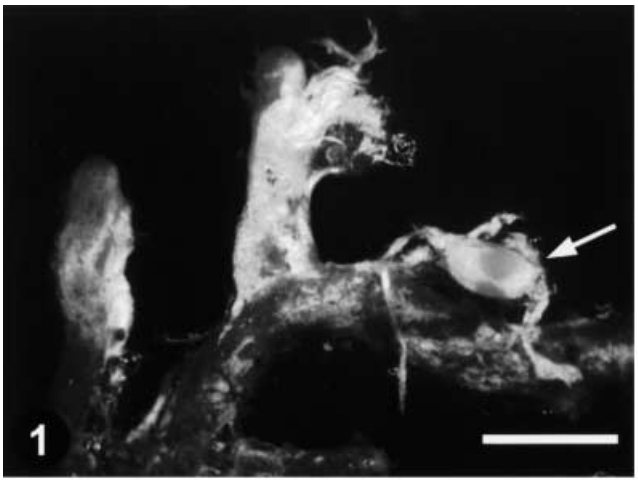
Identification by direct RFLP matching

The following six ectomycorrhizae were identified by direct matching of PCR-amplified rDNA between the mycorrhizae and their associated sporocarps (Table 1).

Amphinema byssoides (Pers.:Fr.) J. Erikss., on *Abies lasiocarpa*, *Picea engelmannii*, *Dryas octopetala* and *Salix barrattiana*. Unramified to monopodial-pinnate. White to yellow or orange, mantle often very thin, becoming lemon yellow in 5% KOH. Emanating hyphae abundant, clamped, hyaline to yellow, often papillate. Hyphoid cystidia seen on sporocarps are absent from mycorrhizae. Very common in the upper subalpine forest and at the ecotone; common in the lower alpine zone. For more detailed descriptions, see Ingleby et al. (1990), Weiss (1991) and Harniman and Durall (1996a).

Cortinarius calochrous (Pers.: Fr.) Fr. subsp. *coniferarum* (M. M. Moser) Brandrud on *Picea engelmannii* (Fig. 1). Monopodial-pinnate. Cottony, white, some areas cream-colored because of yellow intracellular pigments, quickly fuschia in 5% KOH, as are the basal hyphae of sporocarp. Emanating hyphae abundant, clamped, hyaline to pale yellow, 2.5–3.5 µm wide, coalescing to form loose, fan-like hyphal strands. Strands without internal differentiation, also pink in KOH. Outer mantle mainly a prosenchymous reticulum of broadly branching hyphae (mantle type A of Agerer 1991a), some areas with ± parallel hyphae. Inner mantle synenchymous, cells becoming shorter and wider, simple septate, not well-differentiated from Hartig net. Sclerotia common, white, pink in KOH, up to 1 mm in diameter, subglobose to oblong, sometimes bi-lobed, smooth beneath superficial hyphae, covered in loose hyphal strands. Mature sclerotia differentiated into a thin layer of appressed hyphae (similar to emanating hyphae) and a hyaline, pseudoparenchymatous interior, with subglobose to rectangular or triangular cells, 6.0–19×3.6–16 µm. Localized in the upper subalpine forest.

Hydnellum caeruleum (Hornem.) P. Karst., on *Picea engelmannii* (Fig. 2). Monopodial-pinnate to ± coralloid. Cottony, ochre-gray, dingy orange, white or pink, some "carbonizing" (root epidermal cells becoming dark and moribund), olive in KOH (as are basal hyphae and teeth of sporocarps). Emanating hyphae abundant, simple septate, hyaline to slightly vinaceous in H_2O, olivaceous in 5% KOH, 1.7–3.9 µm wide, forming abundant hyphal strands. Strands cottony, concolorous with mycorrhizae, not differentiated internally, 25–1000 µm in diameter, attached to small undifferentiated, vinaceous brown hyphal mats (up to 3×1 cm), which often engulf mycorrhizae. Outer mantle mainly a loose, disorganized prosenchyma of obtusely branched hyphae 2.2–4.1 µm wide, forming a reticulum in some areas. Inner mantle synenchymous, hyphae narrower than in outer mantle, acutely branched, often forming parallel sheets, 1.4–3.5 µm wide. Chlamydospores abundant in hyphal strands and mats, also in emanating hyphae and outer mantle; slightly thick-walled, smooth, hyaline to pale brown, broadly elliptical to subglobose, mainly intercalary, 8–15×5.5–9.0 µm. Hyphae of strands, mats and outer mantle containing dark violet (in H_2O) crystals, which dissolve in 5% KOH releasing a blue-green pigment. Localized in the upper subalpine forest.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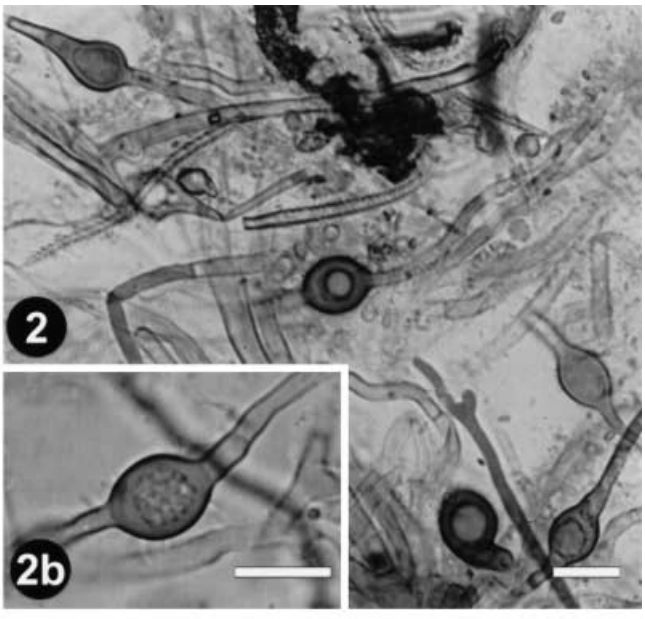
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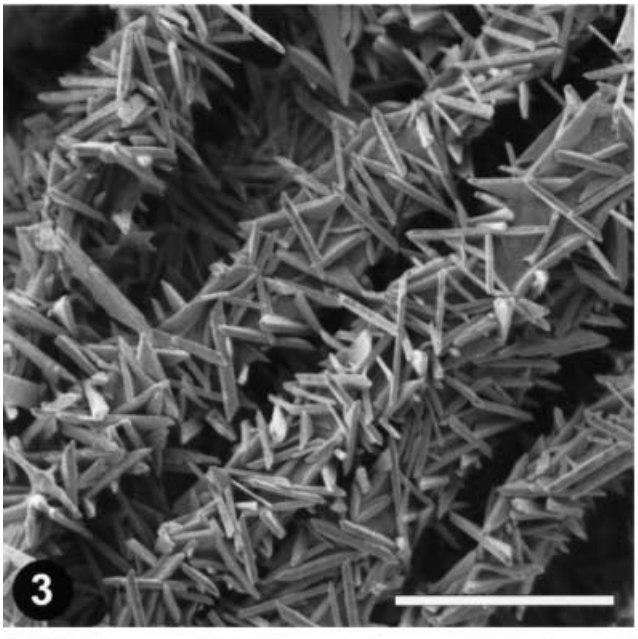
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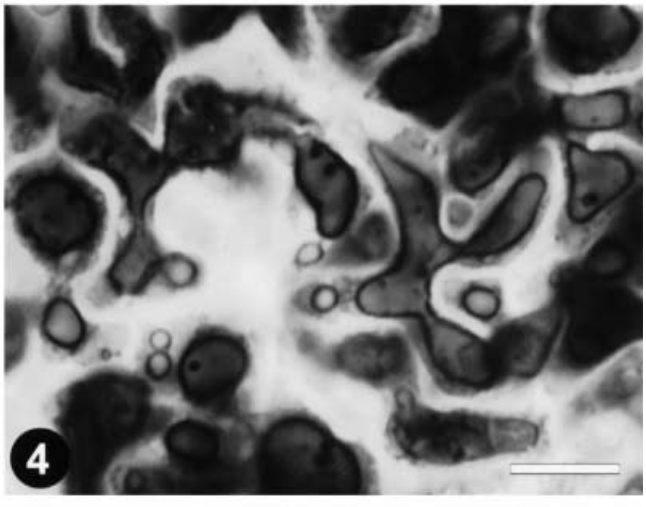
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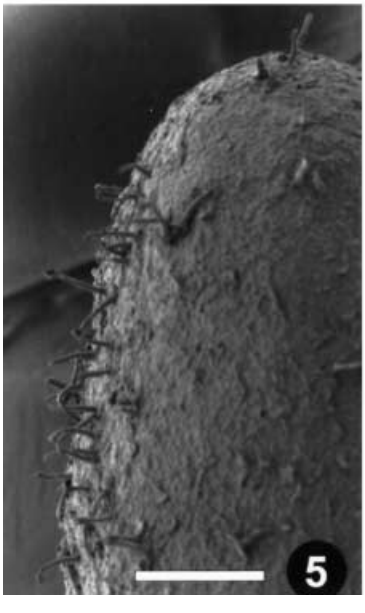
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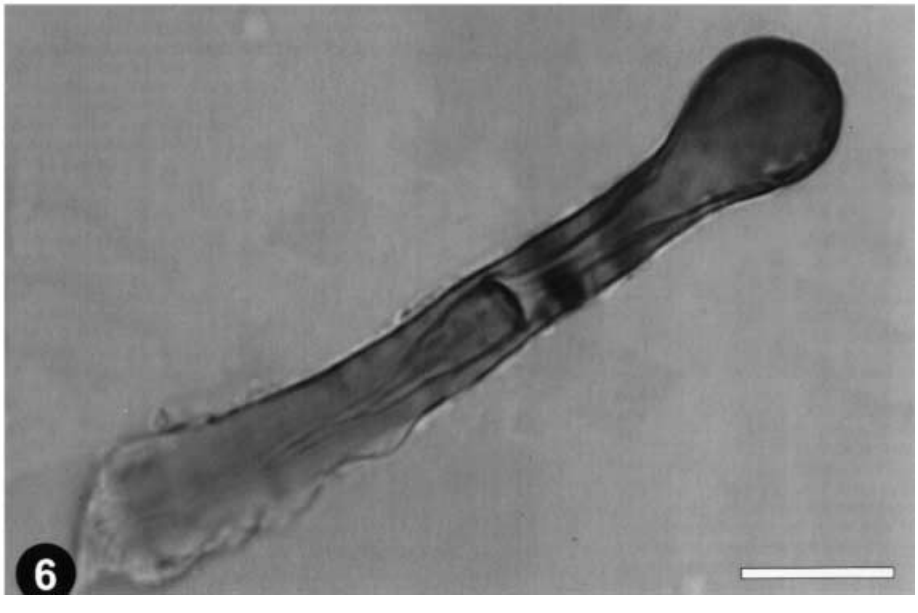
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Laccaria montana Singer on *Picea engelmannii*, *Abies lasiocarpa*, *Salix barrattiana* and *Dryas octopetala*. Monopodial to monopodial pinnate, ± smooth, dull orange-brown (concolorous with sporocarps). Emanating hyphae abundant, 1.5–2.9 µm wide, clamped, hyaline, cylindrical to slightly tortuous. Outer mantle a loose prosenchymous reticulum formed by obtusely branched hyphae, 1.8–4.5 µm wide (usually 2.5 µm), mostly simple septate but clamps present at some septae, often with short side branches, similar to the mantle of *Laccaria proxima* (Ingleby et al. 1990). Inner mantle a synnenchyma of cylindrical to tortuous, acutely branched, hyaline, simple septate hyphae, 1.8–3.5 µm wide, in ± parallel orientation. Common in the upper subalpine forest and localized at the ecotone and in the lower alpine zone.

Piloderma fallax (Lib.) Stalpers on *Picea engelmannii* (Fig. 3). Monopodial-pinnate. Cottony, white to cream. Emanating hyphae abundant (sometimes forming small hyphal pads), mainly hyaline but some containing small amounts of yellow pigment, simple septate, 2.2–2.8 µm wide, densely ornamented with calcium oxalate crystals 1.2–3.3×0.2–0.5 µm. Hyphal strands composed of similar hyphae, loose, cottony, white, up to 400 µm wide, not internally differentiated, but outer hyphae coated with mucilaginous material. Outer mantle a loose, disorganized prosenchyma, hyphae similar to the emanating hyphae, but more variable in width; 1.7–4.3 µm wide. Inner mantle a thin synnenchyma of obtusely branched or parallel, hyaline, simple septate hyphae, without calcium oxalate crystals, 1.4–4.5 µm wide. Localized in the upper subalpine forest.

Russula integra (Vittad.) Fr. on *Abies lasiocarpa* (Fig. 4). Unramified to monopodial-pinnate. Smooth, mantle hyaline (assuming the color of underlying root tissue). Emanating hyphae uncommon, hyaline, simple septate, 2.0–2.8 µm wide, cylindrical to tortuous. Outer mantle a thin synnenchyma of strongly anastomosing, cylindrical hyphae, 3.3–5.2 µm wide, and short rounded cells, 6.5–12.6×4.4–9.6 µm. Central mantle a synnenchyma of subglobose, bent cylindrical and epidermoidal cells, 10–18×2.0–7 µm, many of which are reactive (blue) in sulphovanillin. Inner mantle synenchymous, hyphae hyaline, cylindric to tortuous, branching obtusely, 2.0–5.6 µm wide, often in parallel sheets. Localized at the ecotone.

◀ **Fig. 1–6** Light and scanning electron micrographs of anatomical and morphological features of tree line ectomycorrhizae

Fig. 1 Mycorrhizal system of *Cortinarius calochrous* subsp. *coniferarum* on *Picea engelmannii*. Note sclerotium (*arrow*); *bar* 1 mm

Figs. 2 Chlamydospores in mantle of *Hydnellum caeruleum* on *Picea engelmannii*; *bars* 10µm

Fig. 3 Scanning electron micrograph of mantle hyphae of *Piloderma fallax* on *Picea engelmannii* ornamented with calcium oxalate crystals; *bar* 5 µm

Fig. 4 Sulphovanillin reactive epidermoidal cells in central mantle of *Russula integra* on *Abies lasiocarpa*; *bar* 10 µm

Fig. 5 Scanning electron micrograph of lyocystidia on mantle surface of *cf. Tubulicrinis* mycorrhizae; *bar* 10 µm

Fig. 6 Single amyloid lyocystidia of *cf. Tubulicrinis* mycorrhizae in Melzers reagent; *bar* 100 µm

Identification by anatomical characterization

Comparison of anatomical characters with published descriptions of ectomycorrhizae and with sporocarps allowed for the identification of the following six mycobionts to the species, genus or family level.

Cenococcum geophilum Fr.:Fr on *Abies lasiocarpa*, *Picea engelmannii*, *Dryas octopetala* and *Salix barrattiana*. Unramified to monopodial pinnate. Jet-black. Emanating hyphae abundant, simple septate, stiff, often branching at nearly right angles. Hyphae of outer mantle forming a stellate pattern. Associated with smooth black sclerotia up to 3 mm in diameter. Very common in the upper subalpine forest, at the ecotone, and in the lower alpine zone. For more detailed descriptions see Agerer and Gronbach (1988), Gronbach (1988), Harniman and Durall (1996b).

"Thelephoraceae 1" on either *Abies lasiocarpa* or *Picea engelmannii*. Monopodial to monopodial pinnate. Smooth, brown. Emanating hyphae brownish, slightly thick-walled, clamped, 4.1–5.3 µm wide. Outer mantle synenchymous to pseudoparenchymous, of thick-walled, yellow-brown, mostly rounded triangular but also rectangular or cylindrical cells 9.0–40×6.4–35 µm. Localized areas of mantle becoming blue-green in 5% KOH. Clavate cystidia present on outer mantle; thin-walled, hyaline to pale brown, one or two septate, simple or clamped, 50–120×3.2–5.2 µm, up to 7.8 µm at apex. Inner mantle a tight synnenchyma of ± parallel, hyaline to yellow-brown, cylindrical hyphae (2.2×7.4 µm) with variable branching angles, often clamped. Localized in the upper subalpine forest.

"Thelephoraceae 2" on *Abies lasiocarpa* and *Salix barrattiana*. Monopodial to monopodial pinnate. Smooth, brown. Emanating hyphae uncommon, ± tortuous, pale to dark yellow-brown, thick-walled, clamped, 2.9–6.0 µm wide. Outer mantle a pseudoparenchyma, composed mainly of rounded triangular cells 9–21×7-13 µm (often forming rosettes of 5–8 cells), but similarly sized rectangular and subglobose cells also present, as well as obtusely branched cylindrical cells 2.2–5.0 µm wide. Isolated areas of outer mantle becoming blue-green in 5% KOH. Inner mantle a tight synnenchyma of narrow, cylindrical, pale yellow-brown, acutely branched hyphae, 1.8–5.2 µm wide (mostly 3.0 µm) often forming parallel sheets, clamps common. Common at the ecotone.

"Thelephoraceae 3" on *Picea engelmannii* and *Salix barrattiana*. Monopodial to monopodial-pinnate. Smooth to granulose, blackish. Emanating hyphae sometimes abundant, yellow-brown, clamped or simple septate, thick-walled, 4.0–4.9 (5.9) µm wide. Outer mantle a pseudoparenchyma of dark, red-brown, very thick-walled, subglobose to elliptical or angular cells,

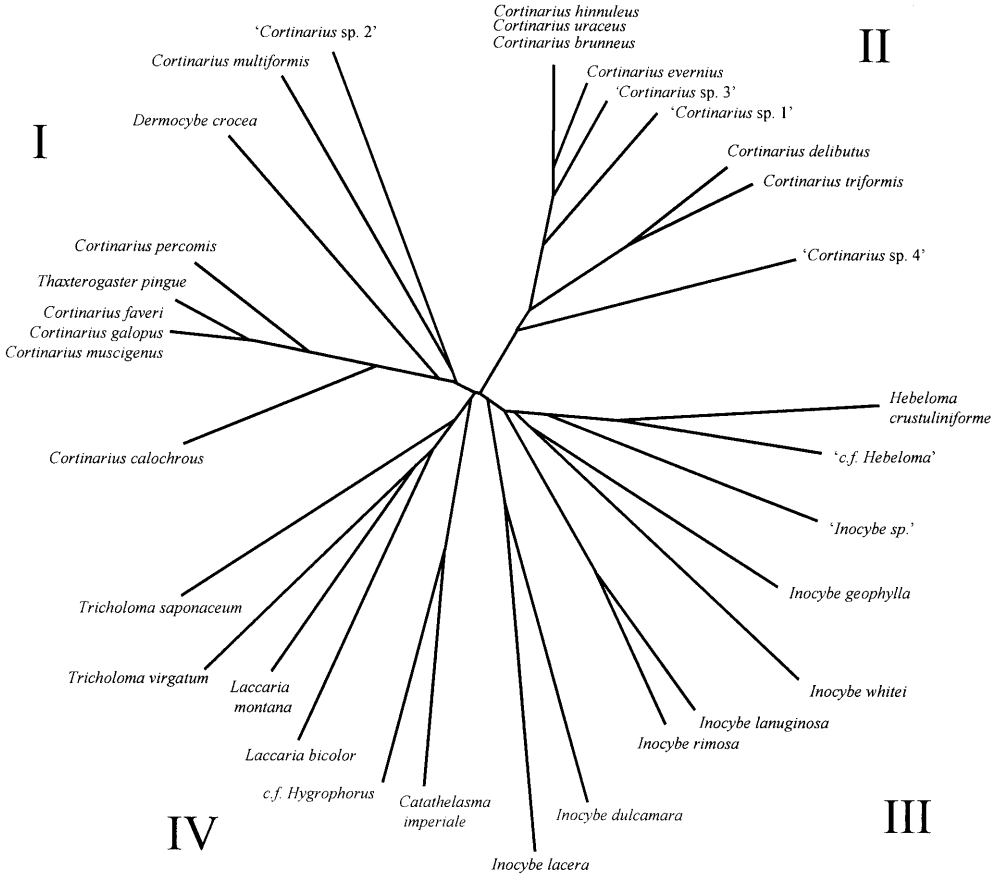

Fig. 7 Neighbor-joining tree based on RFLP data from members of the Cortinariaceae and the Tricholomataceae. Names in quotation marks indicate data from mycorrhizae only, normal type indicates data from sporocarps only and data for *Laccaria montana* and *Cortinarius calochrous* are from both sporocarp and mycorrhizae. *Cortinarius hinnuleus*, *C. uraceus* and *C. brunneus* and *C. faveri*, *C. galopus* and *C. muscigenus* form unresolved polytomies. Bold roman numerals indicate separate clades

11–44×9-33 µm, subglobose cells often more abundant in localized areas forming mounds. Isolated areas of outer mantle becoming blue-green in 5% KOH. Inner mantle a synnenchyma of simple septate, cylindrical to ± contorted, acutely branching, thin-walled, yellow-brown hyphae 1.9–8.0 µm wide. Common in the upper subalpine forest.

"Alpine ascomycete" on *Dryas octopetala* and *Salix barrattiana*. Monopodial. Smooth, brown. Emanating hyphae hyaline to pale yellow, simple septate, 2.4–4.5 (5.4) wide. Outer mantle a pseudoparenchyma of pale brown, interlocking epidermoidal cells ("jig-saw"), 8.0–45×2-12 µm (mantle type M of Agerer 1991a). Inner mantle similar but less regular; cells pale yellow, epidermoidal shape less pronounced, some cylindrical cells (2.7–4.4 µm) also present. Common in the lower alpine zone.

"cf. Tubulicrinis" on *Picea engelmannii* (Figs. 5, 6) Monopodial-pinnate to pyramidal. Smooth, yellow-brown to iridescent green. Emanating hyphae uncommon, yellow-green, clamped, 2.3–3.7 µm wide. Forming yellow to olive-green hyphal strands, ± smooth, 200 µm wide, with thick-walled contorted hyphae on the outer surface. Outer mantle synenchymous to pseudoparenchymous, of thick-walled, yellow-brown cylindrical, short rectangular, triangular or subglobose cells 9–40×4.4–17 µm. Inner mantle a loose synnenchyma of cylindrical, obtusely branching, pale yellow, simple septate, thin-walled hyphae, 2.6–5.2 µm wide. Cylindrical, amyloid lyocystidia common on the surface of mycorrhizae, also on hyphal strands; hyaline to dark olive, 43–63×3-6 µm, with a thick outer wall over a thin-walled capillary lumen which widens (up to 9.3 µm) at the apex (Figs. 5, 66). Localized in the upper subalpine forest.

Phenetic clustering of RFLP data

For mycorrhizae that lacked conspecifics among sporocarps, but which appeared to have Cortinariaceous or Tricholomataceous affinities (described below), RFLP data were compared to that of several members of the Cortinariaceae and Tricholomataceae (collected as sporocarps) by phenetic clustering analysis. The resulting phenogram (Fig. 7) consisted of four clades: (I) *Cortinarius* subgenera *Myxacium* and *Phlegmacium*, *Dermocybe crocea* (Schaeff.:Fr.) M. M. Moser, *Thaxterogaster pingue* Singer and A. H. Sm. and one *Cortinarius* mycorrhiza ("*Cortinarius* sp. 2"), (II) *Cortinarius* subgenus *Telamonia*, *C. delibutus* (subgenus *Myxacium*) and three *Cortinarius* mycorrhizae; ("*Cortinarius* sp. 1, 3 and 4"), (III) *Inocybe* spp., *Hebeloma crustuliniforme* (Bull.) Quél., and the mycorrrhizae ("*Inocybe* sp."), and *"cf. Hebeloma"* and (IV) *Tricholoma* spp., *Laccaria* spp., *Catathelasma imperiale* (Fr.) Singer and the mycorrhiza, *"cf. Hygrophorus"*.

"*Cortinarius* sp. 1" on *Picea engelmannii.* Monopodial-pinnate. Cottony, whitish with faint pinkish tones, often bent. Emanating hyphae abundant, hyaline but with localized intracellular yellow pigment, clamped, 2.2–4.7 µm wide, Hyphal strands narrow, delicate, ± smooth, undifferentiated, up to 200 µm wide, faintly pinkish. Outer mantle a disorganized prosenchyma of hyaline to pinkish, cylindrical, obtusely branched hyphae, 2.4–5.0 µm wide, often with clamp connections. Inner mantle synenchymous; a mixture of rarely clamped, hyaline to pinkish, narrow, cylindrical, acutely branched hyphae [1.0–5.0 (6.0) µm] and subglobose (4.2–14×3.5–11 µm) cells. Localized in the upper subalpine forest.

"*Cortinarius* sp. 2" on *Picea engelmannii.* Monopodial pinnate. Cottony, white, often bent. Emanating hyphae abundant, hyaline, clamped, 2.0–5.0 µm wide, forming undifferentiated, loose cottony, strands up to 300 µm wide. Outer mantle a disorganized prosenchyma of hyaline, cylindrical, obtusely branched hyphae, 2.0–4.6 µm wide, often with clamps. Inner mantle synenchymous; a mixture of simple septate, hyaline, cylindrical (1.6–3.4 µm), and subglobose (4.5–10×3.2–6.5 µm) cells. Common in the upper subalpine forest.

"*Cortinarius* sp. 3" on *Picea engelmannii.* Monopodial pinnate. Cottony, white, often bent. Emanating hyphae abundant, hyaline, clamped, 2.3–5.4 µm wide. Outer mantle a disorganized prosenchyma of hyaline, cylindrical, obtusely to acutely branched hyphae, 2.4–5.4 µm wide (usually 2.8 µm), often with clamps. Inner mantle a disorganized synnenchyma of simple septate, hyaline, cylindrical to tortuous hyphae, 1.6–3.5 µm wide. Localized at the ecotone.

"*Cortinarius* sp. 4" on *Salix barrattiana.* Monopodial pinnate. Cottony, pinkish. Emanating hyphae abundant, pinkish, clamped, 2.2–3.7 µm wide, forming undifferentiated, loose, cottony strands up to 350 µm wide. Outer mantle a disorganized prosenchyma of hyaline to pinkish, cylindrical, obtusely branched hyphae, 1.6–4.3 µm wide, often with clamp connections. Inner mantle synenchymous; a mixture of simple septate, hyaline, cylindrical (1.5–4.4 µm) and epidermoidal (8.7–30×3.7–6.3 µm) cells. Localized in the lower alpine zone.

"*cf. Hebeloma*" on *Picea engelmannii.* Monopodial pinnate. Mantle web-like, whitish to pale cream. Emanating hyphae abundant, hyaline to very pale yellow, clamped, 2.5–8.7 µm wide. Outer mantle a disorganized prosenchyma of hyaline, cylindrical, obtusely branched, simple septate hyphae, 2.4–5.4 µm wide (usually 2.8 µm). Mantle becoming synenchymous towards root surface, but no distinct inner mantle was noted. Localized in the upper subalpine forest.

"*cf. Hygrophorus*" on *Picea engelmannii* and (or) *Abies lasiocarpa*. Monopodial-pinnate, ± smooth, orange-brown. Emanating hyphae hyaline, clamped, 2.4–5.4 µm wide. Hyphal strands up to 175 µm wide, orange-brown, ± flattened, composed of clamped, yellow-orange, ± thick-walled, or hyaline and thin-walled hyphae, 4.6–5.1 µm wide. Outermost mantle a prosenchyma of cylindrical, hyaline, clamped hyphae forming a regular and symmetric reticulum over a pseudoparenchymous inner mantle (similar to mantle type P of Agerer 1991a). Inner mantle cells hyaline, simple septate, subglobose, triangular, rectangular or obtusely branched cylindrical cells, 8.0–27×4.5–9.0 µm. Localized in the upper subalpine forest.

"*Inocybe* sp." on *Dryas octopetala.* Monopodial to monopodial-pinnate. Smooth, tawny to yellow-olivaceous. Emanating hyphae hyaline, clamped, cylindrical to tortuous, narrow (2.6–3.1 µm wide). Outer mantle a prosenchyma of repeatedly branched, simple septate hyphae, 2.0–6.0 µm wide, often wider at branch points (up to 9.1 µm), branching obtuse, forming two-, three-, and four-way intersections, similar to the mantle of *Inocybe lacera* (Ingleby et al. 1990). Septa and some cellular contents staining bright blue in lactophenol cotton blue. Inner mantle a synnenchyma of acutely branched hyaline, simple septate, cylindrical to subglobose or epidermoidal cells 5.9–17×1.9–3.5 µm. Common in the lower alpine zone.

Discussion

Some of the ectomycorrhizal fungi identified are essentially ubiquitous in temperate ectotrophic plant communities, forming symbioses with a wide variety of hosts in diverse habitats; *Cenococcum geophilum*, *Amphinema byssoides* and *Tomentella* spp. can be found throughout the temperate northern hemisphere (Trappe 1964; Jülich and Stalpers 1980; Kernaghan and Currah 1998). The majority of species identified, however, have restricted distributions and are characteristic of high latitudes and elevations.

Laccaria montana exemplifies this latter distribution. It is restricted to arctic, boreal and montane habitats, where it is associated with both conifers and angiosperms (Mueller 1992). The ectomycorrhizae of *L. montana* are anatomically similar to those of *L. proxima* (Boud.) Pat. described by Ingleby et al. (1990) and are similar to *L. bicolor* (Maire) Orton on the basis of phenetic clustering analysis of the RFLP data (Fig. 7).

Russula integra also exhibits a northern/montane distribution. An *Abies* associate, it is known mainly from montane and subalpine coniferous forests in Europe and North America (Romagnesi 1967; Einhellinger 1987; Kernaghan and Currah 1998). The mycorrhizae are anatomically similar to those of *Russula silvicola* Shaffer (Kernaghan et al. 1997), but differ in having epidermoidal rather than polygonal sulphovanillin reactive cells in the mantle (Fig. 44).

Cortinarius calochrous subsp. *coniferarum* is also known from montane and boreal forests in Europe (Moser 1960; Brandrud et al. 1990) as well as from the mountains of western North America (Kernaghan and Currah 1998). The presence of sclerotia associated with the mycorrhizae of *C. calochrous* (Fig. 1) is notable in that sclerotia production by ectomycorrhizal basidiomycetes is most common in members of the Boletales, e.g.

Paxillus involutus (Batsch) Fr. (Gronbach 1988; Ingleby et al. 1990), *Gyrodon lividus* (Bull.: Fr.) Sacc. (Agerer et al. 1993), and *Leccinum* sp. (Ingleby et al. 1990). Within the Cortinariaceae, sclerotia have been described from *Hebeloma crustuliniforme* (Zak 1973; Voiry 1981), *H. sacchariolens* (Ingleby et al. 1990), *Cortinarius subporphyropus* Pilat. (Godbout and Fortin 1983), *C. magellanicus* Speg. (Palfner and Godoy 1998) and *C. alnobetulae* Khün. (Wiedmer and Senn-Irlet 1999). Sclerotia production by species of *Cortinarius* has not yet been reported outside the subgenera *Phlegmacium* and *Myxacium*.

The fuchsia reaction of the mycorrhizae of *Cortinarius calochrous* in 5% KOH can also be seen in the basal hyphae, pileus, and cortina of the sporocarp. This reaction is common in sporocarps of *Cortinarius* subgen. *Phlegmacium* sec. *Calochroi* as well as in other species of *Phlegmacium* (Moser 1960). Similar KOH reactions have been noted in the ectomycorrhizae of *Dermocybe cinnabarina* (Fr.) Wünsche [Brand 1991, as *Cortinarius cinnabarinus* (Fr.)] and *Cortinarius armillatus* (Fr.) Fr. (Thoen 1979).

The other *Cortinarius* mycorrhizae described ("*Cortinarius* spp. 1–4") share general morphological and anatomical features with each other, with *C. calochrous*, and with other *Cortinarius* mycorrhizae described in the literature (Agerer 1987, 1988; Gronbach 1988; Cuvelier 1990; Brand 1991). These include thin-walled, unornamented hyphae, a disorganized prosenchymous outer mantle and clamp connections in the hyphal strands and outer mantle. These mycorrhizae vary, however, in pigmentation, abundance of hyphal strands and, in *C. calochrous*, with respect to the KOH reaction and sclerotia production.

Hydnellum caeruleum is a common northern conifer associate (Harrison 1961; Väre et al. 1996), although it is also known from deciduous forests at lower latitudes (Baird and Kahn 1986). The mycorrhizae of *H. caeruleum* are anatomically similar to those formed by *H. peckii* Banker (Agerer 1993); both are carbonizing and form abundant rhizomorphs and distinctive chlamydospores (Fig. 2). Differences include the silver-white color, lack of olive reaction in KOH, and the yellow, spherical, warted chlamydospores of *H. peckii*. The chlamydospores of *H. caeruleum* resemble immature *H. peckii* chlamydospores, but are single-walled, spherical to ellipsoid and smooth rather than warted. Chlamydospores found in the mantles of other Bankeraceous mycorrhizae are described by Agerer (1991b, 1992).

The dark violet, intra-hyphal crystals found in the mantle and emanating hyphae of *H. caeruleum* have also been described from the sporocarps of *Hydnellum* (including *H. caeruleum*) and *Sarcodon* spp. (Harrison 1964), as well as the mycorrhizae of *H. peckii* (Agerer 1993) and *Albatrellus ovinus* (Schff.:Fr.) Kotl. and Pouz. (Agerer et al. 1996)., They are thought to contain thelephoric acid (Agerer 1993).

Piloderma fallax is found throughout the northern coniferous zone, where it is restricted to acidic soils [Eriksson et al. 1981, as *P. bicolor* (Peck) Jülich] and may be an indicator of late successional forests (Smith et al. 2000). Both sporocarps and mycorrhizae can produce variously shaped crystals of calcium oxalate (Fig. 3), but because crystal formation is dependent on the calcium concentration in the contacting soil (Graustein et al. 1977), mycorrhizae of *Piloderma* are likely to show significant variation in this character.

The mycorrhizae of *Piloderma fallax* have been described by Froidevaux [1975, as *P. byssinum* (P. Karst.) Jülich], Brand (1991, as *P. croceum* J. Erikss. and Hjörts.) and Goodman and Trofymow (1996) as possessing bright yellow pigment in the mantle and hyphal strands. All of the *P. fallax* tissue collected in the present study was essentially white, with only very small amounts of yellow pigment visible under high magnification. Because the yellow sporocarps of *P. fallax* often fade to a whitish color with age (Larsen et al. 1997), these collections may represent mycorrhizae formed by an older fungal thallus. The discrepancy between this description of *P. fallax* mycorrhizae and those of other authors may mean that the abundance of *P. fallax* has been underestimated in previous ectomycorrhizal surveys.

The genus *Inocybe* is well represented in montane habitats (Favre 1955, 1960), although the majority of species are widely distributed (Kuyper 1986). The mycorrhizae identified as "*Inocybe* sp." from the alpine zone produced an RFLP pattern that did not match any of the *Inocybe* sporocarps analyzed (Table 1), but were anatomically similar to the *Inocybe* mycorrhizae described by Ingleby et al. (1990), Cripps and Miller (1996), and Beenken et al. (1996a, b, c, d). The compact reticulate mantle formed by simple septate hyphae producing thin, clamped emanating hyphae and the reaction to lactophenol-cotton blue appear to be good characters for the identification of *Inocybe* mycorrhizae. Phenetic clustering analysis of RFLP data also placed the mycobiont with *Inocybe*, near *I. rimosa* (Bull.:Fr.) Kumm. and *I. lanuginosa* (Fig. 7).

The Thelephoraceae sensu Stalpers includes nine genera, many of which are very widely distributed (Jülich and Stalpers 1980; Ginns and Lefebvre 1993). The three mycorrhizae referred to as "Thelephoraceae", were so designated on the basis of their dark, pseudoparenchymatous mantles, with dark, thick-walled, clamped emanating hyphae, cyanesence in KOH, and in the case of "Thelephoraceae 1", distinctive cystidia. Similar characters have been described from other mycorrhizae identified as *Tomentella*, *Pseudotomentella* or "*Tomentella*-like" (Danielson and Pruden 1989; Köljalg 1992; Agerer 1994, 1996; Agerer et al. 1995; Goodman 1996)."Thelephoraceae 3" is anatomically similar to the unidentified symbiont of *Picea*, "*Piceirhiza nigra*", described by Gronbach (1988). The confirmation of thelephoric acid in "*Piceirhiza nigra*" by HPLC indicates a Thelephoraceous mycobiont and possibly a species of *Tomentella* (Agerer et al. 1995).

The cyanesence of hyphae in KOH seen in "Thelephoraceae 1–3", is likely a pH-mediated change in thele-

phoric acid (Burdsall and Setliff 1974; Gill and Steglich 1987; Agerer et al. 1995). This reaction has also been noted in sporocarps of the Thelephoraceae (*Thelephora*, *Tomentella*, *Tomentellastrum* and *Pseudotomentella*) (Stalpers 1993; Köljalg 1996), as well as in the mycorrhizae of *Tomentella sublilacina* (Ellis and Holw.) Wakef. [Agerer 1996, as *Tomentella albomarginata* (Bourdot and Galzin) M.P. Christ.]. Although we were unable to match the "Thelephoraceae" mycorrhizae to the Thelephoraceous sporocarps analyzed by RFLP (*T. caryophyllea* Fr., *Thelephora americana* Lloyd and *Tomentella umbrinospora* Larsen) (Table 1), the anatomical evidence suggests mycobionts in the genus *Tomentella*.

The mycorrhizae referred to as "alpine ascomycete" were found only in association with *Salix* and *Dryas.* They are anatomically similar to those formed by the hypogeous genus *Tuber*; with very regular epidermoidal mantle cells, but lack the long, pointed cystidia often formed by *Tuber* spp. (Voiry 1981; Blaschke 1987; Rauscher et al. 1995). Species of *Tuber* have been collected under *Salix* (Trappe 1971; Watling 1992; Maia et al. 1996), although the association does not appear to be common. The RFLP data from these ectomycorrhizae do not match that of *Tuber rufum* Pico. (the only species of *Tuber* so far known from Northern Alberta) or of *Geopora arenosa*, another hypogeous ascomycete collected under *Salix* in the region. The lack of information on ectomycorrhizal ascomycetes in alpine habitats limits our ability to identify the mycobiont in this case.

The ectomycorrhizae referred to as "*cf. Tubulicrinis*" are tentatively identified on the basis of their amyloid lyocystidia (Figs. 5, 6). Lyocystidia occur in other corticioid genera including *Litschauerella* and *Tubulicium*, but only in *Tubulicrinalis* are they cylindrical, amyloid and unornamented (Oberwinkler 1965; Hjortstam et al. 1973, 1988). Because no associated sporocarps were collected, positive identification will require DNA sequencing. However, given the diversity and ubiquitous nature of corticioid taxa, new symbiosis involving corticioid fungi should be expected.

The mycorrhizae referred to as "*cf. Hygrophorus*" have a pseudoparenchymatous mantle with an overlying reticulum of cylindrical hyphae. Similar anatomies have been described from Russulaceous mycorrhizae (Agerer 1995; Kernaghan et al. 1997), but the presence of clamp connections clearly precludes a Russulaceous mycobiont. The mycorrhizae are anatomically similar to those of *Hygrophorus pustulatus* (Pers.) Fr. (Gronbach 1988) (with the exception of the outermost mantle). Clustering analysis of the RFLP data from these mycorrhizae places the mycobiont near *Catathelasma imperiale* (Fig. 7) and *Hygrophorous chrysodon* (not shown).

The mycorrhizae referred to as "*cf. Hebeloma*" are anatomically similar to those of *H. crustuliniforme* described by Brunner et al. (1991). Because the RFLP data are also similar (Fig. 7), I suspect the mycobiont involved to be another species of *Hebeloma* occurring on the sites (see Kernaghan and Currah 1998).

Phenetic clustering analysis separated the taxa analyzed into four major clades (Fig. 7) and aided in the identification of mycorrhizae which lacked conspecific sporocarps. However, clustering based on RFLP data from large selections of unrelated taxa is not recommended as unrelated taxa may cluster together because of homoplasy.

The size of undigested ITS regions in ectomycorrhizal fungi tends to vary at the generic level and is, therefore, valuable for the identification of unknowns (Gardes et al. 1991; Henrion et al. 1992; Kernaghan et al. 1997). The decision to double the weight of this character in the phenetic analysis was based on the assumption that insertion/deletion events are of greater evolutionary significance than point mutations. This weighting scheme helped to stabilize the phenogram and to separate genera.

The approach to the identification of ectomycorrhizae used here involves detailed anatomical characterization combined with molecular analysis. The information provided should facilitate subsequent reidentification by other researchers using either anatomical or biochemical characters.

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