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No evidence that nitrogen enrichment affect fungal communities of *Vaccinium* roots in two contrasting boreal forest types

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ABSTRACT

In boreal forests ericaceous shrubs often dominate the forest floor vegetation. Nitrogen enrichment has been shown to decrease shrub abundance and in this study we explored whether it also affects the root associated fungal communities. Fine roots of *Vaccinium myrtillus* were collected in a Norway spruce dominated forest and of *Vaccinium vitis-idaea* in a Scots pine dominated forest. In both forests, nitrogen enrichment was experimentally induced by adding 12.5 and 50 kg N ha^{-1} yr^{-1} for 12 (spruce forest) and four (pine forest) years. Based on terminal restriction fragment length polymorphisms, subcloning and sequencing analyses, the root associated fungal communities were examined. We found 93 fungal species including Asco-, Basidio- and Zygo-mycota. In general, the *Rhizoscyphus ericae* aggregate was the most dominant and this was followed by Herpotrichiellaceae and *Sebacina*. Ordination analysis revealed that nitrogen enrichment did not change species composition of the fungal communities in neither the spruce nor the pine forest, while fungal community structures were clearly discriminated between the dominant shrub species in each forest. Similarly, no fungal species showed a significant response to nitrogen enrichment. Therefore, nitrogen enrichment appears to have no effect on root associated fungi of understorey dwarf shrubs in boreal forests, while it is clear that spruce and pine forests harbor distinctive communities of these fungi.

1. Introduction

In boreal forests, ericaceous dwarf shrubs are an important component of the understorey vegetation. Among these dwarf shrubs, *Vaccinium myrtillus* and *Vaccinium vitis-idaea* are two keystone species in north Europe and Eurasia (Hultén and Fries, 1986). The two species often co-occur but normally become dominant in different forest types as the deciduous *V. myrtillus* is common in relatively more fertile and mesic sites than the ever-green *V. vitis-idaea*. Fungal colonization of the ericaceous roots contributes to the success of the plants as ericoid mycorrhizal fungi (ERF) have been shown to facilitate plant access to complexly bound organic nitrogen (Bajwa and Read, 1985; Bajwa et al., 1985; Leake and Read, 1990; Sokolovski et al., 2002) as well as mineral nitrogen (Kosola et al., 2007).

Like for other terrestrial ecosystems, atmospheric deposition of anthropogenic nitrogen may alter biogeochemical cycles as well as ecosystem structure and function in boreal forests (Gilliam, 2006). It has been demonstrated that the abundance of *Vaccinium* in the understorey vegetation may decrease in response to nitrogen addition (Strengbom et al., 2002; Nordin et al., 2005; Manninen et al., 2009). Since soil nitrogen enrichment is known to have a negative effect on mycorrhizal colonization and diversity (Wallenda and Kottke, 1998 and references therein; Lilleskov et al., 2002), which would affect water and nutrient absorption in host plants (Baxter and Dighton, 2001; Jonsson et al., 2001; van der Heijden and Kuyper, 2001; Hasselquist et al., 2005), the nitrogen induced decline of *Vaccinium* may be correlated to decreased colonization of symbiotic fungi.

For many species of ectomycorrhizal fungi in boreal forests, the effect of nitrogen enrichment is generally negative. Nitrogen enrichment has been found to reduce the abundance and species diversity of aboveground fruiting bodies (Wallenda and Kottke, 1998 and references therein; Peter et al., 2001; Strengbom et al., 2001). Belowground, nitrogen enrichment normally decreases the amount of extramatrical mycelia as well as species diversity of the fungi associated with the roots (Kårén and Nylund, 1997; Lilleskov et al., 2002; Nilsson and Wallander, 2003). In contrast to the numerous studies of nitrogen enrichment effects on ectomycorrhizal fungi, there are only few studies of ERF responses to nitrogen. Moore-parkhurst and Englander (1982) demonstrated that high nitrogen supply to ericaceous plants grown in a greenhouse resulted in declined fungal colonization of the roots. In the field no such effects have been found in response to experimental nitrogen additions (Caporn et al., 1995; Johansson, 2000; Urcelay et al., 2003).

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These results were based on the total fungal abundance as measured by fungal coils in host roots or a fungal cell wall specific chemical (ergosterol). However, as revealed for ectomycorrhizal fungi (e.g., Lilleskov et al., 2002), ERF responses to nitrogen may differ between fungal species. Therefore species identification is needed to infer the effect of nitrogen enrichment on ERF.

Newly developed molecular fingerprinting techniques such as restriction fragment length polymorphisms (RFLP) and denaturing gradient gel electrophoresis (DGGE) allow detection of root associated fungi without culturing (e.g., Allen et al., 2003; Bougoure and Cairney, 2005a,b). These studies have indicated that fungal species composing ERF are more diversified than previously thought (Cairney and Meharg, 2003), and a substantial number of species are shared by non-ericaceous plants (Villarreal-Ruiz et al., 2004; Zijlstra et al., 2005; Selosse et al., 2007; Upson et al., 2007). Although these studies have contributed to reveal the root associated fungi in ericaceous plants, the degree of infection by each fungus remains unclear, since relatively small numbers of environmental DNA samples were analyzed in each study. Because morphological characteristics and known bioindicators such as phospholipid fatty acids (PLFA) are not variable enough for the discrimination of ERF species, DNA fingerprinting is the only available technique to determine them. In ericaceous roots, fungal colonization is normally heavy. Studies indicate that substantial parts of the hair roots are infected (Johansson, 2000; Davies et al., 2003; Urcelay et al., 2003), and several fungi sympatrically colonize a small area of a root (Monreal et al., 1999; Allen et al., 2003). Hence, to determine the community structure of ERF, large number of small root fragments must be analyzed.

Using this sampling strategy, the aim of the current study was to investigate mainly the effect of nitrogen addition, but also effects of forest type and host plant species on colonization and species composition of the fungal community of dwarf-shrub roots in two contrasting boreal forests (Norway spruce and Scots pine dominated) in northern Sweden.

2. Materials and methods

2.1. Study sites

The study was done within the Svartberget experimental forest (64°14′N, 19°46′E, 175 m a.s.l.), 70 km from the Gulf of Bothnia in the province of Västerbotten, northern Sweden. The background atmospheric nitrogen deposition for the area is estimated to be $\leq$3 kg N ha^{-1} yr^{-1} (which is low compared to regions further south in Sweden) and average annual precipitation is 583 mm (1997–2000) (Phil-Karlsson et al., 2003; Nordin et al., 2006).

The Norway spruce (*Picea abies* (L.) Karst.) dominated site is a mesic late successional forest (tree age *ca.* 100 years) with a closed tree canopy. The soil is a well-developed iron podzol of till with an organic layer of *ca.* 20 cm. The understorey vegetation is dominated by ericaceous dwarf shrubs, mainly *V. myrtillus* L. (*ca.* 80% of the field-layer canopy), but also *V. vitis-idaea* L. (*ca.* 10% of the field-layer canopy) and some *Deschampsia flexuosa* (L.) Trin. The bottom layer is dominated by *Hylocomium splendens* (Hedw.) B.S.G. and *Pleurozium schreberi* (Brid) Mitt. The Scots pine (*Pinus sylvestris* L.) dominated site, located *ca.* 1 km from the Norway spruce dominated site, is a dry late successional forest (tree age *ca.* 70 years) with a relatively open tree canopy. The soil is a weakly podzolized sediment of sandy silt with an organic layer of *ca.* 10 cm. The field layer is dominated by *V. vitis-idaea* (*ca.* 80% of the field-layer canopy) and some *Calluna vulgaris* (L.) Hull. (*ca.* 20% of the field-layer canopy). The bottom layer is dominated by lichens (*Cladina rangiferina* (L.) Weber ex Wigg and *Cladina arbuscula* (Wallr.) Hale and Culb.) and *P. schreberi*.

At both forest sites, long-term nitrogen addition treatments of 0 (Control), 12.5 (N1) and 50 (N2) kg N ha^{-1} yr^{-1} were applied to experimental plots sized 1000 (pine site) or 2500 (spruce site) m^2. In the pine forest the plots were of quadratic shape and the distance between plots were 1 m. To avoid spill-over effects from nitrogen addition between plots, all sampling was done at least 5 m from plot borders. In the spruce forest the distances between the rectangular plots (71 × 35 m) were larger than in the pine forest, but sampling was still done at least 10 m from plot borders. Also, in the spruce forest there are no permanent control plots. However, the experimental forest has a homogenous structure and between the randomly placed nitrogen treated plots there are large left-over areas. Thus control samples were collected outside the nitrogen treated plots over five separate areas of the same size as the nitrogen treated plots, and at least 10 m away from plot borders. In both forests, the nitrogen addition treatments were replicated six–seven times ($n = 6$–7). The amount of nitrogen addition in the N1 treatment is similar to the atmospheric nitrogen deposition in southern Sweden (available at http://frodo.smhi.se/website/MATCH_mo_tdnt/main.htm). The N2 treatment was at the start of the experiment chosen to explore ecosystem response of a nitrogen load that within a relatively short time period (<5 years) may cause significant effects. In the long-term the N2 treatment has been found as a good complement to the N1 treatment since it may give an early indication of effects that will appear in N1 plots later on (Nordin et al., in press). Nitrogen was added to plots by spreading (by hand) granulated NH_4NO_3 right after snow melt in late May or early June each year. In the spruce forest nitrogen additions had been going on for 12 years, and in the pine forest for four years, at the time when field sampling was done.

The long-term nitrogen addition treatments had caused significant changes to the understorey vegetation at the time of the current study. In the spruce forest *D. flexuosa* had increased, and at least on N2 plots it had become co-dominant with *V. myrtillus*, while *V. vitis-idaea* had nearly disappeared. Moreover, nitrogen addition had caused increased occurrence of leaf pathogenic fungi and of herbivorous insects on *V. myrtillus*, causing mid- to late summer defoliation of this plant at least some years (see Nordin et al., in press for a complete report on vegetation dynamics during the 12 year experimental period). In the year of the current study, defoliation of *V. myrtillus* was *ca.* 10% in controls and *ca.* 40% in both N1 and N2 plots (Nordin et al., in press). In the pine forest the nitrogen induced vegetation changes had been less dramatic than in the spruce forest and was mainly manifested as a slightly increased abundance of *V. vitis-idaea* (A. Nordin unpublished data). No major pathogen or herbivore damage had been inferred to *V. vitis-idaea* irrespective of the nitrogen treatments (A. Nordin unpublished data).

2.2. Sampling of Vaccinium roots

Root sampling was performed from late August to early September in 2007. To see the effect of nitrogen addition on root associated fungi, we collected the roots of the dominant dwarf-shrub in each forest. In the spruce forest we randomly sampled 9–11 lateral root systems (*ca.* 10 cm, >5 m apart from each other) of *V. myrtillus* from each nitrogen treatment ($n = 5$ for Control and $n = 6$ for N1 and N2; Table 1). Roots were excavated by tracing from aboveground parts of the plants to discriminate between dwarf-shrub species. In the pine forest, *V. vitis-idaea* roots were collected in a similar manner with 9–11 root samples excavated from each control ($n = 6$), N1 ($n = 6$) and N2 ($n = 7$) plot (Table 1).

To explore the effect of dwarf-shrub species on root associated fungal communities in the spruce forest on control plots, we collected the roots of both *V. myrtillus* and *V. vitis-idaea* ($n = 6$; Table 1). However, we did not collect the roots of both dwarf-shrub

Table 1
Summarized fungal communities of the *Vaccinium* roots in two boreal forest types (spruce and pine dominated) with three different nitrogen treatments: 0 kg N ha^{-1} yr^{-1} (Control), 12.5 kg N ha^{-1} yr^{-1} (N1) and 50 kg N ha^{-1} yr^{-1} (N2). Total number of species and percent of the root samples colonized (parenthesis) are shown. Full species list is in Appendix 1.

Host, forest type, treatment	Number of studied plots/total number of samples	Total species richness (minimum root infection (%))		Ascomycota						Basidiomycota				Zygomycota	Unidentified
		All inclusive	Assumed ERF*	*Rhizoscyphus* aggregate*	*Cadophora**	*Phialocephala* complex[a,*]	Other Helotiales*	Herpotrichiellaceae	Others[b]	Atheliaceae[c]	*Sebacina**	Other ectomycorrhizas[d]	Others[e]	*Mortierella*	
V. myrtillus in spruce forest															
Control	5/48	44 (94)	19 (79)	5 (42)	4 (13)	2 (21)	5 (27)	2 (27)	8 (23)	2 (10)	3 (26)	5 (11)	1 (4)	5 (25)	1 (2)
N1	6/60	52 (95)	21 (81)	6 (36)	6 (16)	2 (28)	5 (22)	2 (44)	7 (24)	3 (13)	2 (20)	4 (7)	2 (4)	5 (23)	4 (5)
N2	6/55	42 (93)	19 (78)	5 (35)	5 (23)	1 (27)	6 (23)	3 (37)	7 (15)	2 (6)	2 (13)	2 (7)	1 (2)	4 (10)	1 (3)
Sub total	17/163	71 (94)	26 (79)	6 (37)	7 (18)	2 (25)	8 (24)	3 (37)	11 (21)	3 (10)	3 (19)	9 (8)	3 (3)	6 (19)	4 (4)
V. vitis-idaea in spruce forest															
Control	6/59	45 (93)	25 (90)	6 (20)	5 (9)	3 (17)	7 (27)	2 (19)	3 (10)	2 (12)	4 (68)	2 (3)	3 (7)	5 (16)	1 (2)
V. vitis-idaea in pinus forest															
Control	6/57	24 (98)	16 (89)	3 (57)	5 (35)	2 (7)	3 (30)	2 (47)	2 (17)	1 (10)	3 (31)	0	2 (3)	1 (2)	0
N1	6/57	46 (97)	26 (95)	5 (69)	8 (25)	3 (16)	7 (30)	2 (47)	6 (21)	1 (7)	3 (25)	2 (4)	3 (5)	2 (4)	2 (4)
N2	7/69	39 (97)	18 (91)	3 (68)	6 (29)	3 (13)	5 (27)	3 (33)	6 (29)	1 (10)	1 (28)	1 (1)	3 (6)	3 (13)	1 (1)
Sub total	19/183	60 (97)	28 (92)	5 (65)	8 (30)	3 (12)	9 (29)	3 (42)	8 (23)	1 (9)	3 (28)	3 (2)	5 (5)	4 (7)	3 (2)
Ground total	42/405	93	32	6	8	3	11	5	13	3	4	11	7	8	5

*Analyzed as assumed ERF in this study.
[a] *Phialocephala*, *Acephala* and *Mollisia*.
[b] Including Botryosphaeriales, *Penicillium*, *Candida*, *Galactomyces*, *Hypocrea*, *Cladosporium*, *Debaryomyces*, and unknown Ascomycota.
[c] Including *Amphinema* and *Piloderma*, others are included in other Basidiomycota.
[d] Including *Cortinarius*, *Russula*, *Sistoterma* and Thelephorales.
[e] Unknown Atheliaceae, *Mycena*, *Rhizoctonia* and *Rhodotorula*, Tremellales and unknown Basidiomycota.

species from any of the nitrogen treated plots in the spruce forest (due to insufficient abundance of *V. vitis-idaea*) or from any of the plots in the pine forest (due to insufficient abundance of *V. myrtillus*).

In total across the two forests and three treatments, our sampling strategy resulted in 414 root samples. The samples were put in separate plastic bags with adhering soil and kept in 4 °C (for a month) until further analyses. The effect of the length of the storage period on abundance of fungal species on the roots was tested statistically and found to have no ($P > 0.05$) significant effect.

2.3. Preparation of DNA and genotyping

A small piece (*ca.* 2 mm) of hair root from each sample was washed in an ultrasonicator and further stored at −20 °C. Extraction of DNA was performed using the method described by Nara et al. (2003). Briefly, each sample was pulverized in a 2 ml tube containing a zirconia ball using a homogenizer and subjected to the modified cetyltrimethylammonium bromide (CTAB) method (Nara et al., 2003). The extracted DNA was dissolved in a 50 μl of 0.1× TE buffer. Negative controls, in which root fragments were not pulverized, were used to detect contaminants amplified in the PCR.

2.4. Terminal restriction fragment length polymorphisms (T-RFLP) analysis

The rDNA internal transcribed spacer (ITS) regions were amplified using 5 μl of Multiplex PCR kit (Qiagen, Hilden, Germany), 2 μl of extracted DNA and 200 nM of the ITS1F primer (Gardes and Bruns, 1993) with a 5′ D2 fluorescent label (Sigma–Aldrich, Sweden) and each 100 nM of labeled (5′ D3 fluorescent) and non-labeled ITS4 primers (White et al., 1990). Mixing of labeled and non-labeled primer was to equalize the fluorescent strength between D2 and D3. Thermal cycling program was repeated for 40 cycles followed by the manufacture's instruction of Multiplex PCR kit.

We incubated 2 μl of each PCR product with 1 U of either *Hin*fI or *Alu*I (Fermentas, St. Leon-Rot, Germany, or New England Biolabs, Herts, UK) in the total of 10 μl reaction mixture. We mixed 1 μl of the digested products in a 25 μl of purified formamide (SLS; Beckman Coulter, Fullerton, CA, USA) with 0.31 μl of CEQ 600 size standard (Beckman Coulter). Capillary gel electrophoresis was performed using a CEQ 8000 (Beckman Coulter). The diluted DNA solutions were denatured for 2 min at 95 °C, injected for 50 s at 2.0 kV, and separated for 65 min at 50 °C. The terminal restriction fragment lengths were determined using FRAGMENTS implemented in a CEQ 8000 genetic analysis system (Beckman Coulter).

2.5. Cloning and sequencing analysis to develop a reference library for T-RFLP identification

ITS region of 138 randomly selected root samples were again amplified using non-labeled ITS1F and ITS4 primers. PCR products of these 138 PCR products were randomly divided into five groups, then mixed within groups and cloned using the pGEM-T easy vector system (Promega, Madison, Wisconsin, USA). For each group, approximately 100 colonies were picked up and transformed PCR products were re-amplified using labeled ITS primers and *Taq* DNA polymerase with ThermoPol buffer (New England BioLabs) following the manufacture's instruction. The re-amplified PCR products were digested separately using *Hin*fI and *Alu*I, and digested products were mixed to the ratio of 2:1 to discriminate fragments. A total of 1.6 μl of digested products were used in the capillary gel electrophoresis as described above. To discover more T-RFLP types, we selected 96 samples, which showed few matched fragments with the obtained T-RFLP types, and subjected them to the same cloning and PCR procedures as described above. We grouped them into three before cloning, and *ca.* 100 colonies from each group were determined the T-RFLP types using the same method mentioned above. In total, 779 subcloning samples were subjected to T-RFLP analyses.

The T-RFLP types were determined based on the obtained fragments. When colonies had fragments within ±2 bp in all four fragments (two fluorescent dyed-fragments derived from two restriction enzymes), they were considered as the same T-RFLP type. A ±2 bp matching window was determined from the T-RFLP patterns of cloning samples, which fragments showed continuous variation for four bps in maximum. Small (<60 bp) and large (>700 bp) fragments were neglected due to the difficulty in determining the fragment size. When each unique T-RFLP types matched with the fragment profiles that were generated from root samples, its ITS sequences were determined by the commercially available DNA sequencing service (Eurofins MWG Operon, Ebersberg, Germany) or using Beckman 8000 (Beckman Coulter) based on the manufacture's instruction. We mainly used ITS1F primer to determine the ITS sequence but ITS2, ITS3 and ITS4 were used when necessary.

2.6. Identification of fungi

T-RFLP types were assigned to each root samples when all the fragments of the T-RFLP type were found in the root sample with the matching window of ±2 bp. A spreadsheet software was used to find the matching T-RFLP types in each samples.

To estimate the species of each T-RFLP types, we compared the obtained ITS sequences with the Genbank database using blastn implemented in NCBI. The taxon of each T-RFLP type was basically estimated based on the most similar sequences among the informative sequences (data from isolated cultures and identified sporocarps). However, because many of the Ascomycota fungi matched highly with different fungal taxa, the phylogenetic inference (by the neighbor-joining method with pairwise deletion) was conducted to determine the taxonomic group of the fungus using MEGA 4 (Tamura et al., 2007). When the obtained sequences were highly similar to each other (>98%), these T-RFLP types were considered as a single species.

2.7. Analysis

Since some of the negative controls also showed slight amplifications in PCR, root samples which did not show detectable fragment signals were considered due to the failure of PCR and removed from the analysis (nine of the 414 samples). Root fragments were considered to be infected by the fungus, when the fungal T-RFLP matched with the fragment profiles of the root sample. The percent root infected was calculated in each plot by dividing the number of infected root samples by the total number of root samples. The Simpson's diversity index ($1/D$) was also calculated for each plot, where $D = \sum Pi^2$, where Pi was defined as the frequency of the ith fungus divided by the total frequency of fungi in each plot.

To examine the effect of nitrogen enrichment on percent root infected, species richness per root fragment and Simpson's diversity index, general linear models (GLM; JMP 6.0, SAS Institute, Cary, NC, USA) were used. We started from the saturated model including forest (spruce and pine), nitrogen addition (C, N1 and N2) and interaction between them. (The data on *V. vitis-idaea* in the spruce forest (from controls) were not included in the analyses.) Models were simplified by eliminating non-significant ($P > 0.05$) terms from the models one by one, and P-values after model simplification are given. When the data were obtained for each root sample (ex., the number of fungi in a root sample), plot was included in the models as a nested and random factor.

Detrended correspondence analysis (DCA) was used to visualize the species composition of ERF communities on the different N treatment plots. The analysis was performed using PC-ORD 5.19 (MjM Software, Oregon, USA).

Species specific preferences of fungi to nitrogen addition treatments, host plant and forest type were assessed using Fisher's exact tests. For the analysis, we pooled the frequency of each fungus within each host plant and nitrogen treatment. Since the minimum requirement of the frequency of each fungus for statistically significant results of the Fisher's exact test was three to five depending on the comparisons, the less frequent fungi were removed from the analysis. To control false discovery rate and reduce familywise error rate associated with multiple testing, a sharpened procedure of Benjamini–Hochberg correction was applied instead of classical Bonferroni correction, as implemented in Verhoeven et al. (2005).

We retrieved a diverse assortment of Ascomycota, Basidiomycota and Zygomycota from *Vaccinium* roots (see Results). Although we consider all retrieved fungi possibly associated with *Vaccinium* roots, known ericaceous root associated fungi were grouped and analyzed separately from the rest of the fungi in some analyses. In the present study, this "assumed ERF" consisted of all fungi in the order Helotiales and *Sebacina*. Some Helotiales fungi normally classified as DSE, i.e., *Phialocephala* spp., were included in the "assumed ERF". These fungi have been found to form hyphal coils in ericaceous roots (see Jumpponen and Trappe, 1998 and references therein) and probably should be considered as mycorrhizal fungi (e.g., Jumpponen, 2001).

Hereafter, taxonomic names (such as Helotiales, *Sebacina*) are used to indicate groups of fungi, otherwise specified.

3. Results

3.1. Observed fungi

A total of 173 T-RFLP types were obtained from 779 cloning samples subjected to the development of a reference library for T-RFLP identification of fungal species on the *Vaccinium* roots, Of these, 116 T-RFLP types were matched with the fragment profiles generated from root samples. Among them, three T-RFLP types were found in the negative controls. Their ITS sequences matched with *Malassezia restricta* or *Trichosporon porosum* (yeast fungi) and were removed from further analysis. Among the other 113 T-RFLP typs, each of 20 T-RFLP types were pooled with one of the other T-RFLP types since ITS sequences of these T-RFLP types were highly (>98%) similar to those. Finally, 93 T-RFLP types were used as putative species in this study.

PCR amplification of root extracted DNA was successful in 405 of 414 samples. An average of 30.4 terminal fragments were obtained from each root sample. There was no significant difference among the number of terminal fragments between forest types or nitrogen treatments ($P > 0.34$ in a GLM analysis). By dividing the number of matched fragments by the total number of fragments in a root sample, an average of 48% of the fragments were assigned to either of the fungi.

Among 93 species, 46, 34 and 8 species belonged to Ascomycota, Basidiomycota and Zygomycota, respectively (Table 1). Taxonomic position of remaining five fungi were left unknown since their ITS sequences were not determined. In Ascomycota, 28 species were considered belonging to Helotiales based on the closest blast matches. When phylogenetic inference was conducted for Helotiales species with reference to the Genbank deposited sequences, 6, 8 and 3 species were considered to belong to the *Rhizoscyphus ericae* aggregate (*R. ericae* + *Meliniomyces*; hereafter, *Rhizoscyphus* aggregate), *Cadophora*, the *Phialocephala* complex (*Phialocephala* + *Acephala* + *Mollisia*), respectively. Taxonomic positions of the remaining 11 Helotiales species were left ambiguous since they matched with very few identified species or diversified taxa. Other than Helotiales species, five species matched with *Capronia* sequences. However, they also matched with the fungi in different taxonomic groups from Herpotrichiellaceae. Since phylogenetic position of these sequences did not match with their taxonomic position, a conserved taxon, Herpotrichiellaceae, was given to these species. The remaining 13 species belonged to diversified fungal taxa including soil yeasts or airborne fungi (e.g., *Cladosporium* and *Tumularia*) (Appendix 1). In Basidiomycota, 17 of 34 fungi matched highly with confirmed or estimated ectomycorrhizal fungi including Atheliaceae (*Piloderma* and *Amphinema*), *Cortinarius*, *Russula*, *Sebacina*, *Sistoterma* and Thelephorales. Another nine species belonged to Tremellales, most of which are *Cryptococcus* and *Mallasezeria*. Others were similar to pathogenic or saprophytic fungi (*Rhizoctonia*, *Rhodotorula*, *Mycena* and unknown Atheliaceae). All the species in Zygomycota were included in the genus *Mortierella*.

3.2. Infection and diversity

Presence of fungi was confirmed in most of the root fragments (93–98%) (Table 1). The observed infection rates did not differ between the nitrogen treatments ($P = 0.92$). In addition we tested whether infections rates would differ between the forest types with their respective dominant host plants (*V. myrtillus* in the spruce forest and *V. vitis-idaea* in the pine forest) and found no significant difference ($P = 0.092$). When assumed ERF was used for the same analyses, the infection rate was still high (78–95%; Table 1) and the infections rate was significantly higher ($P = 0.0008$) for *V. vitis-idaea* roots in the pine forest (92% in average) than for *V. myrtillus* roots in the spruce forest (79%).

Multiple infections within a root fragment were frequently observed; 75% (all inclusive) or 50% (assumed ERF) of the root fragments were infected with more than one fungus. The average species richness (all inclusive) within a root fragment varied between 2.2 and 3.1 species. No significant differences were observed between nitrogen treatments ($P = 0.50$) or between the spruce and pine forests ($P = 0.21$). When assumed ERF was used for the same analyses, the effect of nitrogen treatment was not significant ($P = 0.82$), but average species richness was significantly higher ($P = 0.0004$) for *V. vitis-idaea* in the pine forest (1.8–2.0 species per root fragment) than for *V. myrtillus* in the spruce forest (1.3–1.4 species per root fragment). Considering the presence of undetected fungi, both the observed infection rates and the observed species richness per root fragments should be considered as minimum values.

A total of 24–52 fungal species were found in each treatment plot (Table 1). There was no great difference between nitrogen treatments (42–52 species per treatment) in the spruce forest, but in the pine forest the number of fungi in the control treatment (24 species) was less than in N1 (46 species) and N2 (39 species). This difference is largely depending on the non-"assumed ERF". We found only eight species from non-"assumed ERF" in the control treatment, while 20 and 21 species were found in each of N1 and N2 in the pine forest (Table 1). Simpson's diversity index ($1/D$) varied between 7.7 and 14.0 (all inclusive) and 2.8 and 5.0 (assumed ERF), but the differences were not significant neither between the nitrogen treatments ($P > 0.72$) nor between the forests ($P > 0.10$).

3.3. Fungal community

In both forests, the most abundant fungi associated with *Vaccinium* roots were the *Rhizoscyphus* aggregate and Herpotrichiellaceae,

which were in general followed by *Cadophora* and *Sebacina* Infection of *Sebacina* was particularly high in *V. vitis-idaea* in the spruce forest. Generally, fungal communities were similar between the nitrogen treatments, but different between the forests. For example, irrespective of nitrogen treatment Herpotrichiellaceae sp1 was the dominant fungus on *V. myrtillus* (in the spruce forest) and *Rhizoscyphus* aggregate sp1 on *V. vitis-idaea* (in the pine forest) (Appendix 1).

Detrended correspondence analysis (DCA) on the total root fungal communities illustrated a separation between the forests and within the spruce forest also a separation between host plant species along the primary axis, which had an eigen value of 0.367 and explained 13.7% of the variance (Fig. 1). By contrast, nitrogen treatment did not appear to discriminate the fungal communities (Fig. 1). A similar result was obtained when the assumed ERF were used for the same analysis (figure not shown). Furthermore, nitrogen treatment did not discriminate the fungal communities even when the two forests with their respective dominant host plant were analyzed separately (figures not shown). The explanatory variables of the first two axes were low in all these analyses, indicating the great variation among the plots.

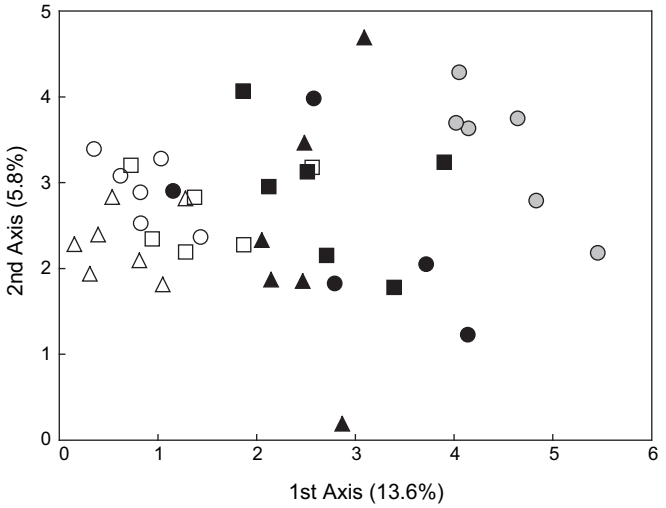

Fig. 1. Detrended correspondence analysis of fungal communities of *Vaccinium myrtillus* in the spruce forest (closed symbols), *V. vitis-idaea* in the spruce forest (shaded) and in the pine forest (open). Shapes of symbols indicate nitrogen treatment, circle: control, square: N1 (12.5 kg N ha^{-1} yr^{-1}), triangle: N2 (50 kg N ha^{-1} yr^{-1}).

3.4. Fungal preference

To examine fungal species specific responses to nitrogen enrichment, forest or host plant, Fisher's exact test with Benjamini–Hochberg correction was applied for each fungus. According to the minimum requirement of the observation for the Fisher's exact test, 17–37 fungal species were subjected to the comparison of each effect. When the response to nitrogen addition was assessed in each forest, no biased occurrence of species was observed among the nitrogen treatments (Table 2). Next, to examine host plant specificity, the frequency of each fungus was compared between *V. myrtillus* and *V. vitis-idaea* from control treatment in the spruce forest. Only one fungus, *Sebacina* sp3 was significantly more abundant in *V. vitis-idaea* than in *V. myrtillus* (Table 2). Finally, to see the preference for either of the two forests, data on fungal infection of *V. vitis-idaea* in the control treatments were compared between the forests. Three fungi (*Sebacina* sp2 and sp3 and sp4) preferred the spruce forest and five fungi (*Rhizoscyphus* aggregate sp1, *Cadophora* sp1, Herpotrichiellaceae sp1, Ascomycota sp1 and *Sebacina* sp1) preferred the pine forest (Table 2).

4. Discussion

4.1. Fungal communities on Vaccinium roots

In previous studies of fungal communities on *Vaccinium* roots based on cultured isolates, most of the obtained fungi belonged to Helotiales or *Capronia* (Herpotrichiellaceae) (Allen et al., 2003; Midgley et al., 2004). Since culturing may mask unculturable fungi (Berch et al., 2002; Bougoure and Cairney, 2005a,b), these studies probably do not reflect *in situ* fungal communities. In contrast to the culture based method, the direct PCR approach which applies PCR directly on field-obtained samples, is advantageous because it can detect and discriminate the diversified fungal taxa. Together with other studies (Allen et al., 2003; Bougoure and Cairney, 2005a,b; Bougoure et al., 2007), this study confirms the usability of the direct PCR approach by showing the diversified Asco- and Basidio-mycota species on *Vaccinium* roots. At the same time, however, the presented data probably underestimate the diversity and infection rate of root associated fungi as nearly half of the PCR fragments were not assigned to any fungi (T-RFLP types) in this study.

Applying direct PCR on a large number of short root fragments (2 mm) demonstrated that most of the *Vaccinium* root fragments (*ca.* >90%) were colonized by fungi, in which the *Rhizoscyphus* aggregate was the most dominant followed by Herpotrichiellaceae and *Sebacina*. *Rhizoscyphus* aggregate and *Sebacina* have been repeatedly found in ericaceous roots, and are known as the major components of ERF (Allen et al., 2003; Bougoure et al., 2007). *Rhizoscyphus* has been shown to improve host plant uptake of various nitrogen forms (Sokolovski et al., 2002; Grelet et al., 2009), while host benefits from association with *Sebacina* is unclear. The anatomical characteristics and abundant infection of *Sebacina* (Selosse et al., 2007) may imply a central physiological and/or ecological importance of these fungi to the ericaceous plants. Herpotrichiellaceae (simulants of *Capronia*) are also frequently observed in ericaceous roots and occasionally form hyphal coils in roots (Allen et al., 2003), although the physiological importance of these fungi is not known.

We also found a number of fungi whose interactions with ericaceous plants are ambiguous. These fungi include yeasts (frequently found in soils, air, water and plants including species of *Penicillium*, *Cryptococcus*, *Rhodotura*, *Rhizoctonia*, Tremellales), possible pathogenic fungi such as *Mortierella* and known ectomycorrhizal fungi (*Cortinarius*, Russlaceae, and Thelephorales). In the here studied *Vaccinium* roots, these fungi were not always minor and sometimes they were found in more than 10% of the root fragments (Table 1; Appendix 1). Also other studies have found

Table 2
Number of fungal species which showed specific responses to nitrogen addition, host plant and forest type, as revealed by the Fisher's exact test with Benjamini–Hochberg correction.

Effect	Responded fungi	Tested fungi[a]	Comparisons
Nitrogen enrichment			Nitrogen treatments in each forest
Spruce forest	0	37	
Pine forest	0	24	
Host plant	1	22	*V. myrtillus* and *V. vitis-idaea* on control treatment in spruce forest
Forest type	8	17	*V. vitis-idaea* on control treatment in spruce forest and pine forest

[a] Number of fungi for which frequency exceeded the minimum requirement for the Fisher's exact test.

similar fungi in studies of ericaceous roots (e.g., Bougoure et al., 2007). Although nothing is known about the ecology of these fungi in ericaceous roots, interaction of these fungi with other plants has been reported. For example, plant growth, phosphorus uptake and resistance to pathogenic fungi were improved by introducing yeasts (*Candida valida*, *Rhodotorula glutinis* or *Trichosporon asahii*) (El-Tarabily, 2004) and *Mortierella* (Osorio and Habte, 2001) to soils. Although the physiological location of these introduced fungi were not confirmed in these studies, the interaction could take place in the roots, as colonization of an induced Ascomycota yeast (*Williopsis*) was observed inside maize roots, enhancing plant growth (Nassar et al., 2005). These studies indicate that we should not underestimate the importance of these functionally unknown fungi in ericaceous roots.

4.2. Factors affecting ERF

In the present study, no statistical evidence was observed that experimental nitrogen addition affected the fungi we defined as assumed ERF. Similar results were obtained from field studies on *C. vulgaris* in European heathlands (Caporn et al., 1995; Johansson, 2000) and *Ledum palustre* in Alaskan tundra (Urcelay et al., 2003), showing that ERF infection of these two plants were not affected by nitrogen addition of 70–120 kg N ha^{-1} yr^{-1} during two to three years. In these studies, fungal infection was measured using microscopic observations or root ergosterol analysis, and the fungal species specific responses to nitrogen addition could not be addressed. Thus the combination of different responses by different fungi could possibly result in no difference of the ERF infection from nitrogen addition. By showing the infection rate of each fungal species, the present study eliminates this possibility as none of the individual ERF species showed a significant response to nitrogen addition. However, in the current study we were only able to detect responses of major species and the responses of minor species remain unclear, since biased occurrence of less frequent fungi cannot be detected by the used statistical approach.

In boreal forest, nitrogen addition has been related to *Vaccinium* decline resulting in graminoid expansion (i.e., Nordin et al., 2005, in press in the here studied spruce forest). The decline of *Vaccinium* has been considered owing to the increased occurrence of pathogenic leaf fungi and herbivorous insects on *Vaccinium* subjected to nitrogen addition (Nordin et al., 1998; Strengbom et al., 2002, 2005). Severe defoliation may decrease fungal abundance and diversity on plant roots, due to reduced carbon allocation to roots (Gehring and Whitham, 1994). Contrary to our expectation, the nitrogen induced defoliation of *V. myrtillus* reported for some of the years from the here studied spruce forest (Nordin et al., in press) did not appear to affect ERF. Instead, we suggest that the conserved potential symbiotic fungi may facilitate the re-establishment of pathogen/herbivore damaged *Vaccinium* under circumstances of decreased nitrogen input.

Moreover, nitrogen addition could have been expected to affect ERF irrespective of host defoliation, since ectomycorrhizal fungal community structure appears sensitive to nitrogen enrichment (e.g., Lilleskov et al., 2002). However, when looking at the major fungi associated with *Vaccinium* roots, Ascomycota and *Sebacina* (Basidiomycota), their representation are relatively minor among ectomycorrhizal fungi (e.g., Ishida et al., 2007). Because the response to nitrogen seems to be specific for each fungal taxon (Nygren et al., 2008), and not all ectomycorrhizal fungal taxa respond to nitrogen, the non-response of ERF may be due to the characteristics specific for the major ERF taxa (*Rhizoscyphus*, and *Sebacina*). Alternatively, the host plant response to nitrogen may cause the difference in response between ecto- and ericoid-mycorrhizal fungi. In boreal forest, nitrogen addition improves tree growth, which causes decline of carbon transfer to ectomycorrhizal fungi (Olsson et al., 2005). On the other hand, nitrogen enrichment may not induce such a change of carbon transfer in dwarf shrubs as their growth and photosynthesis appear to be less responsive to nitrogen addition (Leith et al., 1999). No change in ERF in response to nitrogen addition may thus be due to no change in host plant carbon transfer.

While nitrogen addition showed no effect on ERF, fungal communities were clearly separated between the forests (Fig. 1). Although our data could not discriminate the effects of host plant from that of forest type, from comparing the number of fungi that showed a preference either to forest type (eight species) or host plant (one species) (Table 2), it appears like environmental factors in each forest seems to be more important in structuring ERF communities than host plant identity. The relatively low degree of host effect may be due to that the studied two host plants are congeneric. In the study of ectomcyorrhizal fungi, congeneric host pairs showed the highest similarity of fungal community (Ishida et al., 2007). On the other hand, the studied two forests differ markedly; going from the mesic fertile spruce forest to the dry nutrient poor pine forest. The effect of ecosystem type was observed by Bougoure et al. (2007) who reported distinctive fungal communities associated to *C. vulgaris* roots along a forest succession gradient going from an open heathland to a pine forest. Although we cannot determine which environmental factor (or past history of environmental impacts) that contribute the most to the distinctive differences in ERF community between the forests, ambient soil fertility may be important, as it is known as the main factor determining vegetation structure and function in boreal ecosystems (Tamm, 1991). Therefore, we do not rule out a possible indirect effect of ambient nitrogen on structuring ERF communities, even if the relatively long-term ($\leq$12 yrs) nitrogen additions did not affect ERF in our experiment.

In conclusion, we found no evidence for the effect of nitrogen addition on infection rate, species diversity or community structure of *Vaccinium* root associated fungi in boreal spruce or pine forest. Although nitrogen enrichment induces *Vaccinium* defoliation due to infection by nitrogen favored pathogenic leaf fungi or to herbivory (Strengbom et al., 2002, 2005) and ultimately dwarf-shrub decline (Nordin et al., 2005, in press), we suggest that conserved potential ERF may help re-establishment of *Vaccinium* if nitrogen input decreases. While no effect of nitrogen addition, the fungal community differed between the spruce and pine forest, and also between *V. myrtillus* and *V. vitis-idaea* in the spruce forest. Since these forests harbored different environmental factors and dominant dwarf shrubs, distinctive ERF communities were formed between these forests.

Acknowledgements

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Appendix 1. Fungal species and frequency in the *Vaccinium* root samples. Roots were collected in two types of boreal forest (spruce and pine dominated) with three different nitrogen treatments: 0 kg N ha^{-1} yr^{-1} (C), 12.5 kg N ha^{-1} yr^{-1} (N1) and 50 kg N ha^{-1} yr^{-1} (N2) in northern Sweden. Figures in the parentheses under treatments indicate the total number of samples summarized across replications within treatments.

Tentative identification	Spruce forest				Pine forest			Accession number	Length (bp)	One of the closest match in Genbank[a] (accession number, percentile identity)
	V. myrtillus				*V. vitis-idaea*					
	C	N1	N2	C	C	N1	N2			
	(48)	(60)	(55)	(59)	(57)	(57)	(69)			
Ascomycota										
Rhizoscyphus sp1	9	6	3	3	25	28	36	AB476464	844	*Rhizoscyphus ericae* (EU221877, 99%)
Rhizoscyphus sp2	8	13	12	4	11	16	18	AB476504	533	*Meliniomyces variabilis* (EF093171, 98%)
Rhizoscyphus sp3	1	6	2	5	0	1	0	AB476510	859	*Meliniomyces* bicolor (AY394885, 91%)
Rhizoscyphus sp4	0	1	0	1	5	2	1	AB476506	524	*Meliniomyces* sp. (EF093175, 96%)
Rhizoscyphus sp5	3	1	1	1	0	1	0	AB476490	538	*Rhizoscyphus ericae* aggregate (AM084704, 94%)
Rhizoscyphus sp6	2	1	2	2	0	0	0	AB476537	521	*Rhizoscyphus ericae* aggregate (AM084704, 93%)
Cadophora sp1	2	6	9	1	11	5	16	AB476468	573	*Phialophora* sp. (DQ069046, 97%)
Cadophora sp2	3	3	4	1	7	3	3	AB521979	838	Uncultured fungus (EU292395, 93%)
Cadophora sp3	2	0	1	2	3	7	2	AB521980	564	*Leohumicola verrucosa* (AY706320, 90%)
Cadophora sp4	1	1	0	1	1	2	2	AB476540	548	*Phialophora* sp. (DQ069046, 98%)
Cadophora sp5	0	1	2	1	0	2	1	AB476467	551	*Phialophora* sp. (DQ069046, 97%)
Cadophora sp6	0	1	1	0	1	1	2	AB476482	869	Uncultured fungus (EU292395, 94%)
Cadophora sp7	0	1	0	0	0	1	0	AB476539	535	*Phialophora* sp. (DQ069046, 97%)
Cadophora sp8	0	0	0	0	0	1	0	AB521978	565	*Cystodendron* sp. (DQ914671, 85%)
Phialocephala sp1	9	16	15	6	4	5	6	AB476481	818	*Phialocephala europaea* (AY078133, 97%)
Phialocephala sp2	1	1	0	2	0	3	2	AB476519	513	*Mollisia minutella* (EU314679, 99%)
Phialocephala sp3	0	0	0	3	1	1	2	AB476494	829	*Phialocephala sphaeroides* (AY524844, 92%)
Helotiales sp1	6	5	0	7	13	12	9	AB476471	525	*Hyphodiscus hymeniophilus* (DQ227258, 93%)
Helotiales sp2	4	4	2	3	1	0	0	AB476538	531	*Leptodontidium elatius* (AF475152, 93%)
Helotiales sp3	1	2	3	2	0	2	2	AB476502	513	*Hymenoscyphus epiphyllus* (DQ431180, 98%)
Helotiales sp4	0	0	0	0	3	1	5	AB521981	478	Uncultured fungus (FJ475725, 100%)
Helotiales sp5	2	0	4	1	0	1	0	AB476497	517	*Lachnum papyraceum* (AB267643, 96%)
Helotiales sp6	1	1	1	1	0	1	0	AB476536	523	*Cystodendron* sp. (DQ914672, 94%)
Helotiales sp7	0	1	0	1	0	1	1	AB476496	524	*Hymenoscyphus monotropae* (AF169309, 97%)
Helotiales sp8	0	0	0	0	0	0	2	AB476495	694	*Alatospora acuminata* (AB041243, 82%)
Helotiales sp9	0	0	1	0	0	1	0	AB476533	517	*Gyoerffyella* sp. (EF093184, 99%)
Helotiales sp10	0	0	0	1	0	0	0	AB521982	543	*Scleropezicula alnicola* (AF141168, 83%)
Helotiales sp11	0	0	1	0	0	0	0	AB476523	516	*Cadophora* sp. (EF493279, 94%)
Herpotrichiellaceae sp1	12	26	19	10	26	27	22	AB476465	838	Uncultured Herpotrichiellaceae (FJ553268, 94%)
Herpotrichiellaceae sp2	1	2	1	0	1	1	1	AB476488	800	Uncultured fungus (EU292643, 85%)
Herpotrichiellaceae sp3	0	0	0	0	0	0	2	AB476498	579	*Exophiala eucalyptorum* (EU035417, 83%)
Herpotrichiellaceae sp4	0	0	1	0	0	0	0	AB476525	553	*Capronia* sp. (EU139158, 85%)
Herpotrichiellaceae sp5	0	0	0	1	0	0	0	AB476541	572	*Exophiala eucalyptorum* (EU035417, 88%)
Botryosphaeriales sp1	1	1	3	2	2	2	6	AB476478	685	Uncultured fungus (EF040857, 98%)
Galactomyces sp1	2	8	0	0	0	0	0	AB476526	339	*Galactomyces geotrichum* (DQ907937, 98%)
Penicillium sp1	1	0	2	0	0	2	4	AB476520	542	*Penicillium glabrum* (EU128630, 98%)
Cladosporium sp1	0	0	2	3	0	2	0	AB476515	517	*Cladosporium cladosporioides* (FJ537128, 99%)
Candida sp1	2	2	0	1	0	0	0	AB476517	410	*Candida sake* (AJ549822, 99%)
Hypocrea sp1	1	2	1	0	0	0	0	AB476527	575	*Hypocrea* sp. (EU294196, 99%)
Debaryomyces sp1	0	2	1	0	0	1	0	AB476542	604	*Debaryomyces hansenii* (GQ376084, 99%)
Ascomycota sp1	4	3	1	0	8	7	11	AB521974	788	Uncultured fungus (DQ309103, 95%)
Ascomycota sp2	0	0	0	0	0	0	3	AB476516	561	*Tumularia* aquatica (FJ000399, 97%)
Ascomycota sp3	0	1	0	0	0	1	1	AB521976	515	*Acremonium* sp. (FJ430713, 91%)
Ascomycota sp4	1	0	1	0	0	0	0	AB521977	745	Uncultured ascomycete (EF619860, 96%)
Ascomycota sp5	0	0	0	0	0	0	1	AB476477	538	*Scleroconidioma sphagnicola* (DQ182416, 94%)
Ascomycota sp6	1	0	0	0	0	0	0	AB521975	557	Uncultured fungus (DQ309228, 90%)
Basidiomycota										
Amphinema sp1	0	1	1	0	0	0	0	AB476486	560	Uncultured fungus (DQ309228, 90%)
Atheliaceae sp1	1	0	0	0	0	0	0	AB476544	617	*Athelia arachnoidea* (U85791, 95%)
Atheliaceae sp2	0	0	0	0	0	0	1	AB476470	647	*Fibulorhizoctonia* sp. (U85789, 98%)
Piloderma sp1	4	6	2	3	6	4	7	AB476522	595	*Piloderma* (EF493279, 98%)
Piloderma sp2	2	1	0	4	0	0	0	AB476480	587	*Piloderma byssinum* (DQ469281, 99%)
Cortinarius sp1	1	0	0	0	0	1	0	AB476499	652	*Cortinarius acutus* (FJ039692, 92%)
Cortinarius sp2	0	1	0	0	0	0	0	AB476548	581	*Cortinarius caesiobrunneus* (EU292288, 99%)
Russula sp2	0	0	3	0	0	0	0	AB476543	659	*Russula bicolor* (FJ845435, 99%)
Russula sp1	0	1	0	1	0	0	0	AB476463	724	*Russula decolorans* (DQ367913, 100%)
Russula sp3	2	0	0	0	0	0	0	AB476475	664	*Russula paludosa* (AJ971402, 98%)
Russula sp4	0	1	1	0	0	0	0	AB476483	660	*Russula vinosa* (AY061724., 99%)
Russula	1	0	0	1	0	0	0	AB521985	696	*Russula gracilis* (FJ845431, 94%)
Sebacina sp1	3	6	4	1	15	11	19	AB476469	621	*Sebacina vermifera* (EU625992, 89%)
Sebacina sp2	8	6	3	25	2	2	0	AB476472	569	Sebacinaceae sp. (FJ197211, 86%)
Sebacina sp3	0	0	0	12	1	2	0	AB476487	629	Sebacinaceae sp. (FJ197211, 86%)
Sebacina sp4	3	0	0	7	0	0	0	AB476493	622	Uncultured Sebacinale (EF127237, 93%)

(*continued on next page*)

Appendix 1. (*continued*)

Tentative identification	Spruce forest				Pine forest			Accession number	Length (bp)	One of the closest match in Genbank[a] (accession number, percentile identity)
	V. myrtillus				*V. vitis-idaea*					
	C	N1	N2	C	C	N1	N2			
	(48)	(60)	(55)	(59)	(57)	(57)	(69)			
Thelephora sp1	1	1	0	0	0	0	0	AB476476	605	*Thelephora terrestris* (FN393122, 99%)
Thelophorales sp1	0	0	0	0	0	1	0	AB476545	629	*Hydnellum peckii* (DQ367901, 93%)
Mycena sp1	2	1	0	2	0	0	0	AB476547	642	*Mycena* cf. *vitilis* (DQ384588, 86%)
Mycena sp2	0	0	0	0	1	1	1	AB521984	670	*Mycena tenax* (EU846251, 90%)
Rhodotorula sp1	0	0	0	0	0	0	2	AB476503	585	*Rhodotorula pustula* (AF444531, 90%)
Rhodotorula sp2	0	1	0	0	0	1	0	AB476524	578	*Rhodotorula mucilaginosa* (FJ515212, 100%)
Rhodotorula sp3	0	0	0	1	1	0	0	AB476528	538	*Rhodotorula chungnamensis* (AY479978, 84%)
Tremellales sp1	2	2	2	5	0	2	0	AB476505	747	*Malassezia globosa* (AY743630, 96%)
Tremellales sp2	0	2	0	1	0	1	0	AB476507	702	*Malassezia globosa* (AJ437693, 98%)
Tremellales sp3	0	0	0	0	0	0	2	AB476491	473	*Tremella foliacea* (AF042450, 90%)
Tremellomycetes sp1	0	2	0	0	0	0	0	AB476513	631	*Mrakia nivalis* (AF144484, 77%)
Cryptococcus sp1	0	0	0	0	0	0	4	AB476492	485	*Cryptococcus* sp. (DQ317359, 98%)
Cryptococcus sp2	0	0	0	0	0	0	2	AB476529	486	*Cryptococcus* aff. *victoriae* (FN298668, 100%)
Cryptococcus sp3	0	1	0	0	0	0	0	AB476500	588	*Cryptococcus magnus* (FN400937, 100%)
Cryptococcus sp4	0	0	1	0	0	0	0	AB476512	613	*Cryptococcus terricola* (EU266561, 99%)
Malassezia sp1	0	0	1	0	0	0	0	AB476535	604	*Malassezia sympodialis* (AY743657, 96%)
Basidiomycota sp1	0	0	0	0	0	1	1	AB521983	560	Uncultured fungus (FJ475792, 100%)
Basidiomycota sp2	0	0	1	1	0	0	0	AB476473	630	*Sistotrema coronilla* (GQ411514, 75%)
Zygomycota										
Mortierella sp1	5	8	1	3	1	0	1	AB476501	599	*Mortierellales* sp. (EF031099, 99%)
Mortierella sp2	3	6	3	0	0	1	5	AB476532	597	*Mortierella macrocystis* (AJ878782, 97%)
Mortierella sp3	3	1	1	1	0	0	5	AB476479	627	*Mortierella macrocystis* (EU240133, 84%)
Mortierella sp4	2	3	2	0	0	2	0	AB476518	585	*Mortierella* sp. (EU240133, 98%)
Mortierella sp5	1	0	0	2	0	0	0	AB476530	603	*Mortierella gamsii* (DQ093723, 99%)
Mortierella sp6	0	0	0	2	0	0	0	AB476531	605	*Mortierella* sp. (AY842393, 96%)
Mortierella sp7	0	0	0	1	0	0	0	AB521986	651	*Mortierella* cf. *hyalina* (AY157495, 81%)
Mortierella sp8	0	1	0	0	0	0	0	AB521987	640	*Mortierella* sp. (EU240040, 80%)
Unidentified										
Unidentified sp1	1	1	2	0	0	0	1			
Unidentified sp2	0	1	0	0	0	0	0			
Unidentified sp3	0	1	0	0	0	1	0			
Unidentified sp4	0	1	0	1	0	0	0			
Unidentified sp5	0	0	0	0	0	1	0			

[a] Among the closely matched sequences in the Genbank, informative sequences (data from cultured isolates or identified sporocarps) were selected. When informative sequence was not available, the closest match was shown.

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