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Is rarity of pinedrops (*Pterospora andromedea*) in eastern North America linked to rarity of its unique fungal symbiont?

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Abstract Like other myco-heterotrophic plants, *Pterospora andromedea* (pinedrops) is dependent upon its specific fungal symbionts for survival. The rarity of pinedrops fungal symbiont was investigated in the eastern United States where pinedrops are rare. Wild populations of eastern pinedrops were sampled, and the plant haplotypes and fungal symbionts were characterized with molecular techniques; these data were compared to those from the West with phylogenetic analyses. The frequency of the fungal symbiont in eastern white pine forests was assessed using a laboratory soil bioassay and in situ pinedrops seed baiting. Only one plant haplotype and fungal symbiont was detected. The plant haplotype was not unique to the East. The fungal symbiont appears to be a new species within the genus *Rhizopogon*, closely related to the western symbionts. This fungal species was not frequent in soils with or without pinedrops, but was less frequent in the latter and in comparison to the fungal symbionts in western forests. Seed baiting resulted in few germinants, suggesting that mycelial networks produced by the eastern fungal symbiont were rare. Results suggest that eastern pinedrops rarity is influenced by the distribution and rarity of its fungal symbiont.

Keywords Myco-heterotrophy · *Pterospora andromedea* (pinedrops) · *Rhizopogon* · Fungal specificity · Rarity · Distribution

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Introduction

Mycorrhizal symbioses are often generalized as mutually beneficial for the plant and fungal symbionts, but this is not always correct in that mycorrhizal symbioses can fall along a continuum from mutualism to parasitism (Johnson et al. 1997; Jones and Smith 2004). An extreme example in this mutualism-parasitism continuum is the parasitic mycorrhizal relationship between many non-photosynthetic plants and their fungal symbionts. These non-photosynthetic or myco-heterotrophic plants (Leake 1994), develop a unique relationship with their mycorrhizal fungi to acquire fixed carbon from photosynthetic hosts via a shared mycorrhizal fungus (Björkman 1960; McKendrick et al. 2000).

The myco-heterotrophic strategy evolved independently and repeatedly, resulting in over 400 achlorophyllous myco-heterotrophic species (Leake 1994; Merckx and Freudenstein 2010). Several researchers have used molecular techniques to demonstrate the associations of many myco-heterotrophic plants with a narrow range of closely related species of fungi, even over large geographical distances (Cullings et al. 1996; Taylor and Bruns 1999a; Kretzer et al. 2000; Bidartondo and Bruns 2001, 2002; Taylor et al. 2002; Yagame et al. 2008; Bougoure et al. 2009; Barrett et al. 2010). The specificity of myco-heterotrophic plants for their fungal host follows patterns of specificity seen with other parasite-host systems, such as plant rusts and other plant pathogens (Thompson 1994), and contrasts with the majority of autotrophic plants which can associate with a diverse

range of mycorrhizal fungal species (Trappe 1977; Molina et al. 1992, and see Bruns et al. 2002a).

The myco-heterotrophic plant pinedrops (*Pterospora andromedea* Nutt.; Ericaceae, Monotropoideae) forms typical monotropoid mycorrhizae with a narrow range of basidiomycete ectomycorrhizal fungi in the genus *Rhizopogon* subgenus *Amylopogon* (Cullings et al. 1996; Bidartondo and Bruns 2001). Fungi in the genus *Rhizopogon* are disturbance tolerant, mammal dispersed, and form truffle-like hypogeous fungi that are otherwise almost exclusively associated with Pinaceae (Molina et al. 1992; Molina and Trappe 1994). Five plastid DNA haplotypes of pinedrops have been identified and these haplotypes show preference for either *R. salebrosus* or *R. arctostaphyli*, even when they co-occur (Bidartondo and Bruns 2002). In the Sierra National Forest where pinedrops is common, these fungal symbionts are common and found frequently in the soil spore bank (Kjøller and Bruns 2003). Soil spore banks are analogous to soil seed banks, referring to resistant spores that are found in soils awaiting germination cues following disturbance (Baar et al. 1999).

Like other myco-heterotrophic plants, pinedrops produce large quantities of wind dispersed dust-like seeds that contain reduced endosperms (Leake 1994; McKendrick et al. 2000). An in vitro germination study showed that pinedrops seeds would only germinate when stimulated by a diffusible chemical produced by its fungal symbiont, or a closely related species (Bruns and Read 2000). An in situ seed germination study showed further fungal specificity requirements in that only the pinedrops seeds associated with the mycorrhizal fungi of their maternal seed source (i.e. *R. arctostaphyli* or *R. salebrosus*) could develop into mycorrhizal seedlings (Bidartondo and Bruns 2005). These methods for sowing seeds that bait for the respective fungal symbiont can also be used for assessing symbionts' presence and distribution. The biology of pinedrops clearly shows that the fungal symbiont plays a crucial role in survival, recruitment and distribution of this plant species.

Pinedrops is endemic to North America where it is found in mixed conifer/deciduous forests. The species is limited to either western or eastern forests, with no occurrences in the middle of the continent. Western occurrences extend throughout the Sierra Nevada and Rocky Mountain ranges from Canada to Mexico, and eastern occurrences extend throughout the Great Lake states across Canada to New England and New York (Bakshi 1959). Although western pinedrops is common, the species appears to be relatively rare and in decline in the East (Schori 2002). Specifically, in Ontario Canada pinedrops are now recorded in only four counties. Only two towns in Vermont now hold one occurrence each. Only three locations in New York have recent records of the plants, all of which are in the same county.

The research on pinedrops and its fungal symbionts has been focused in the western United States where the plant and its fungal symbionts are common (Cullings et al. 1996; Bruns and Read 2000; Bidartondo and Bruns 2001, 2002, 2005; Kjøller and Bruns 2003). To conserve and manage for eastern pinedrops a better understanding of this plant and its fungal symbionts are needed. Here we use molecular techniques to identify eastern pinedrops haplotypes and fungal symbionts from plant and fungal tissue collected from pinedrops in the field. These data are compared to those from the West with phylogenetic analyses. We further investigate the fungal symbionts with regards to their frequency in eastern forests using soil bioassay and in situ seed sowing methods. We predicted that the fungal symbionts of eastern pinedrops are less frequent than their counterparts in western forests.

Materials and methods

Field sites and experimental plots

Field sites were located within mixed deciduous conifer forests that were dominated by *Pinus strobus*. Two types of sites were used: (1) sites with an active population of pinedrops (i.e. inflorescences observed within the last 3 years) and (2) sites in which pinedrops have never been reported; hereafter referred to as pinedrops (PD) and non-pinedrops (NPD) sites, respectively. Due to the rarity of pinedrops in the eastern United States only a limited number of study sites were available. Sites were established in New York and Michigan with three PD and NPD sites in each state. The New York sites were all located in the only known location with active population in the state, in Letchworth State Park (77° 55′N by 42° 40′W), Livingston county (Livingston-1, -2 and -3). The Michigan sites were located in Emmet (84° 15′N by 45° 40′W), Mecosta (85° 30′N by 43° 35′W), and Keweenaw (88° 15′N by 47° 20′W), each with one PD and NPD site. In New York, the PD sites were all separated by ~1 km and in Michigan by at least 249 km. In both states, NPD sites were located between 1 and 1.6 km from a NPD site.

Each site was defined by a 30×100 m sampling area; from which five randomly selected 5×5 m plots were established, hereafter referred to as NPD plots. In addition, four and five 5×5 m plots containing flowering pinedrops were established at the Emmet and Keweenaw PD sites, respectively. At the Livingston-1 PD site, remnant inflorescences from the previous year were used to establish three additional 5×5 m plots. Plots containing flowering or remnant inflorescences of pinedrops are hereafter referred

to as PD plots. PD plots were not established at the Livingston-2 and -3 PD site or the Mecosta PD site as flowering or remnant inflorescences of pinedrops were absent during the time frame of this study. Pinedrops do not necessarily flower every year, remaining underground and undetected even though they are present at a site (Schori 2002). See Fig. 1 for a schematic of the sampling design and Table 1 for a summary of the number and types of study sites and plots used for the field soil bioassay with *Pine* and the in situ seed baiting, with their respective number of samples.

Collection of plant and soil material

Root tips from flowering pinedrops were collected in August of 2004 from the Emmet and Keweenaw PD sites in Michigan. Seven pinedrops plants from Emmet and 16 plants from Keweenaw were sampled. Root tips were collected by carefully removing soil from one small spot at the base of the inflorescence until part of the root ball was exposed, and from this area a few root tips were harvested. Root tips were placed in 2X CTAB buffer (Gardes and Bruns 1993) and stored at 4°C. Also, mature cracked seed capsules were collected from these plants and placed in sealed plastic bags and stored at 4°C.

From each site, soil samples were collected in the center of each 5×5 m plot using a 5 cm diameter by 15 cm deep soil corer. A total of 72 soil cores were taken in August of 2004 (NPD plots: 12 sites×5 plots per site=60 soil cores; PD plots from three sites: 3+4+5 PD plots=12 soil cores). Soil cores were put into labeled paper bags and left to air dry at room temperature for 3 weeks to select for fungal species with resistant spores (Kjøller and Bruns 2003).

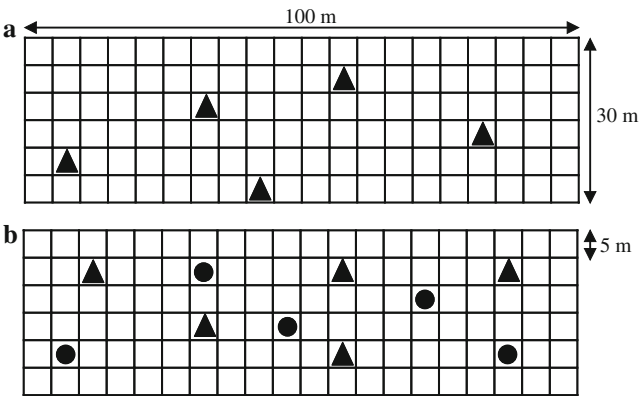

Fig. 1 Schematic of the sampling design used in the field sites; **a** Livingston-1, -2 and -3 non-pinedrops (NPD), and Livingston-2 and -3 pinedrops (PD), Mecosta PD and NPD, and Emmet and Keweenaw NPD sites, and **b** Livingston-1, Emmet, and Keweenaw PD sites; showing NPD (▲) and PD (●) plots

Field soil bioassay with pine

From each soil core, two pots (Ray Leach Tubes - 3.8 cm diameter by 21 cm deep, Stuewe & Sons, Inc., Corvallis, OR, USA) were prepared, containing either 0.5% or 50% field soil, for a total of 144 pots (72 soil cores×2 dilutions=144 pots). The field soils were diluted with extra field soil from their respective site that was pooled, mixed 1:1 with sand, and then sterilized (autoclaved twice). The sterilized soil/sand mix was also used for 60 negative control pots, with five pots for each site (12 sites×5 pots=60).

Purchased eastern white pine (*Pinus strobus* L.) and red pine (*Pinus resinosa* Ait.) seeds (Sheffield's Seed Co., Inc., Locke, NY, USA) were prepared for sowing. The purpose of using red pine was to determine if the fungal symbiont was restricted to white pine given that red pine was occasionally observed near the PD sites. Seeds of eastern white pine were surface sterilized in 30% hydrogen peroxide for 10 min, rinsed and soaked in deionized water for 24 h, and cold stratified at 4°C in sterilized peat for 60 days. Seeds of red pine were prepared as stated above, but not cold stratified. Seeds from both pine species were planted into each pot in August 2004. If a seedling did not germinate or died, pots were re-planted for up to 4 months into the study. All of the pots, including controls, were randomized by site, and placed into racks. Upon seedling germination, approximately 1 cm layer of sterile sand was added on top of the soil of each pot to allow easy identification of sporocarp production (no sporocarps were observed). Seedlings were grown in laboratory conditions at room temperature with 12 h of wide spectrum fluorescent light (94 μmol m^{-2} s^{-1}) per day and watered as necessary.

After 8 months, the seedlings were harvested for their ectomycorrhizal root tips. Not all of the pots contained seedlings; 23 pots were missing one *Pinus* species, and 13 pots had neither *Pinus* species, with six in 0.5% dilution pots and seven in 50% dilution pots. Missing data only resulted in one plot in the Keweenaw NPD site and Lewiston-1 NPD site not being represented.

Seedling root systems were placed in a 0.5 mm soil sieve, carefully rinsed with water and then viewed with a dissecting microscope. For each seedling, live root tips were removed and sorted into morphological groups (morphotypes) following Agerer (1987–2002). Particular attention was given to root tips having *Rhizopogon*-like morphology - coralloid root tips with white hyphae and rhizomorphs. A representative sample of each morphotype from each seedling, and root tips confirmed as mycorrhizal but unable to be morphotyped (e.g. early stage of development), were placed in 2X CTAB buffer and stored at 4°C. Seven of the 60 control seedlings had mycorrhizal fungi at the time of harvest. Four control seedlings were colonized by fungi not observed on any other seedlings.

Table 1 Number and type (PD, pinedrops; NPD, non-pinedrops) of study sites and plots used for collection of soil samples for the field soil bioassay with *Pinus* and out-planting of *Pterospora andromedea* (pinedrops) seed packets for the in situ seed baiting

Study sites	Number of plots		Field soil bioassay no. of soil samples		In situ seed baiting no. of seed packets	
	NPD	PD	NPD	PD	NPD	PD
Livingston-1 PD	5	3	5	3		
Livingston-2 PD	5		5			
Livingston-3 PD	5		5			
Livingston-1 NPD	5		5 (4)[a]			
Livingston-2 NPD	5		5			
Livingston-3 NPD	5		5			
Mecosta PD	5		5			
Mecosta NPD	5		5			
Emmet PD	5	4	5	4	25	20
Emmet NPD	5		5		25 (24)[b]	
Keweenaw PD	5	5	5	5	25 (24)[b]	25
Keweenaw NPD	5		5 (4)[a]		25 (0)[b]	

[a] Actual number of soil samples represented by the harvested bioassay *Pinus* seedlings

[b] Actual number of seed packets retrieved from the field

The three remaining seedlings were colonized by *Wilcoxina rehmii, Tuber* sp. 1, or the eastern *Rhizopogon*. It is presumed these seedlings were inoculated through splashing from neighboring pots during watering. It is possible that treatment seedlings were also inoculated by the eastern *Rhizopogon* through splashing, but then our low estimate of this species in soils is all the more impressive as it represents an upper value.

In situ seed baiting

Pinedrops seed packets were constructed following Rasmussen and Whigham (1993). About 100 seeds from either the Emmet or Keweenaw PD site were placed on 5× 5 cm pieces of nylon mesh with a pore size of 55 μm. The nylon mesh was folded in half and placed within a 35 mm photographic slide frame and the edges were sealed with hot glue. Seed packets were planted at the PD and NPD sites of Emmet and Keweenaw. In each of the 5×5 m plots, 5 packets were planted with one in the center, and the others in one of the plot corners (Emmet-PD: 9 plots× 5 packets=45 packets, ~ 4,500 seeds; Keweenaw-PD: 10 plots×5 packets=50 packets, ~ 5,000 seeds; Emmet and Keweenaw NPD sites: 5 plots×5 packets=25 packets, ~ 2,500 seeds per site). Seed packets were planted horizontally in the soil just below the litter layer - at least 4 cm deep. For easy retrieval of seed packets, one end of a cord was fastened to the packet and the other to a field flag.

After 1 year all of the seed packets were harvested. Seed packets out-planted at the Keweenaw NPD site were not retrievable due to selective logging activities just prior to the collection date. Also, one seed packet from the Emmet NPD site and the Keweenaw PD site were missing, and thus the respective plots had only four replicates instead of five. Harvested packets were sealed in plastic bags and stored at 4°C. The seed packets were processed over a 2 week time period.

Under a dissecting microscope, the number of seeds germinated, imbibed and desiccated was counted for each seed packet. Germinated seeds were characterized by a cracked seed coat and slight emergence of embryo from the seed coat. Seeds with turgid intact seed coats were considered imbibed and dried shriveled seeds were considered desiccated. When possible, a sub-sample of the hyphae and seed germinants from inside each packet were collected and placed in 2X CTAB buffer and stored at 4°C.

Molecular identification of fungi and plant haplotypes

DNA was extracted and amplified from pinedrops root tips, soil bioassay pine root tips and hyphae and germinants from the seed packets following Gardes and Bruns (1993). From all of the DNA extracts, the internal transcribed spacer (ITS) region of rDNA was amplified with the fungal specific primers ITS1-f and ITS4 or ITS4-b (Gardes and Bruns 1993; White et al. 1990). From the pinedrops root tip DNA extracts, the plastid *trn* L intron of DNA was amplified with the chloroplast specific primers *trn*-c and *trn*-d (Taberlet et al. 1991), a region useful for identifying haplotypes in pinedrops (Bidartondo and Bruns 2002).

The restriction endonucleases *Alu*I, *Dpn*II, and *Hin*fI were used to generate restriction fragment length poly-

morphisms (RFLPs) from the fungal PCR products only. Unique RFLP types generated from any of the samples were prepared for sequencing using the fungal primers specified above. Fungal and plant PCR products were cleaned with a Qiagen PCR Purification kit (Valencia, CA, USA). ITS1-f was used in the final sequencing reaction mix for fungal ITS sequences and *trn*-c was used for plant chloroplast sequences, and the sequences were generated at Cornell University's DNA Sequencing Facility using an Automated 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were manually edited using Sequence Editor Version 1.03 (Applied Biosystems). Sequences were subjected to a Blast search (Altschul et al. 1990) to find the closest sequence matches in the GenBank database (National Center for Biotechnology Information, GenBank; http://www.ncbi.nlm.nih.gov/blast).

To compare pinedrops' eastern and western fungal symbionts and plant haplotypes, sequences were manually aligned within an *Amylopogon* database (Bidartondo and Bruns 2002) or within a western pinedrops haplotype database (Bidartondo, unpublished data); representative haplotype sequences are published and available from GenBank: AF377248-AF377252; Bidartondo and Bruns 2002). The *Amylopogon* database contains rDNA ITS sequences from holotype and paratype *Rhizopogon* section *Amylopogon* specimens and sequences of pinedrops fungal symbionts. The pinedrops haplotype database contains plastid DNA *trn* L intron sequences of all currently known pinedrops haplotypes. Phylogenetic distance analyses were done in PAUP* Version 4.0b10 (Swofford 2002) using the neighbor-joining method. Bootstrap analyses were performed using 500 replicates with the Fast option.

Data analysis

The frequency of the fungal symbiont was calculated for each treatment of the following treatment pairs: PD and NPD sites, PD and NPD plots, eastern white pine and red pine and 0.5% and 50% soil dilutions. To determine if the fungal symbiont was associated with a treatment within the treatment pairs, Fisher's exact tests were performed. No statistical tests were suitable for analysis of the pine treatments, due to not meeting the requirement of independent samples. From the in situ seed baiting, the mean number of seeds for each of the three seed categories (germinated, imbibed and desiccated) were calculated for each plot. To test for significant differences between PD and NPD sites and PD and NPD plots within PD sites for each of the seed categories, separate two-sample t-tests were done using unpooled or separate variances. All statistics were performed in Minitab 14 (Minitab Inc, State College, PA, USA).

Results

Fungal symbiont, plant haplotype and autotrophic host

The fungi associated with the roots of 10 out of the 23 plants sampled were successfully analyzed, with two from Emmet and eight from Keweenaw. The remaining 13 plants sampled were not analyzed due to failed DNA amplification. Two fungal RFLP patterns were found: seven samples with pattern one, and three with pattern two. The two patterns were the same for enzymes *Alu*I and *Dpn*II, but slightly different for *Hin*fI (*Alu*I: 454/337, *Dpn*II: 244/242/234, *Hin*fI: pattern one 249/229/123/111 and pattern two 249/229/129/111). To determine if the two fungal RFLP patterns represent different species or intraspecific sequence variation, the sequences were compared. Less than 0.3% sequence variation was found. This suggests that the two patterns represent intraspecific variation (Kårén et al. 1997; Horton 2002), thus only one fungal species was associated with eastern pinedrops. Representative sequences for each of the RFLP patterns were submitted to GenBank (accession numbers: DQ426676, DQ426677).

Based on the GenBank Blast query and phylogenetic analyses, it was determined that this fungal symbiont is a different species than the western symbionts, *R. arctostaphyli* and *R. salebrosus*, but is a closely related *Rhizopogon* species within the subgenus *Amylopogon*. The closest GenBank sequence matches were with *Rhizopogon subbadius*, *R. subpurpurascens* and *R. salebrosus* with 96% sequence similarity (see Supplementary Table 1). The *Rhizopogon* section *Amylopogon* neighbor-joining tree in Fig. 2 shows a separate lineage for this eastern *Rhizopogon* species. This lineage also includes a sequence from pinedrops sampled from Quebec, Canada (Bidartondo, unpublished data; representative sequence from Quebec is published and available from Genbank: AF442136; Bidartondo and Bruns 2002). All of the branches with pinedrops symbionts were strongly supported with bootstrap values greater than 85.

Plant haplotypes were successfully analyzed for seven pinedrops root tip samples from Emmet, and 11 from Keweenaw. Resulting sequences were identical and the GenBank Blast query resulted in a 100% sequence match to a western pinedrops haplotype (accession number: AF377250, Bidartondo and Bruns 2002). The pinedrops haplotype neighbor-joining tree supports these findings, with the eastern pinedrops clade supported by a bootstrap value of 86, and containing some of the western samples (Supplementary Fig. 1).

An intermingling root sample collected within the root ball of a Keweenaw pinedrops was a mycorrhizal root of eastern white pine, identified based on Blast analysis (99% sequence similarity; accession number: AF479874, Whittle

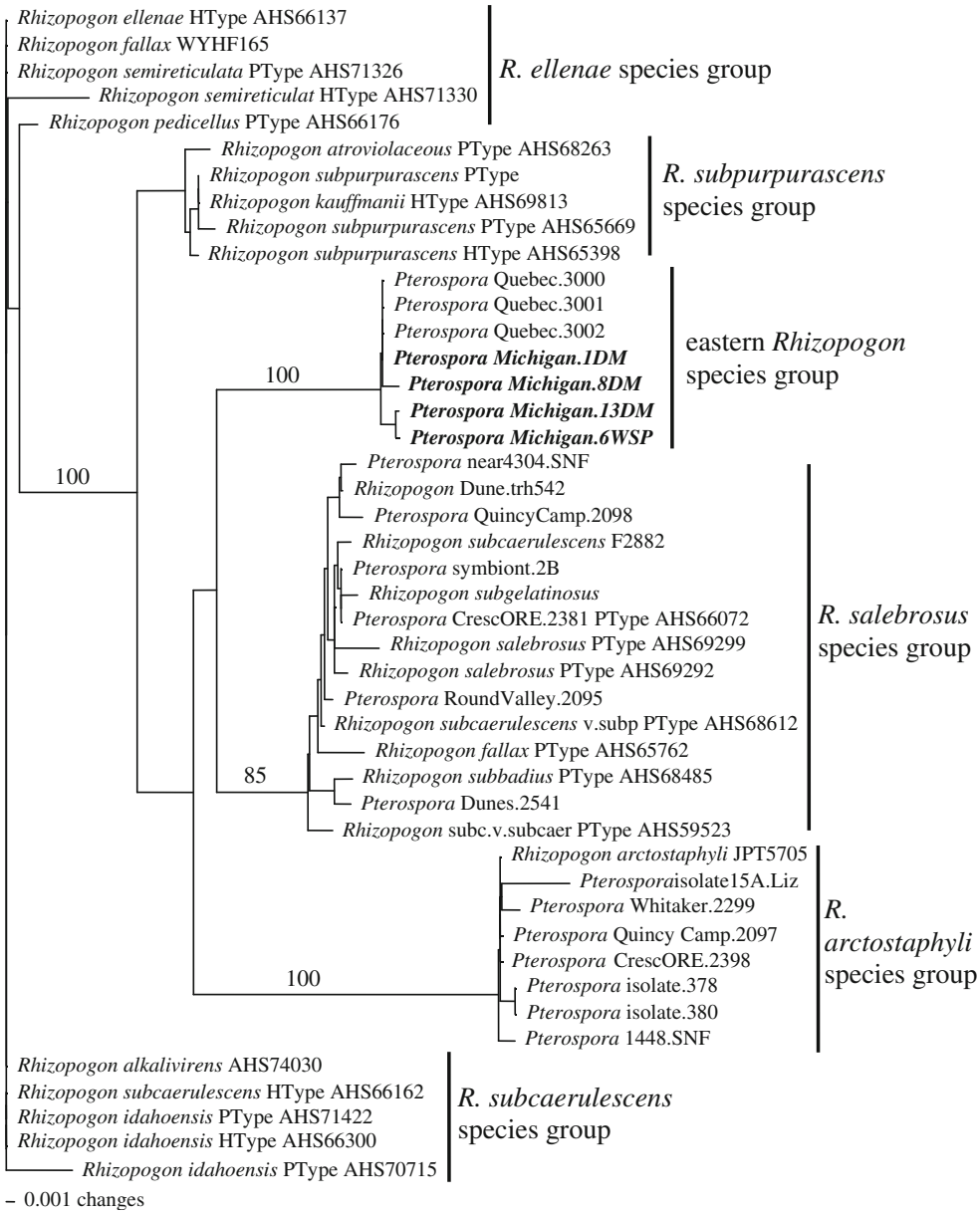

Fig. 2 *Rhizopogon* section *Amylopogon* neighbor-joining tree. Sequences are from the ITS region of rDNA. Fungal sequences from *Pterospora andromedea* (pinedrops) roots are labeled *Pterospora* followed by the location and plant number. The type specimen sequences are noted by a species name followed by HType (holotype) or PType (paratype), and GenBank accession number. The sequence labeled *Pterospora* Michigan.13DM is from the roots of a *Pinus strobus* tree that were collected within the mycorrhizal roots of pinedrops. Numbers on branches represent bootstrap values from 500 replicates. Sequence data from this study was combined with Bidartondo and Bruns (2002)

and Johnston 2002). The fungus on these roots was the eastern *Rhizopogon* species (see Fig. 2).

Field soil bioassay with pines

Out of 233 seedlings (116 eastern white pine and 117 red pine), 76% were colonized by mycorrhizal fungi. These mycorrhizal seedlings often had more than one species on their root system. A total of 355 mycorrhizal root tip samples were collected and subjected to DNA analysis. Of these samples, 67% were successfully amplified and analyzed with RFLPs. Fifty-two unique RFLP types were found and the 13 types most frequently occurring were successfully sequenced. Of these, 11 yielded GenBank matches with 97% or greater sequence similarity (Supplementary Table 1). Of the *Rhizopogon*-like morphotype samples, 88% were successfully analyzed. The *Rhizopogon*-like morphotype was found on 25 seedlings. Only 11 of these seedlings were colonized by the eastern *Rhizopogon* species. The remaining seedlings were colonized by *Suillus americanus* (see Supplementary Table 1).

Of the 233 seedlings, only 4.7% were colonized by the eastern *Rhizopogon*. If converted to the number of soil samples, 10% showed evidence that the eastern *Rhizopogon* propagules were present. There was a non-significant trend for higher frequency of eastern *Rhizopogon* in PD sites and plots than in NPD sites and plots (Table 2). The frequency of eastern *Rhizopogon* between the pine species was similar, although more frequent on eastern white pine

Table 2 Occurrence of the eastern *Rhizopogon* and association with a treatment within the soil bioassay treatment pairs. *P* value based on Fisher's exact test

Soil Bioassay Treatment	Proportion	Frequency	*P* value
PD site	6/42 soil samples	0.14	0.23
NPD site	1/28 soil samples	0.04	
PD plot	3/12 soil samples	0.25	0.09
NPD plot	4/58 soil samples	0.07	
Pinus strobus	7/116 seedlings	0.06	–
Pinus resinosa	4/117 seedlings	0.03	
0.5% soil dilution	5/66 pots	0.08	0.44
50% soil dilution	2/65 pots	0.03	

(Table 2). There was no significant effect of soil dilution on eastern *Rhizopogon* frequency (Table 2).

In situ seed baiting

Of the estimated 12,000 out-planted pinedrops seeds, a total of 6,598 seeds were retrieved from the packets, with 1097 from Emmet-NPD, 3055 from Emmet-PD, and 2382 from Keweenaw-PD. Of these seeds, 0.97% germinated, 56% were imbibed, and 43% were desiccated. Of the 118 packets retrieved, only 12.7% contained germinants, with the minimum of one and maximum of 16 germinants in any one packet. The germinants found were all at the very beginning stages of germination and were characterized by a cracked seed coat and slight emergence of the embryo from the seed coat. All packets with germinants came from PD sites, with five germinants in two packets from Emmet, and 59 germinants in 13 packets from Keweenaw. For the imbibed and desiccated seed categories, statistically significant differences were found between PD and NPD sites with a higher mean for imbibed seeds in PD sites, and a higher mean for desiccated seeds in the NPD sites (Table 3). Within the PD sites, six of the 15 packets were from PD plots, although no statistically significant differences were found between PD and NPD plots for each of the seed categories (Table 4).

Attempts to amplify fungal DNA from seed germinants found in the seed packets were unsuccessful. The DNA from hyphal samples taken within the seed packets was successfully amplified, but samples contained more than one fungal species; fungal identification was not pursued further.

Discussion

Fungal symbionts

Most myco-heterotrophic plants are highly specific in their fungal associations (Hynson and Bruns 2010); pinedrops is not an exception to this extreme specificity. In this study only one fungal symbiont was found to associate with eastern pinedrops, and although distinctly different from the western fungal symbionts, they are closely related species, as shown by the phylogenetic analysis. The western pinedrops haplotype is also associated with the eastern *Rhizopogon* species and the western fungal associates are apparently missing in eastern forests.

Roots of pinedrops sampled from three sites separated by hundreds of kilometers (Quebec, southern Michigan and northern Michigan) all yielded the same fungal symbiont, a potentially new *Rhizopogon* species closely related to, but distinct from, those in the *R. arctostaphylii* and *R. salebrosus* species groups. Bioassays with pine planted in soil collected from 12 sites, six in New York and six in Michigan, yielded only one *Rhizopogon* species, the eastern symbiont. However, we cannot rule out the possibility of other species associating with eastern pinedrops. Unfortunately, no pinedrops could be sampled from New York due to the absence of mature plants during the time frame of this study. Also, pinedrops in the remaining New England sites (New Hampshire and Vermont) were not sampled due to their strict protection. While additional *Rhizopogon* spp. may be found in the East with additional sampling, it is likely the number of

Table 3 Difference between sites with pinedrops (PD) and without pinedrops (NPD) for each seed category in *Pterospora andromedea* (pinedrops) seed packets placed in the field for one year. *P* value based on *t*-test

Seed category	Site treatment	No. of plots	Mean	SE	*P* value
Germinated	PD	19	1.1	0.4	–
	NPD	5	0.0	0.0	
Imbibed	PD	19	66.7	6.5	0.01
	NPD	5	39.7	5.8	
Desiccated	PD	19	31.8	6.8	0.01
	NPD	5	70.5	8.8	

Table 4 Difference between pinedrops sites' plots with pinedrops (PD) and without pinedrops (NPD) for each seed category in *Pterospora andromedea* (pinedrops) seed packets placed in the field for one year. *P* value based on *t*-test

Seed category	Plot treatment	No. of plots	Mean	SE	*P* value
Germinated	PD	9	0.9	0.5	0.30
	NPD	10	1.3	0.6	
Imbibed	PD	9	65.6	10.1	0.44
	NPD	10	67.6	9.0	
Desiccated	PD	9	32.6	10.1	0.46
	NPD	10	31.2	9.5	

species and their abundance will remain low compared to the Pacific Northwest.

Plant haplotypes

Only one pinedrops plastid DNA haplotype was found. This haplotype is not unique, nor is it uncommon in the West (Bidartondo, personal communication). However, the current sample size is limited and it cannot be ruled out that more haplotypes are present in the East today.

Eastern *Rhizopogon* is relatively rare in the spore bank

Only 11% of the field soil bioassay seedlings had mycorrhizal root tips with the *Rhizopogon*-like morphology, with 6% of the mycorrhizal seedlings colonized by the eastern *Rhizopogon*. Kjøller and Bruns (2003) found a *Rhizopogon*-like morphology on 50% of their California *Pinus muricata* soil bioassay seedlings. In their Sierra National Forest site, where pinedrops are common, root tip isolates of *R. salebrosus* and *R. arctostaphylii* had frequencies of 20% and 50%, respectively. The fungal symbiont of pinedrops is less frequent as resistant spores in the soils of sampled eastern white pine forests than sampled western forests. In eastern forests, resistant spores of the eastern *Rhizopogon* were more frequently encountered where pinedrops occurred. Only one of the soil samples from the NPD sites yielded pine seedlings colonized by the eastern *Rhizopogon* species. Further, within the PD sites, plots containing pinedrops yielded higher frequencies of white pine colonized by the eastern *Rhizopogon* spores than the NPD plots. These results suggest that eastern pinedrops rarity is influenced by the distribution and rarity of its fungal symbiont.

Many of the pine-associated fungi that dominate the spore banks observed here (*Rhizopogon*, *Suillus*, *Tuber*, *Wilcoxina* and *Cenococcum*) are common dominants on roots of other pine species in disturbed sites and in bioassays experiments (Deacon and Fleming 1992; Visser 1995; Baar et al. 1999; Yu et al. 2001; Ashkannejhad and Horton 2006; Izzo et al. 2006), but not in relatively undisturbed settings (Gardes and Bruns 1996; Horton et al. 1998; Taylor and Bruns 1999b). These fungi are thought to be adapted to establishment from spore banks following disturbance rather than competing with other fungi in mature forests through mycelial networks. The life history of such disturbance fungi has been compared to the ruderal strategy of plant life histories (Grimes 1977; Dighton and Mason 1984; Last et al. 1987).

If the eastern *Rhizopogon* is a disturbance species, causes for its rarity in the spore bank could be related to the decline in disturbance in eastern white pine forests. Many of these forests were heavily logged in the past (Karamanski 1989; Leahy and Pregitzer 2003), and presumably *Rhizopogon* may have been more abundant in the first decades of post-harvest stand development. Some eastern white pine forests are still being logged today. For example, the Keweenaw PD site was selectively logged for hardwoods about 5 years prior to this study. Fortunately, the eastern white pines at these sites were not logged. Interestingly, the pinedrops occurrence at this site is the highest we have observed in eastern forests, with over 40 inflorescences observed in 2004 (Hazard, personal observation). High levels of flowering following disturbance have been observed in the snowplant (*Sarcodes sanguinea*), another myco-heterotrophic species related to pinedrops that is also associated with *Rhizopogon* spp (Bruns et al. 2002b). Perhaps the disturbance from logging aided eastern *Rhizopogon* early in the last century.

The eastern *Rhizopogon* was found colonizing both eastern white pine and red pine seedlings. Results from laboratory experiments may indicate a broader host range of fungi than field-based studies, leading to the concept of ecological specificity (Harley and Smith 1983; Molina et al. 1992). Evidence supports the specificity of the eastern *Rhizopogon* for eastern white pine in nature. Eastern pinedrops are consistently associated with eastern white pine and not other pine species (Hinds 2000; Schori 2002; Hazard 2006).

Eastern *Rhizopogon* in the vegetative state

From the in situ seed baiting experiment, 13% of the pinedrops seed packets had germinated seeds but none had evidence of a functioning mycorrhizal symbiosis. Whether these germinants would have developed mycorrhizal root

systems is unknown. A study by Bidartondo and Bruns (2005) resulted in 10% of their pinedrops seed packets containing seeds with cracked seed coats, 5% with emergent embryos, and 1% developing into mycorrhizal root systems.

We observed only a few germinants in any one packet. The seed packets containing germinants in the California study by Bidartondo and Bruns (2005) had high percentages (~50%) of germinated seeds (Bidartondo, personal communication). Having few germinants could be attributed to several variables such as low seed viability, poor environmental conditions, insufficient time given for germination, and poor fungal symbiont vigor. However, given the specificity requirements for germination observed in western pinedrops (Bruns and Read 2000; Bidartondo and Bruns 2005), we feel the simplest explanation is that mycelial networks produced by the eastern *Rhizopogon* were rare in our plots and that germination was stimulated by suilloids (see Bruns and Read 2000).

Conclusions

Only one pinedrops haplotype and fungal symbiont was found in this study. The fungal symbiont was an undescribed species in the genus *Rhizopogon* subgenus *Amylopogon*. No GenBank sequences, sporocarp herbarium records, or published literature on this fungus could be found. Attempts were made to locate sporocarps in the field sites, but none were recovered. This was not surprising due to the hypogeous nature of the sporocarp, sporadic fruiting, removal of most sporocarps by mammals (North et al. 1997) and the putative rarity of this species in eastern forests. This fungal species was not frequent in the soil spore banks of eastern white pine forests with or without pinedrops, but was less frequent in the latter. In comparison, the fungal symbiont of pinedrops was less frequent in the soils of sampled eastern white pine forests than previously sampled western forests. These results suggest that the rarity of the fungal symbiont is a contributing factor to the rarity of eastern pinedrops. Strategies to conserve and manage for eastern pinedrops and other myco-heterotrophic plants of concern need to consider the status of the fungal symbiont in order for efforts to be successful.

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References

Agerer R (ed) (1987–2002) Colour atlas of ectomycorrhizae. Einhorn-Verlag, Germany

Ashkannejhad S, Horton TR (2006) Ectomycorrhizal ecology under primary succession on coastal sand dunes: interactions involving *Pinus contorta*, suilloid fungi and deer. New Phytol 169:345–354

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Baar J, Horton TR, Kretzer AM, Bruns TD (1999) Mycorrhizal colonization of *Pinus muricata* from resistant propagules after a stand-replacing wildfire. New Phytol 143:409–418

Bakshi TS (1959) Ecology and morphology of *Pterospora andromedea*. Bot Gaz 120:203–217

Barrett CF, Freudenstein JV, Taylor DL, Kõljalg U (2010) Rangewide analysis of fungal mycoheterotrophic *Corallorhiza striata* complex (Orchidaceae) reveals extreme specificity on ectomycorrhizal *Tomentella* (Thelephoraceae) across North America. Am J Bot 97:628–643

Bidartondo MI, Bruns TD (2001) Extreme specificity in epiparasitic Monotropoideae (Ericaceae): widespread phylogenetic and geographical structure. Mol Ecol 10:2285–2295

Bidartondo MI, Bruns TD (2002) Fine-level mycorrhizal specificity in the Monotropoideae (Ericaceae): specificity for fungal species groups. Mol Ecol 11:557–569

Bidartondo MI, Bruns TD (2005) On the origins of extreme mycorrhizal specificity in the Monotropoideae (Ericaceae): performance trade-offs during seed germination and seedling development. Mol Ecol 14:1549–1560

Björkman E (1960) *Monotropa hypopitys* L. – an epiparasite on tree roots. Physiol Plant 13:308–327

Bougoure J, Ludwig M, Brundrett M, Grierson P (2009) Identity and specificity of the fungi forming mycorrhizas with the rare mycoheterotrophic orchid *Rhizanthella gardneri*. Mycol Res 113:1097–1106

Bruns TD, Bidartondo MI, Taylor DL (2002a) Host specificity in ectomycorrhizal communities: what do the exceptions tell us? Integr Comp Biol 42:352–359

Bruns TD, Kretzer A, Horton TR, Stendell ER, Bidartondo MI, Szaro TM (2002b) Current investigations of fungal ectomycorrhizal communities in the Sierra National Forest. In: Verner J (ed) Proceedings of a symposium on the Kinds River sustainable forest ecosystems project: progress and current status. Pacific Southwest Research Station, Forest Service, USDA, pp 83–89

Bruns TD, Read DJ (2000) *In vitro* germination of nonphotosynthetic, myco-heterotrophic plants stimulated by fungi isolated from the adult plants. New Phytol 148:335–342

Cullings KW, Szaro TM, Bruns TD (1996) Evolution of extreme specialization within a lineage of ectomycorrhizal epiparasites. Nature 379:63–66

Deacon JW, Fleming LV (1992) Interactions of ectomycorrhizal fungi. In: Allen MF (ed) Mycorrhizal functioning, an integrative plant-fungal process. Chapman and Hall, New York, USA, pp 249–300

Dighton J, Mason PA (1984) Mycorrhizal dynamics during forest tree development. In: Moore D, Casselton LA, Wood DA, Frankland JC (eds) Developmental biology of higher fungi. Cambridge University Press, UK, pp 253–268

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Gardes M, Bruns TD (1996) ITS-RFLP matching for the identification of fungi. In: Clapp JP (ed) Methods in molecular biology 50: Species diagnostic protocols: PCR and other nucleic acid methods. Humana Press Inc., USA, pp 177–186

Grimes JP (1977) Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. Am Nat 111:1169–1194

Harley JL, Smith SE (1983) Mycorrhizal Symbiosis. Academic, USA

Hazard C (2006) Investigations into the mycorrhizal ecology of *Pterospora andromedea*. Master Thesis, State University of New York – Environmental Science and Forestry, USA

Hinds HR (2000) Flora of New Brunswick, A manual for the identification of the vascular plants of New Brunswick, 2nd edn. University of New Brunswick, Fredericton, New Brunswick, Canada

Horton TR (2002) Molecular approaches to ectomycorrhizal diversity studies: variation in ITS at a local scale. Plant Soil 244:29–39

Horton TR, Cázares E, Bruns TD (1998) Ectomycorrhizal, vesicular-arbuscular and dark septate fungal colonization of bishop pine (*Pinus muricata*) seedlings in the first 5 months of growth after wildfire. Mycorrhiza 8:11–18

Hynson NA, Bruns TD (2010) Fungal hosts for mycoheterothophic plants: a nonexclusive, but highly selective club. New Phytol 185:598–601

Izzo A, Nguyen DT, Bruns TD (2006) Spatial structure and richness of ectomycorrhizal fungi colonizing bioassay seedlings from resistant propagules in a Sierra Nevada forest: comparisons using two hosts that exhibit different seedling establishment patterns. Mycologia 98:374–383

Johnson NC, Graham JH, Smith FA (1997) Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytol 135:575–585

Jones MD, Smith SE (2004) Exploring functional definitions of mycorrhizas: Are mycorrhizas always mutualisms? Can J Bot 82:1089–1109

Karamanski TJ (1989) Deep woods frontier: A history of logging in northern Michigan. Wayne State University Press, Michigan, USA

Kårén O, Högberg N, Dahlberg A, Jonsson L, Nylund J-E (1997) Inter- and intraspecific variation in the ITS region of rDNA of ectomycorrhizal fungi in Fennoscandia as detected by endonuclease analysis. New Phytol 136:313–325

Kjøller R, Bruns TD (2003) *Rhizopogon* spore bank communities within and among California pine forests. Mycologia 95:603–613

Kretzer AM, Bidartondo MI, Grubisha LC, Spatafora JW, Szaro TM, Bruns TD (2000) Regional specialization of *Sarcodes sanguinea* (Ericaceae) on a single fungal symbiont from the *Rhizopogon ellenae* (Rhizopogonaceae) species complex. Am J Bot 87:1778–1782

Last FT, Dighton J, Mason PA (1987) Successions of sheathing mycorrhizal fungi. Trends Ecol Evol 2:157–161

Leahy MJ, Pregitzer KS (2003) A comparison of presettlement and present-day forests in northeastern lower Michigan. Am Midl Nat 149:71–89

Leake JR (1994) Tansley Review No. 69. The biology of mycoheterotropic ('saprophytic') plants. New Phytol 145:523–537

Merckx V, Freudenstein JV (2010) Evolution of mycoheterotrophy in plants: a phylogenetic perspective. New Phytol 185:605–609

McKendrick SL, Leake JR, Taylor DL, Read DJ (2000) Symbiotic germination and development of myco-heterotrophic plants in nature: ontogeny of *Corrallorhiza trifida* and characterization of its mycorrhizal fungi. New Phytol 145:523–537

Molina R, Massicotte H, Trappe JM (1992) Specificity phenomena in mycorrhizal symbioses: Community ecology consequences and practical implications. In: Allen MF (ed) Mycorrhizal functioning: An integrative plant-fungal process. Chapman and Hall, New York, USA, pp 357–423

Molina R, Trappe JM (1994) Biology of the ectomycorrhizal genus, Rhizopogon. I. Host associations, host-specificity and pure culture syntheses. New Phytol 125:653–675

North M, Trappe J, Franklin J (1997) Standing crop and animal consumption of fungal sporocarps in Pacific Northwest forests. Ecology 78:1543–1554

Rasmussen HN, Whigham DF (1993) Seed ecology of dust seeds in situ: a new study technique and its application in terrestrial orchids. Am J Bot 80:1374–1378

Schori A (2002) *Pterospora andromedea* Nutt. (Pinedrops) New England Plant Conservation Program, Conservation and Research Plan for New England. New England Wildflower Society, ME, USA

Swofford DL (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods), Version 4. Sinauer Associates, MA, USA

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 17:1105–1109

Taylor DL, Bruns TD (1999a) Population, habitat and genetic correlates of mycorrhizal specialization in the 'cheating' orchid *Corallorhiza maculata* and *C. mertensiana*. Mol Ecol 8:1719–1732

Taylor DL, Bruns TD (1999b) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. Mol Ecol 8:1837–1850

Taylor DL, Bruns TD, Leake JR, Read DJ (2002) Mycorrhizal specificity and function in myco-heterotrophic plants. In: van der Heijden MGA, Sanders I (eds) Mycorrhizal Ecology. Springer, Germany, pp 375–413

Thompson JN (1994) The coevolutionary process. University of Chicago Press, IL, USA

Trappe JM (1977) Selection of fungi for ectomycorrhizal inoculation in nurseries. Annu Rev Phytopathol 15:203–222

Yu TE J-C, Egger KN, Peterson LR (2001) Ectendomycorrhizal associations - characteristics and functions. Mycorrhiza 11:167–177

Visser S (1995) Ectomycorrhizal fungal succession in jack pine stands following wildfire. New Phytol 129:389–401

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols – a guide to methods and applications. Academic, USA, pp 315–322

Whittle CA, Johnston MO (2002) Male-driven evolution of mitochondrial and chloroplastidial DNA sequences in plants. Mol Biol Evol 19:938–949

Yagame T, Yamato M, Suzuki A, Iwase K (2008) Ceratobasidiaceae mycorrhizal fungi isolated from nonphotosynthetic orchid *Chamaegastrodia sikokiana*. Mycorrhiza 18:97–101