# Ectomycorrhizal fungi of whitebark pine (a tree in peril) revealed by sporocarps and molecular analysis of mycorrhizae from treeline forests in the Greater Yellowstone Ecosystem

**Katherine R. Mohatt, Cathy L. Cripps, and Matt Lavin**

**Abstract:** Whitebark pine (*Pinus albicaulis* Engelm.) is unique as the only stone pine in North America. This species has declined 40%–90% throughout its range owing to blister rust infection, mountain pine beetle, fire suppression, and global climate change. However, intact mature and old growth forests still exist in the Greater Yellowstone Ecosystem (GYE) at high timberline elevations. This study addresses the urgent need to discover the ectomycorrhizal (ECM) fungi critical to this tree species before forests are further reduced. A study of mature whitebark pine forests across five mountain ranges in the Northern GYE confirmed 32 ECM species of fungi with the pine by sporocarp occurrence in pure stands or by identification of mycorrhizae with ITS-matching. Boletales and Cortinariales (*Cortinarius*) comprise 50% of the species diversity discovered. In Boletales, *Suillus subalpinus* M.M. Moser (with stone pines), *Suillus sibericus* Singer (stone pines), *Rhizopogon evadens* A.H. Sm. (five-needle pines), *Rhizopogon* spp. (pines) and a semi-secotioid *Chroogomphus* sp. (pines) are restricted to the hosts listed and are not likely to occur with other high elevation conifers in the GYE. The ascomycete generalist, *Cenococcum geophilum* Fr., was the most frequent (64%) and abundant (51%) ECM fungus on seedling roots, as previously reported for high elevation spruce-fir and lower elevation lodgepole pine forests in the GYE. The relative importance of the basidiomycete specialists and the ascomycete generalist to whitebark pine (and for seedling establishment) is not known, however this study is the first step in delineating the ECM fungi associated with this pine in peril.

*Key words:* mycorrhizae, basidiomycota, ITS DNA sequence, *Pinus albicaulis*, stone pine.

**Résumé :** Le pin albicaule (*Pinus albicaula* Engelm.) est l'unique représentant du pin arolle en Amérique du Nord. Le déclin de cette espèce atteint 40–90 % sur l'ensemble de l'aire de distribution, sous l'effet de la rouille vésiculeuse, du dendrochtone du pin, de la suppression des feux et du changement climatique global. Cependant, il existe toujours des forêts matures et surannées dans l'écosystème du Grand Parc Yellowstone (GYE), à l'altitude limite des arbres. Les auteurs répondent au besoin urgent de découvrir les champignons ectomycorhiziens critiques pour cette essence, avant que ces forêts ne soient encore plus réduites. La présence des sporocarpes en peuplements purs et les concordances ITS ont permis d'identifier 32 espèces de champignons ECM associées au *P. albicaula* mature, dans trois sites montagneux du nord du GYE. Les Bolétales et les Cortinariales (*Cortinarius*) constituent 50 % de la diversité en espèces observée. Chez les Bolétales, le *Suillus subalpinus* M.M. Moser (avec pins albicaules), le *Suillus sibericus* Singer (pins arolles), le *Rhizopogon evadens* A.H. Sm. (pins à cinq aiguilles), le *Rhizopogon* spp. et le *Chroogomphus* semisécotioïde (pins), sont restreints aux hôtes énumérés et ne sont pas susceptibles de se retrouver chez les conifères de haute altitude du GYE. L'ascomycète ubiquiste, *Cenococcum geophilum* Fr., est le plus fréquent (64 %) et le plus abondant (51 %) sur les racines des plantules, ce qu'on a déjà mentionné pour les forêts de sapins et d'épinettes de hautes élévations, et de pins lodgepoles de basses élévations, dans le GYE. On ne connaît pas l'importance relative des basidiomycètes spécialisés et des ascomycètes généralistes du pin albicaule (incluant l'établissement des plantules), tout de même, cette étude constitue le premier pas dans la détermination des champignons ECM associés à ce pin en péril.

*Mots-clés :* mycorhizes, basidiomycota, séquence ADN ITS, *Pinus albicaula*, pin arolle.

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## Introduction

Whitebark pine (*Pinus albicaulis* Engelm.) is unique as the only stone pine in North America. This species has declined 40%–90% throughout its range, owing to blister rust infection, mountain pine beetle, fire suppression, and global climate change (Kendall and Keane 2001; Tomback and Kendall 2001). The historical range for this species includes high elevation areas of the Sierras, Cascades, Intermountain ranges, and Rocky Mountains of western USA and southern Canada (Arno and Hoff 1990). Where it occurs, whitebark pine either dominates the treeline as a shrubby krummholz form (western part of range) or forms extensive forests of large trees as seral or climax vegetation (Rocky Mountains). Typical treeline habitats are harsh, cold, and windy with a

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short growing season (Arno and Hoff 1990). Other species of stone pine are scattered throughout Europe (*Pinus cembra* L.), Korea (*Pinus koraiensis* Siebold and Zucc.), Eastern Asia (*Pinus pumila* (Pall.) Regel.), and Siberia (*Pinus siberica* Mayr). All are five-needle pines with indehiscent cones and large wingless seeds and share a common taxonomy in subsection Cembra (section *Strobus*). Distribution and deposition of the large, meaty seeds is accomplished primarily by Clark's nutcracker (*Nucifraga columbiana* Wilson) for whitebark pine and by related corvid birds for other stone pines (McCaughey and Schmidt 2001). Seeds can be cached up to 12 km from the source (Hutchins and Lanner 1982; Tomback and Linhart 1990) and seedling clusters result when more than one seed germinates in a cache (Tomback 2001).

Pines require ectomycorrhizal (ECM) fungi for normal growth and survival (Smith and Read 1997; Read 1998), and a few previous studies have confirmed whitebark pine to be ectomycorrhizal (Johnson and Kendall 1994; Perkins 2004; Cripps and Mohatt 2005). However, no concerted effort has been made to discover the ECM species associated with whitebark pine before forests decline further. It is not known whether these pine forests rely on a unique suite of fungi and whether any (most) are restricted to the pine family, the genus *Pinus,* five-needle pines, stone pines, or the tree species itself. Certain genera and species of ECM fungi are known to be restricted to the genus *Pinus* (Bruns et al. 2002), and are therefore unlikely to occur with other trees in high elevation habitats. Fungi such as *Suillus plorans* (Roll.) Singer, *Suillus placidus* (Bon.) Singer, and *Suillus sibericus* Singer associate almost strictly with five-needle pines and occur with stone pines in the Alps and in Asia (Moser 2004). *Suillus placidus* and *S. sibericus* may be further restricted to subgenus *Strobus* (Wu et al. 2000) and are in the same phylogenetic clade (Kretzer et al. 1996). These specific associations of ECM fungi with pines lend support to a co-evolutionary history (Wu et al. 2000). If certain mycorrhizal fungi are unique to whitebark pine, pines in general, or stone pines, these species are likely to decline along with the tree species. In addition, the isolated nature of forests at the treeline and the unusual survival strategies of stone pines could have implications for the distribution of its fungal associates.

Large mature forests of whitebark pine still exist at high elevations in the Greater Yellowstone Ecosystem (GYE, Yellowstone National Park and adjacent lands in the Rocky Mountains) and these stands remain relatively unscathed by destructive forces, although their pristine status is changing. In this region, whitebark pine is a keystone species (Schwandt 2006) and forms extensive forests that provide habitat for flora and fauna in harsh, high elevation treeline sites (Lanner 1980) and that are important in watershed dynamics for snow-holding capacity in spring (Tomback and Kendall 2001; Weaver 2001). Where forests overlap with the range of the grizzly bear (*Ursus arctos horribilis* Ord.) in the GYE, they are considered critical bear habitat and the seeds are a crucial source of high fat content food before hibernation (Mattson and Jonkel 1990). Biologists link survival of the grizzly bear in this area with that of cone-producing whitebark pine.

The purpose of this study is to initiate discovery of the fungi that associate with whitebark pine before forests are further reduced. Whitebark pine forests in the GYE are ideal study sites because of their keystone nature, extensive pure stands, reasonable accessibility, and relative health. However, these forests exist in harsh, open subalpine habitats and are often devoid of fruiting fungi, particularly during periods of low precipitation (Keck 2001). Substantial rain events during this study provided a unique opportunity to collect fruiting ECM fungi in high elevation mature and old-growth whitebark pine forests (150–300+ years) in the GYE. Ectomycorrhizal sporocarps were collected in whitebark pine forests over five well-separated mountain ranges in the GYE, and their association with whitebark pine was confirmed either by the purity of the stand or by molecular identification of ectomycorrhizae. Many of the species are poorly known and each was completely described (Mohatt 2006). Sequences of the ITS region of identified ECM sporocarps from within the system were used for molecular comparison with those from mycorrhizal roots; comparison with NCBI GenBank database was used when necessary. In addition, mycorrhizae on seedling roots were examined quantitatively to provide information on the fungal species present in early tree establishment within this system. Information on native ECM fungi associated with whitebark pine is also of value to the extensive efforts currently underway to restore whitebark pine forests using a combination of management strategies such as fire, logging, and the planting of rust-resistant nursery grown seedlings (Burr et al. 2001; Keane and Arno 2001; Perkins 2004; Schwandt 2006).

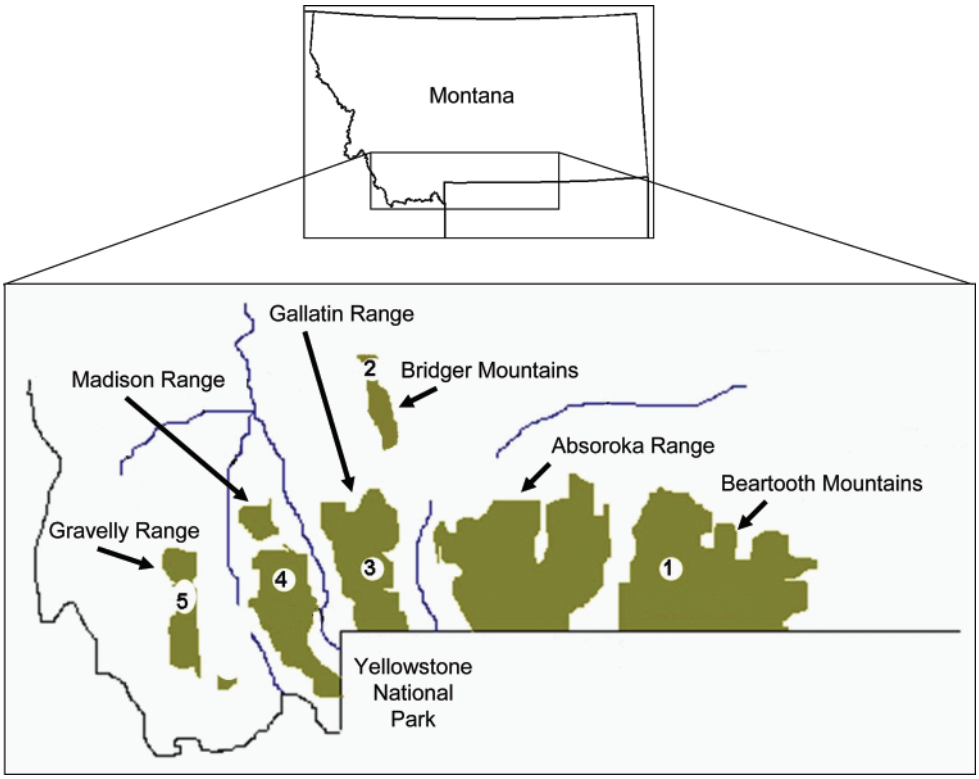
## Methods

### Site descriptions

Sites in five different mountain ranges in the northern GYE were sampled at various intensities between August 2004 and September 2005 (Fig. 1). All sites include dominant stands of mature whitebark pine (150–300+ years) with understories composed of varying levels of regenerating whitebark pine and most have *Vaccinium scoparium* Leiberg ex Cov. covering the forest floor (Fig. 2). *Abies lasiocarpa* (Hook.) Nutt. (subalpine fir) and *Picea engelmannii* Parry ex Englm. (Engelmann spruce) are present in some forests typically as understory trees and efforts were made to avoid these and collect in pure whitebark pine areas. All sites are between 2440–3110 m a.s.l. and received approximately 73–123 cm of precipitation annually for 2004 and 2005. The area covered at each site was approximately 1.5 ha (4 acres), except for the Gravelly site which contained two 1.5 ha subsites totaling 3 ha, and the New World site which was composed of five 1.5 ha subsites totaling 7.5 ha. The size of each area searched was dependent on the extent of pure stands.

New World Mine district (Site 1) is located northeast of Cook City Montana (45°06′N, 109°94′W, 2590–3109 m a.s.l.). Total precipitation at this site was 123 cm in 2004 and 115 cm in 2005 (NRCS Web site). Whitebark pine stands are located on both south- and east-facing slopes. Soils are composed of sedimentary materials, glacial till, and limestone with 30%–50% subsoil angular rock fragment (Davis and Shovic 1984; Tomback et al. 2001). Sacagawea saddle (Site 2) is located in the northwest part of the Bridger

**Fig. 1.** Study sites containing whitebark pine forests in the Greater Yellowstone Ecosystem, located in five different mountain ranges. 1. New World Mine district, 2. Sacagawea Saddle, 3. Golden Trout Lakes, 4. Big Sky Ski Area, and 5. Gravelly Range.




Mountains north of Bozeman, Montana (45°89′N, 110°98′W, 2700 m a.s.l.). Total precipitation at this site was 117 cm in 2004 and 109 cm in 2005. Soils are medium to coarse-grained granite with 35%–50% subsoil angular rock fragment content (Davis and Shovic 1984). Golden Trout Lakes (Site 3) is located across Gallatin Canyon from Big Sky, Montana (45°9′N, 110°98′W, 2590 m a.s.l.). The nearest SNOTEL station (NRCS, snow telemetry; NRCS Web site) is distant, so accurate climate data is unavailable for this site, but it is most similar to that of Lone Mountain (Big Sky). Soils are composed of coarse grained granite with thin deposits of glacial till and a subsoil of 35%–70% subrounded rock fragment (Davis and Shovic 1984). Big Sky ski area (Site 4) is located near the town of Big Sky, Montana (45°3′N, 111°44′W, 2438 m a.s.l.). Total precipitation at this site was 75 cm in 2004 and 74 cm in 2005 (NRCS Web site). Soils are composed of a medium textured surface layer with some clay in the subsurface layer, granite, with a subsoil subrounded rock content of 35%–50% (Davis and Shovic 1984). The Gravelly site (Site 5) is located in the Gravelly Mountains northwest of Yellowstone National Park (44°92′N, 111°83′W, 2590–2630 m a.s.l.). Total precipitation at this site was 77 cm in 2004 and 86 cm in 2005 (NRCS Web site). Soils are derived from sandstone and limestone (D. Svoboda, personal communication, 2006 Beaver Head-Deerlodge National Forest).

### Sporocarp collection and identification

Each site was visited one to seven times from August 2004 to October 2005. Visits were timed to follow significant precipitation events in each area, since these habitats are typically devoid of fruiting fungi in dry conditions (Keck 2001). The study covered two peak fruiting periods (August–September), and one spring fruiting period (July). Sites that contained more extensive areas of pure whitebark pine, such as the New World, were visited more often. Pure or nearly pure areas of the whitebark pine forests were searched for above- and below-ground sporocarps of ECM fungi. Hypogeous fungi were sought out by sight (cracks in the soil surface) and not by raking sensitive habitats. Resupinate sporocarps were neither searched for nor discovered. Collecting was not restricted by transects or plots to maximize the area covered and to increase the diversity of ECM fungi discovered, since fruiting ECM fungi are scarce in these habitats. Each collection was processed separately, given a collection number, and notes were included as to the purity of whitebark pine stands surrounding each collection. Given the limited sampling strategy it is likely that some ECM fungi were missed.

Since many of the taxa are poorly known, sporocarp collections were photographed and described in detail while fresh (Mohatt 2006). For most species, at least one sample of fresh tissue was placed in 2% CTAB (a storage and lysis buffer) for molecular analysis and to build a reference sequence database for comparison to sequences from ectomycorrhizae for their identification. Sporocarps were dried on a Sigg dehydrator at a setting of 2 until dry and placed in the MONT Herbarium fungal collection, Montana State University, Bozeman Montana (Mohatt 2006). For most species, microscopic work was performed on dry specimens reconstituted in 70% EtOH followed by addition of 2.5% KOH. Tissue and spores were observed at 100× with a Leica com-

**Fig. 2.** Mature whitebark pine forest with *Vaccinium* understory in the New World Mine District, Greater Yellowstone Ecosystem.

pound microscope (Leica Microsystems, Wetzlar, Germany). Complete descriptions and technical literature used to identify species are in Mohatt (2006).

### Seedling collection and ECM sample preparation

Singles and clusters (assumed to be germinated from bird caches) of whitebark pine seedlings were harvested from mature forests periodically throughout the growing season of 2005, across four of the sites surveyed for fruiting ECM fungi (excluding Big Sky). Between 1 and 9 seedlings were collected during visits to each site resulting in a total 57 seedling units (9 of these 57 units were clusters of 2–5 seedlings). Seedlings were 3–9 years old with diameters of 2–9 mm and each supported 3–9 whorls. Efforts were made to limit the impact of destructive sampling by thinning areas that hosted numerous seedlings.

Seedlings were harvested by digging up nearly the entire root system with a trowel or shovel. Whole seedlings were placed in plastic bags, labeled, watered, and sealed for transport to the laboratory in a cooler. Whole root systems were soaked in cool water for 2–24 h, gently washed over screens, and examined for ectomycorrhizae under the dissecting microscope (Brundrett et al. 1996). Mycorrhizae were sorted into morphotypes based on colour, texture, shape, mantle details, and presence or absence of rhizomorphs. Morphotypes were not pooled across sites or among seedlings on a site, but were analyzed separately. At least one sample (consisting of 1–8 root tips) of each morphotype per seedling was used in the molecular analysis. Samples of ectomycorrhizae suspected of being *Cenococcum geophilum* were examined with a microscope for a star-shaped mantle pattern, but the genetic diversity of *Cenococcum* was not examined in detail (Jany et al. 2002). The large distinct cystidia (O.K. Miller, Jr., unpublished data, 2005) and amyloid tissue were used to confirm identification of the *Chroogomphus* species (Agerer 1987–2006), and several root samples were taken in close proximity to identified ECM sporocarps. Samples of each morphotype were placed in 2% CTAB and stored at 4 °C in preparation for molecular analysis.

### Polymerase chain reaction

DNA was extracted from fresh tissue of ECM sporocarps collected in whitebark pine forests and ectomycorrhizae from young whitebark pine seedlings and stored in 2% CTAB using materials supplied in the FastDNA® kit (Qbiogene Inc., Irvine, Calif.) and a modified version of the FastDNA® protocol. Two different methods for DNA extraction were used as the FastDNA® protocol worked well for sporocarps but yielded poor quality and (or) impure DNA for ectomycorrhizae. The FastDNA® protocol was modified to include the use of Qiagen spin columns in place of the binding matrix (Qiagen Inc., Valencia, Fla.), which

resulted in a significantly higher percentage of samples amplified in polymerase chain reaction (PCR). Because the modified method is more expensive, it was only used for ECM samples. Extracted DNA was stored at –20 °C until needed.

Polymerase chain reaction (PCR) was used to amplify the ITS region of sporocarps and ectomycorrhizae (Mullis and Faloona 1987; Saiki et al. 1988). Reactions were carried out in 25 μL volume with 1 μL of purified template DNA (dilutions of 1:10 to 1:100 were also used for ectomycorrhizae samples that failed to amplify undiluted), 1 μL of 10 μmol/L ITS1-F primer and ITS4-B primer (Gardes and Bruns 1993, 1996*a*), 7 μL of sterile ddH2O, and 14 μL of Jumpstart ReadyMix (Sigma™, St. Louis, Mo.). In cases where samples did not amplify (see Mohatt 2006), the less taxon-specific reverse primer ITS4 was used in place of ITS4-B (White et al. 1990), but this primer set did not reveal additional taxa. Reactions were performed in an Eppendorf Mastercycler Gradient thermocycler (Brinkman Instruments, Westbury, N.Y.) using the following conditions: initial denaturation 95 °C for 5 min; 40 cycles of denaturation 94 °C for 1 min, annealing 50 °C for 30 s (optimized for our lab), and extension 72 °C for 1 min, final extension 72 °C for 10 min, and 4 °C hold (Gardes and Bruns 1993). PCR products were purified using the QIAquick Gel Extraction kit and protocol (Qiagen Inc.). Sequencing was performed at Davis Sequencing (Davis, Calif.) and the University of California at Berkeley, California, sequencing facility.

### ITS sequence matching for identification of mycorrhizae

Owing to the large taxonomic diversity of sequences and incomplete sequencing of some samples, only the ITS1 region was used in the analysis. Izzo et al. (2005*a*, 2005*b*) state that since the ITS1 region typically has the highest variability of the entire ITS region it contains adequate variability for the analysis of most ECM fungi. This should pertain particularly to our data where ectomycorrhizae were primarily identified by matches to *identified sporocarps within our system.* This is particularly valid for *Rhizopogon* and *Suillus* species which have high variability in the ITS1 region (Kretzer et al. 1996; Manian et al. 2001). For some groups, variation in the ITS1 region of our own sporocarps was assessed (*Russula* cf. *torulosa*, *Tricholoma moseri*) to identify mycorrhizae to species, but the ITS1 variation for a larger sampling is not known. *Cortinarius* species lack sufficient variability in the ITS1 region (Kåren et al. 1997) and *Inocybe* species are more effectively identified by other parts of the genome, so these taxa were identified only to genus by ITS matching. Sequences from ectomycorrhizae that did not match with a sequence from sporocarps collected in whitebark pine forests were submitted to a BLAST search for matching to the most similar identified sequence in the NCBI GenBank database for genus-level identification (Altschul et al. 1990, 1997). The percent similarity of matches to sporocarps or GenBank accessions is presented for the morphotypes. Matches above 97% were considered confirmed, since matches are primarily to identified sporocarps *from within the system*. A phylogenetic analysis was used to display sporocarp–mycorrhizae relationships, as is now common for field data (Gardes and Bruns 1996*b*), and the resulting tree is in Mohatt (2006), but is not presented here. Sequences of sporocarps and uncultured mycorrhizae are in the GenBank database.

### Quantification of ECM fungi on seedling roots

A quantitative analysis of ECM types or species on roots was done to characterize the importance of particular genera or species on seedlings. The relative abundance of each morphotype on each seedling was estimated by counting the number of colonized root tips of each morphotype and dividing by the total number of colonized root tips on each seedling (Horton and Bruns 2001; Cripps 2004). Dead and shriveled mycorrhizae were not identifiable and were not included in the analysis (10%–90% of root tips). The relative frequency was calculated by dividing the total number of seedlings with a particular morphotype by the total number of seedlings examined (Horton and Bruns 2001). An importance value for all the morphotypes encountered was calculated by summing relative abundance and relative frequency data (Horton and Bruns 2001; Cripps 2004; Horton et al. 2005).

## Results

### ECM fungi fruiting in whitebark pine forests

Collection of sporocarps in whitebark pine forests during the field seasons of 2004 and 2005 yielded 111 collections (104 identified), 44 species of ECM fungi, in 22 genera, 15 families, and across three phyla. Of the 44 species of ECM fungi collected in whitebark forests, 24 were collected in pure stands of whitebark pine, confirming their putative association with this tree species (Table 1), and these represented almost two thirds of all collections. Detailed morphological and microscopic descriptions of all fungi collected in whitebark pine forests are in Mohatt (2006) along with a dichotomous key provided for their identification.

### ECM fungi confirmed on seedling roots

At least 13 distinct fungal taxa were identified on seedling roots collected from four sites using molecular analysis. ITS1 sequences from ectomycorrhizae on seedling roots compared with those from sporocarps of 26 mycorrhizal species collected in whitebark pine forests revealed 6 from seedling roots that match, or have a close affinity to, taxa collected above ground. Confirmed taxa are *Russula* cf. *torulosa/queletii, Tricholoma moseri*, *Suillus subalpinus* M.M. Moser, *Suillus tomentosus* var. *discolor*, *Rhizopogon evadens*, and *R. milleri* (Table 2). Three additional taxa (*Cortinarius* sp. 1: cf. *duracinus*, *Cortinarius* sp. 2 and *Inocybe* sp.) were identified to genus with a BLAST search. Two thelephoroid taxa were discovered using a BLAST search (Table 2), but their resupinate sporocarps are yet to be discovered in whitebark pine forests in the GYE. Species could not be determined, since ITS1 variability is not sufficient for delineation within this group (Pritsch et al. 2000). *Cenococcum geophilum* and the secotioid *Chroogomphus* species were confirmed on roots by the microscopic details of their mycorrhizal morphology (see methods).

Addition of molecular data brings the total of ECM fungi confirmed with whitebark pine by sporocarps presence in pure stands or on roots to 32 species (Table 1). Of these,

**Table 1.** Fruiting ECM fungi collected in whitebark pine forests in the Northern GYE 2004–2005 by site, and confirmed association in pure stands or on seedling roots as ectomycorrhizae.

| | Site | | | | | Confirmed |
|---|---|---|---|---|---|---|
| Species | 1 | 2 | 3 | 4 | 5 | Stand/roots |
| Basidiomycota | | | | | | |
| Agaricales | | | | | | |
| Amanitaceae | | | | | | |
| *Amanita "alpina"* A.H. Smith nom. prov. | + | + | + | – | – | +/– |
| Hygrophoraceae | | | | | | |
| *Hygrophorus gliocyclus* Fr. DQ517417 | + | + | – | – | + | +/– |
| *Hygrophorus marzuolus* (Fr.) Bres. | + | – | + | – | – | +/– |
| *Hygrophorus olivaceoalbus* (Fr.:Fr.) Fr. DQ517418 | + | – | – | – | – | +/– |
| *Hygrophorus subalpinus* A. H. Smith | + | – | + | – | – | +/– |
| *Hygrophorus* sp. (aff. *H. piceae*) | – | + | – | – | – | +/– |
| Tricholomataceae | | | | | | |
| *Leucopaxillus paradoxis* (Cost. & Durfour) Boursier | – | – | – | – | + | +/– |
| *Tricholoma moseri* Singer DQ517420 | +* | – | – | – | + | +/+ |
| Cortinariaceae | | | | | | |
| *Cortinarius* cf. *clandestinus* A.H. Smith DQ517406 | + | – | – | – | + | +/– |
| *Cortinarius duracinus* Fr. DQ517404, DQ517412 | +* | + | – | – | – | +/+ |
| *Cortinarius "flavobasalis"* McKnight & Moser DQ517402 | + | – | – | – | + | +/– |
| *Cortinarius "flavoroseus"* nom. prov. DQ517403 | + | | – | – | + | +/– |
| *Cortinarius* aff. *fulminoides* (Moser) Moser DQ517405 | – | + | – | – | + | +/– |
| *Cortinarius* cf. *subolivescens* A.H. Smith | + | – | – | – | + | +/– |
| *Cortinarius* sp. | * | – | – | – | – | –/+ |
| *Dermocybe crocea* (Schff.) Moser | + | – | – | – | – | +/– |
| *Inocybe* sp. | * | – | – | – | – | –/+ |
| Russulales | | | | | | |
| *Lactarius deliciosus* (L.:Fr.) Gray | + | + | – | + | – | +/– |
| *Russula* cf. *torulosa* Bres. DQ517369, -371, -372 | +* | – | – | – | + | +/+ |
| *Russula* sp. 2 DQ517367 | – | + | –* | – | – | –/+ |
| *Russula* sp. 3 DQ517364 | – | – | – | – | + | +/– |
| Boletales | | | | | | |
| *Chroogomphus* sp. DQ517399, DQ517400 | +* | – | – | + | – | +/+ |
| *Rhizopogon* cf. *evadens* A.H. Smith DQ517388 | +* | + | – | – | + | +/+ |
| *Rhizopogon* cf. *milleri* A.H. Smith DQ517378, -379, -380 | +* | – | – | + | + | +/+ |
| *Suillus subalpinus* M.M. Moser DQ517390 | +* | + | + | – | – | +/+ |
| *Suillus sibiricus* (Singer) Singer DQ517393 | + | + | – | – | + | +/– |
| *Suillus tomentosus* var. *discolor* DQ517394 | +* | – | – | – | – | –/+ |
| *Suillus* sp. | –* | – | – | – | – | –/+ |
| Phallales–Gomphales | | | | | | |
| *Hysterangium separabile* Zeller | + | + | – | + | – | +/– |
| Thelephorales | | | | | | |
| Thelephoroid type 1 (*Pseudotomentella* sp.) | –* | – | – | – | – | –/+ |
| Thelephoroid type 2 (*Tomentellopsis* sp.) | –* | – | – | – | – | –/+ |
| Ascomycota | | | | | | |
| *Cenococcum geophilum* Fr. | –* | –* | –* | –* | –* | –/+ |
| Total species | | | | | | 32 |

**Note:** Asterisks (*) represent identified ectomycorrhizae; (+) represent sporocarps; (–) represent "no recorded occurrences". Site 1, New World Mine district; Site 2, Sacagawea Saddle; Site 3, Golden Trout Lakes; Site 4, Big Sky Ski Area; and Site 5, Gravelly Range. GenBank accession numbers are for sequences of sporocarps used in mycorrhizal identification.

half are in the Cortinariales (28%) or Boletales (22%). The majority of all species discovered are epigeous species (85%), and the remainder hypogeous (9%) or secotioid (3%), and one lacks a fruiting body (*C. geophilum*). Several species of hypogeous or secotioid genera (*Endogone*, *Gautieria*, *Geopora*, *Hydnotrya*, *Thaxterogaster*) collected in GYE whitebark pine forests could not be confirmed as occurring with whitebark pine (Mohatt 2006).

## Diversity and Importance of ECM species on whitebark pine seedlings

The number of ECM taxa on a single seedling averaged

**Table 2.** Fungal taxa identified on roots of whitebark pine seedlings collected in the northern GYE using molecular analysis of the ITS1 region or mantle morphology.

| Taxon | GenBank accession No. | Taxon match | Similarity (%) | No. base pair overlap |
|---|---|---|---|---|
| *Cenococcum geophilum* | NA | Mantle morphology | NA | NA |
| *Chroogomphus* sp. | NA | Mantle morphology* | NA | NA |
| *Cortinarius* sp. 1 | DQ517411 | DQ517404 (*C. duracinus*)* | 98 | 207/210 |
| *Cortinarius* sp.2 | DQ517409 | *Cortinarius* clade | 95 | 208/218 |
| *Inocybe* sp. | DQ517416 | DQ068957 (*Inocybe* sp.)* | 98 | 211/214 |
| *Rhizopogon* cf. *evadens* | DQ517374 | DQ517388 (*R. evadens*)* | 99 | 241/242 |
| *Rhizopogon* cf. *milleri* | DQ517381, DQ517382 | DQ517378, DQ517379, DQ517380 (*R. milleri*)* | 97 | 270/276 |
| *Russula torulosa/queletii* | DQ517368 | DQ517372 (*R. cf. torulosa*)* | 100 | 237/237 |
| *Suillus subalpinus* | DQ517390 | DQ517390, DQ517391, DQ517392 (*S. subalpinus*)* | 100 | 268/268 |
| *Suillus* sp. | DQ517395, DQ517396 | *Suillus* clade* closest to *S. variegatus* | 99 | 131/132 |
| *Thelephoroid* type 1 | DQ517360, DQ517361, | AF274770 (*Pseudotomentella sp.*) BLAST | 99 | 214/216 |
| *Thelephoroid* type 2 | DQ517359 | AM086445 (*Tomentellopsis sp*) BLAST | 97 | 249/255 |
| *Tricholoma moseri* | DQ517420, DQ517421 | DQ517419 (*T. moseri*)* | 99 | 289/291 |

**Note:** Percent similarities are matches to sporocarps from whitebark pine forests (asterisk) or GenBank database. Bootstrap values and weighted indices for phylogenetic analysis are in Mohatt (2006).

2.2 ± 1.3, with a range of 1–5 morphotypes. A few seedling clusters were also collected, and the number of seedlings comprising a cluster ranged from 2–5 seedlings. When each cluster is analyzed as one unit, there appears to be a trend of overall ECM richness that increases with the number of seedlings in a cluster, but data are not sufficient to verify this statistically (only nine clusters analyzed). However, a two sample *t* test revealed that the average number of morphotypes per individual seedling in a cluster was not significantly higher ($p > 0.41$) than the average number of morphotypes found on seedlings growing individually (Mohatt 2006).

The majority of all root tips on whitebark pine seedlings showed evidence of either previous (dried mycorrhizae) or current mycorrhization. From 13 to 17 morphotypes were recognized on the 716 ECM root tips from 57 seedling units. This is considered a conservative estimate of ECM species richness as some morphotypes could be composed of multiple species, and this is particularly a concern for the cortinarioid and suilloid types. Of the total root tips, 51% were Ascomycota (*Cenococcum*), 40% were identified Basidiomycota (12 types), and the remaining 9% (64 root tips) consisted of at least 4 morphotypes that remain unknown (no results from the two primer sets used). Relative frequency and abundance of each fungal group (species) from across sites were summed to obtain importance values (Fig. 3). *Cenococcum geophilum* was the most frequent and abundant species, and occurred on 51% of the infected root tips and 64% of all seedlings examined. Other groups that ranked high in importance value on seedlings were cortinarioid, suilloid, russuloid, and thelephoroid types (Fig. 3).

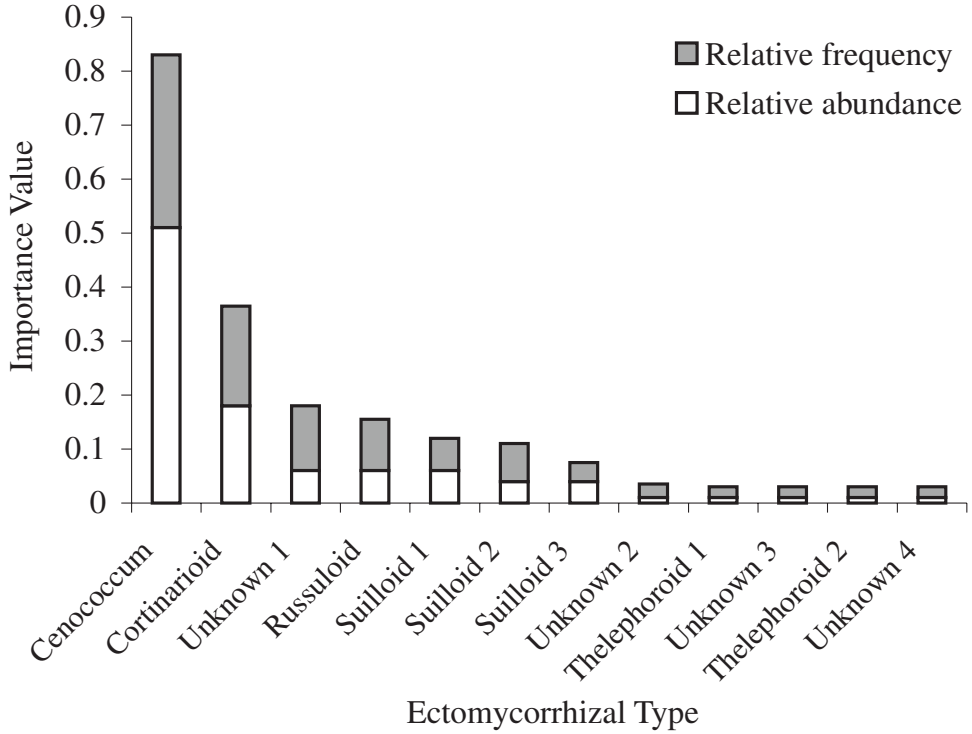
## Discussion

Thirty-two species of ECM fungi are confirmed to occur with whitebark pine in GYE forests, and this low diversity could reflect the limited sampling for the two season field study; however, it is an important initial step towards determining the ECM fungi associated with whitebark pine. Additional sampling, particularly of thelephoroids, *Cortinarius* species, fungi on mature roots, and sporocarps over a larger range and longer time scale could increase this number. Molina et al. (1992) suggest that pines might associate with as many as 2000 species of ECM fungi, although the number with five-needle or stone pines would be a subset of these. While pines share a large number of ECM fungi with other coniferous trees, they also have developed a strong genus-level specificity for particular mycorrhizal fungi (Molina et al. 1992).

Although sampling was limited, the low number of ECM fungi recorded with whitebark pine is comparable with the low number reported with other pine species in harsh western habitats, and for other conifer forest types at lower elevations in the GYE. Only 10 ECM species have been detected with high elevation ancient bristlecone pines (*P. longaeva* Bailey) in California (Bidartondo et al. 2001), whereas there were 15–19 ECM species with pinyon pines (*Pinus edulis* Engelm.) in Arizona (Gehring et al. 1998). For other forest types within the GYE, 22 types of ECM fungi are reported with *Pinus contorta* Engelm. at lower elevations (all basidiomycota plus *C. geophilum*) and 18 in mixed lodgepole–spruce forests (Cullings et al. 2000; Cullings and Makhija 2001). Another GYE study detected 66 species of ECM fungi in undisturbed lodgepole pine forests, but roots were dominated by one suilloid species, one Tricholomataceae species, *Cortinarius* species, and *C. geophilum* (Byrd et al. 2000). The ECM fungi detected in most of these studies result from molecular root probes, and methods did not include sequence matches to identified sporocarps from these habitats. In the whitebark pine forests, most fungal morphotypes on seedlings (70%) were identified by molecular matches to sporocarps *from within the system*. The low diversity of ECM fungi reported from these western coniferous forests could reflect simple systems composed of extensive monocultures of one or two tree species, semi-arid or harsh conditions that limit fungal diversity, or a sampling bias that suggests a need for additional sampling.

In terms of species richness, the most recorded groups for whitebark pine in the GYE are the Boletales (*Suillus*, *Rhizopogon*, and *Chroogomphus*) and the Cortinariales (only *Cor-*

**Fig. 3.** Importance values of pooled ECM morphotypes on whitebark pine seedlings collected across four sites in the Northern GYE. Importance values calculated by adding the relative abundance and frequency data.




*tinarius*, and not *Hebeloma* or *Inocybe*). These two groups comprise half of the taxa discovered, half of the collections, and are common as mycorrhizae below ground (occur on 32% of root tips). A list of ECM taxa compiled from diverse sources of taxonomic literature citing whitebark pine as a putative host revealed 55 possible associates, and over 70% are in Boletales (*Rhizopogon*, *Suillus*, *Gomphidiaceae*) or Cortinariales (primarily the genus *Cortinarius*) (Mohatt 2006). These informal reports from Washington, Idaho, Oregon, Wyoming, and the Columbia Basin (Moser et al. 1994, 1995; Moser and Ammirati 1999; Moser 2002, 2004; R. Fogel unpublished data, 2005; J. Trappe, unpublished data, 2005) suggest that the diversity of Boletales and Cortinariales may extend to whitebark pine forests in these states, but more definitive studies are necessary to confirm this.

Within Boletales, the suilloid clade is one of the most diverse groups discovered in GYE whitebark pine forests. This clade, composed primarily of *Rhizopogon*, *Suillus*, and *Chroogomphus*, in this study, is almost exclusively restricted to the Pinaceae, and most species exhibit host specificity on a finer level (Bruns et al. 2002). Two *Suillus* species from GYE whitebark pine forests, *S. sibericus* and *S. subalpinus*, occur primarily with five-needle pines. *Suillus sibericus* is reported with stone pines in Europe and Asia (Moser 2004), and is now confirmed with whitebark pine in western USA. *Suillus placidus* occurs with stone pines in the Alps and the Altai mountains of Asia and also with *Pinus strobus* L. in eastern USA. The related *S. subalpinus* is confirmed with whitebark pine in the GYE, where it was first described by Moser (1997). *Suillus plorans*, a strict symbiont of stone pines in Europe and Asia (Moser 2004), has not been found in GYE forests to date. All four common stone pine associates (*S. subalpinus*, *S. placidus*, *S. sibericus*, and *S. plorans*) are glandular dotted species; a group restricted to the genus *Pinus*. Those with whitebark pine are not expected to occur with other conifers in the GYE, with the possible exception of *Pinus flexilis* James at much lower elevations.

*Rhizopogon* species (Boletales, suilloid clade) are a common component of whitebark pine forests and are also exclusive to the *Pinaceae*. *Rhizopogon evadens* A.H. Smith appears only to be associated with the genus *Pinus* (Grubisha et al. 2002) and, along with *Rhizopogon milleri* A.H. Smith, has been reported from whitebark pine forests outside GYE. *Rhizopogon* species are particularly important in the regeneration of young pine seedlings (Gobert and Plassard 2002; Steinfeld et al. 2003), and this genus was common on roots of whitebark pine seedlings in our study, even those from open avalanche paths. Small and large mammals, such as squirrels and deer, are important in the distribution of *Rhizopogon* and *Suillus* spores (Izzo et al. 2005*b*; Ashkannejhad and Horton 2006), and these genera can also persist as sporebanks in soil (Kjøller and Bruns 2003, Ashkannejhad and Horton 2006). In addition, *Rhizopogon* sporocarps are a possible food source for grizzly bears in the GYE, and spores have been documented in bear scat from whitebark pine forests in the GYE (Mattson et al. 2002).

*Chroogomphus* species (Boletales, suilloid clade) are primarily restricted to pines (Miller and Aime 2001; Miller 2003), and the semisecotioid species discovered in whitebark pine forests appears molecularly closest to *Chroogomphus leptocystis* (Singer) O.K. Mill. (C. Aime, unpublished data, 2005) known from five-needle white pine forests (*Pinus monticola* Dougl. ex D. Don) in Idaho (Miller 2003). The fungus is recorded from two distant mountain

ranges, and is not the secotioid fungus *Chroogomphus loculatus* Trappe and Miller from Oregon (Castellano et al. 1999). *Chroogomphus helveticus* (Pilat) Kuthán and Singer occurs with the stone pine in Europe and the closely related *C. sibiricus* with *P. siberica* in Asia (Moser 2004).

In this study, *Cortinarius* (Cortinariales) was found to be the most diverse ECM genus in whitebark pine forests, comprising one fourth of all fungal species confirmed to be in association with whitebark pine in the Northern GYE, accounts for one fourth of all collections, and is a dominant type on roots. This is not surprising since *Cortinarius* species are a significant component of ECM communities in western forests (Kernaghan 2001; Moser 2004). Some *Cortinarius* species are suspected of having a preference for particular hosts, but owing to the high number of species and lack of taxonomic knowledge, the ecology of this genus remains poorly known in western USA. Two of our species occur with the stone pine, *P. cembra*, in the Alps; however, other *Cortinarii* from GYE whitebark pine forests also occur in subalpine spruce–fir forests (Moser 2004). Two of the GYE species (*C. "flavoroseus"* and *C. "flavobasalis"*) are restricted to high elevation western habitats and often depend on meltwater from remnant snowbanks in the spring for fruiting (Cripps 2007).

The number of ECM morphotypes on the roots examined averaged two per seedling, and most root systems of the young whitebark pines were not well developed in terms of fine roots. For these seedlings, 13 identified taxa account for 91% of the ECM fungi found on root tips with 4 types (9%) unidentified. In comparison with other pine hosts, 1-year-old *P. muricata* seedlings in California were colonized by 7 species of ECM fungi (Baar et al. 1999), and *P. sylvestris* seedlings in Sweden hosted at least 11 ECM species (Jonsson et al. 1999). The GYE whitebark pine seedlings were older (3–9 years) than for these other studies. However, diversity can remain low on roots of mature pines in harsh western habitats. For example, of the 10 ECM taxa occurring on roots of mature (ca. 1000 years old) *P. longaeva*, another high elevation western pine, 4 accounted for 94.5% of all mycorrhizae. The low diversity with bristlecone pine is attributed to harsh environmental conditions that severely limit the development of the belowground fungal community (Bidartondo et al. 2001).

By far the most frequent (64%) and abundant (51%) ECM fungus on the whitebark pine seedlings examined was *C. geophilum*, an asexual ascomycete that reproduces via sclerotia or hyphal contact in the soil (and not spores). For some seedlings in this study, it was the only ECM fungus present on roots. It is a generalist with a global distribution and forms associations with a wide variety of tree genera, including *Abies*, *Betula*, *Eucalyptus*, *Fagus*, *Quercus*, *Picea*, *Pinus*, and *Populus* (Agerer 1987-2006). It can be the most frequently encountered ECM fungus on roots at alpine treeline (Kernaghan and Harper 2001) and in the alpine zone (Cripps and Eddington 2005). *Cenococcum geophilum* is also common at lower elevations in conifer forests in Yellowstone National Park (Cullings and Makhija 2001) and in disturbed and undisturbed lodgepole forests near that area (Byrd et al. 2000) according to root core studies. In a Wyoming study, only 3 of 83 first-year spruce and fir seedlings at treeline hosted this fungus, while 52% of juvenile trees (≥2-years-old) were colonized by *C. geophilum* (Hasselquist et al. 2005). The researchers considered this to be an indirect indication of the importance of *C. geophilum* for conifer survival at the timberline, and suggested that its absence could explain high mortality levels in first year conifer seedlings compared with that of older seedlings. Colonization levels of the juvenile spruce and fir in the same study are comparable to that of our 3–9 year old whitebark pines, also at the treeline. Hasselquist et al. (2005) also showed, in a greenhouse study, that *Cenococcum geophilum* was beneficial, under limited-water conditions, for spruce and fir seedlings, and this species has been found to increase the drought tolerance of Norway spruce in situ (Nilsen et al. 1998). Whitebark pine is known to have a high early seedling mortality rate (McCaughey 1993; Tomback et al. 1993, 2001; McCaughey and Tomback 2001); however, the role of ECM fungi and in its early establishment has not been examined.

In the present study, most seedlings were sampled from within the canopy zone of mature whitebark pine forests, but a few were from open avalanche slopes. A comparison of *C. geophilum* on whitebark pine seedlings from avalanche paths and adjacent mature forests in the GYE showed the fungus to be less frequent in open areas at treeline, but data are limited (Mohatt 2006). Whitebark pine seeds are commonly bird-dispersed, and planted away from mature forests on open slopes, which might preclude immediate association with *C. geophilum*. In the high elevation spruce–fir forests of Wyoming, discussed previously, *C. geophilum* levels declined with seedling distance to mature trees (Hasselquist et al. 2005). This suggests the necessity of adult trees in close proximity as a source of this fungus for seedlings and reflects a fungal life cycle that lacks spores for long distance dispersal.

The ecological consequences of hosting generalists, such as *Cenococcum*, versus more host-selective fungi (*Rhizopogon* and *Suillus*), particularly for seedling establishment, are not known. *Rhizopogon* and *Suillus* species were documented on whitebark pine seedlings within the canopy zone, and in open avalanche paths and on severe burns where seeds were likely planted at distances from mature forests by nutcrackers (Cripps et al. 2006; Mohatt 2006). *Rhizopogon* and *Suillus* species depend on large and small mammals for dispersal away from the canopy zone and can persist in soil as spore banks (Bidartondo et al. 2001, Ashkannejhad and Horton 2006). It has been suggested that some pioneer tree species tend to associate with specialist fungi that are of value because they are not shared by competing tree species (Bruns et al. 2002; Rusca et al. 2006). If this holds true for whitebark pine forests, it implies that invading understory fir and spruce would not be able to successfully tap into an established mycorrhizal system of fungi specialized for pine. However, the disadvantage of hosting specialist fungi is that they may not be available under some conditions, and for whitebark pine, they may be lost as the tree species declines. Other studies suggest that many species of ECM fungi colonize multiple tree hosts in the mixed conifer forests of North American (Horton and Bruns 1998, Massicotte et al. 1999, Cullings et al. 2000). While fungal species with a broader host range might sus-

tain whitebark pine, it is also possible that they could speed replacement of whitebark pine by association with invasive spruce and fir. At least for the Boletales portion of the mycorrhizal community delineated here, it is unlikely that whitebark pine shares these associates with other high elevation conifers in the GYE, and this group is a common colonizer of pine seedlings. Lower elevation pines, such as the lodgepole pine, could play a role as a reservoir of some ECM pine associates in the GYE; however, it is not clear whether these fungi could also survive at treeline elevations where whitebark pine exists. If whitebark pine, as a declining species, is unique as a host for certain ECM fungi, this also has implications for conservation of these species. These initial results can suggest future avenues of research on the diversity and ecology of ECM fungi with whitebark pine, and investigations relevant to the extensive efforts currently underway for restoration of declining whitebark pine forests (Burr et al. 2001; Keane and Arno 2001; Schwandt 2006).

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