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The Effect of Latitude, Season and Needle-Age on the Mycota of Scots Pine (*Pinus sylvestris*) in Finland

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The seasonal and latitudinal influences on the diversity and abundance of mycota of *Pinus sylvestris* needles were investigated. A sample of 1620 needles resulted in a total of 3868 fungal isolates, which were assigned to 68 operational taxonomic units (OTUs). The majority of these OTUs (65%) belong to Ascomycota and only 0.03% was grouped as Basidiomycota. The dominant and most frequently isolated OTU was *Hormonema dematioides*. Other well-known species with a saprotrophic nutritional mode such as *Lophodermium* spp. were also observed. The abundance of fungi increased from fall to spring. Frequencies varied significantly in Northern and Southern Finland suggesting that factors associated with latitudinal differences have an impact on the abundance of fungi.

Keywords *Pinus sylvestris*, harsh environment, mycota, needle age, cryptic lifecycle, needles, *Hormonema dematioides*
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1 Introduction

Mycota of conifer needles includes endophytes and epiphytes that colonize the interior and exterior surface of living needles respectively (Hyde and Soytong 2008). Fungal endophytes typically live asymptotically inside the plant tissues for the whole or at least a significant part of their life cycle (Petrini 1991, Saikkonen et al. 1998, Hyde and Soytong 2008). It may be difficult to determine whether a fungus is an endo- or epiphyte as some fungi are able to occur in both habitats (Osono and Mori 2004, 2005). Some epiphytic fungi colonize internal tissues especially at leaf senescence, while certain endophytes have an epiphytic phase in their life cycle (Petrini 1991). The newly emerging needles of conifers are endophyte-free, but quickly overtime they are infected horizontally by fungal spores (Helander et al. 1993, 1994, Saikkonen et al. 1998, 2004). Frequencies of these fungi are affected by dry weather conditions, which are known to be unfavourable to the germination of fungal spores. Consequently fungal abundances vary according to seasonal precipitation and temperature (Osorio and Stephan 1991, Elamo et al. 1999). The extensive diversity and abundance of endophytes in woody plants have led to increased interest in the studies of their interactions and importance to the host plants (Arnold et al. 2003, Arnold 2007, Sieber 2007, Jumpponen and Jones 2009, Aly et al. 2010, Gazis and Chaverri 2010, Saikkonen et al. 2010, Rocha et al. 2011). The most intensively examined woody plant families have been Betulaceae, Fagaceae, Cupressaceae and Pinaceae (Saikkonen 2007, Sieber 2007 and references therein). Most of the dominant fungal species of the conifers belong to the class Leotiomycetes (Carroll and Carroll 1978, Kowalski 1993, Hata and Futai 1996, Ganley and Newcombe 2006) and the order Helotiales (Sieber 2007).

In Finland most of the studies have concentrated on endophytic fungi of grasses (Saikkonen et al. 2006, Wäli et al. 2008). In woody plants, most of the studies have focused on leaves of different species of birch (Helander et al. 1993, 2006, 2007, Elamo et al. 1999, Lappalainen et al. 1999, Lappalainen and Yli-Mattila 1999, Ahlholm 2002a, Saikkonen et al. 2003). The functional role of these fungi to invertebrate herbivores of *Betula* spp. have equally been investigated (Lappalainen and Helander 1997, Lappalainen et al. 1999, Ahlholm 2002b).

Very few studies have been conducted on endophytes of symptomless needles of Norway spruce (*Picea abies* (L.) H. Karst.) (Müller and Hallaksela 1998, 2000, Müller et al. 2001, 2007) and their role as primary decomposers of forest litter (Müller et al. 2001, Korkama-Rajala et al. 2008). Fungal endophytes of needles of Siberian larch (*Larix sibirica* Ledeb.) (Kauhanen et al. 2006) and Scots pine (*Pinus sylvestris* L.) have also been studied in Finland (Helander et al. 1994, Ranta et al. 1994, Helander 1995) with special emphasis on the affect of the pollution and the acid rain to their abundance.

The most frequently reported fungi of Scots pine needles are *Lophodermium pinastri* (Schrad. ex Hook.) Chev., other *Lophodermium* spp., and an agent of needle cast, *Cyclaneusma minus* (Butin) DiCosmo, Peredo & Minter (Kowalski 1982, 1993, Helander et al. 1994, Helander 1995). These fungi are weak parasites colonising living symptomless Scots pine needles as endophytes (Kowalski 1982, 1993) and they were present in most of the sites studied (see Sieber 2007). Also *Cenangium ferrucinosum* Fr.:Fr. is considered as specific endophyte of Scots pine (Helander 1995).

The ecology, species composition and changes in abundance of endophytes of Scots pine needles during the growing season have been studied in Finland (Helander et al. 1994) but not the impact of geographical locations. Some authors have also studied the seasonal changes in the diversity and frequency of fungal endophytes of different *Pinus* species (Kowalski 1993, Martín et al. 2004, Zamora et al. 2007, Guo and Wang 2008). Needle-age dependent changes for the endophyte assemblage in needles of Scots pine have also been studied (Kowalski 1993, Helander et al. 1994). The colonization rate of Scots pine needles by endophytes tends to increase with needle age (Kowalski 1993, Helander et al. 1994). A similar colonization pattern has been observed in other *Pinus* (Hata and Futai 1996, Hata et al. 1998, Guo and Wang 2008) and conifer species (Barklund 1987) as well as in deciduous trees (Herre et al. 2007, Osono 2008). Hata et al. (1998) suggested that the frequency of endophyte of *Pinus* spp.

needle depends on increased chance of infection, time after needle flush, improved habitat conditions and competition with other fungi.

Climate and resource availability are major factors that also control the geographical distribution and abundance of fungi (Arnolds 1997, Botella and Diez 2011). Diversity of endophytes of coniferous foliage has been reported to decrease with increasing latitude, which was suggested to correlate with lower tree species richness (Sokolski et al. 2007). The composition of fungal assemblages in temperate (Carroll and Carroll 1978, Göre and Bucak 2007) and tropical areas (Joshee et al. 2009) are also affected by the seasonal factors. Fungal diversity also varies as a function of latitude and annual rainfall (Wilson 2000, Arnold and Lutzoni 2007). Isolation success of fungi of Scots pine needles is affected also by the season, inoculum size, moisture, temperature and other climatic factors (Kowalski 1993).

The aim of this study is to examine the impact of latitudinal, seasonal and needle-age effects on the fungal diversity and frequencies of Scots pine needles in Finland. No distinction was made on the fungal isolates as endophytes or epiphytes. Our hypothesis is that fungal species biodiversity decreases in harsher environment (e.g. cold temperatures) in the symptomless needles of *Pinus sylvestris*.

2 Materials and Methods

2.1 Study Sites and Sampling

Scots pines needle samples from 27 different Scots pine trees located at three different geographical areas (North, Central and Southern Finland) were collected. For each geographical area, three study sites (three trees in every site) were chosen at the same latitude with approximately 50 km distance between each sampling site. In Northern Finland (66° northern latitude, temperature sum 900–950 degree days (d.d.), the average temperature at winter in 2006–2007 was –11 °C) the needles were collected from Rovaniemi (R), Meltaus (M) and Kivalo (K). In the central Finland (62°N, 1150–1250 d.d., –8 °C) the needles were collected from Pieksämäki (P), Varkaus (V) and Suonenjoki (S) and in Southern Finland (60°N, > 1250 d.d., –3 °C) from Mäntsälä (Mä), Pikkala (Pik) and Viikki (Vi). Two branches from each tree from every site were collected such that each branch had needles from the first and second year. A total of 10 needles from the 1st year and the 2nd year cohort per branch were randomly chosen (altogether 20 needles per tree). Samples were collected in three different seasons; the fall: October 2006 (monthly rainfall: North 45 mm, Central 119 mm and South 190 mm), winter: February 2007 (N 21 mm, C 19 mm, S 18 mm) and spring: April 2007 (N 20 mm, C 23 mm and S 45 mm). A total of 810 pine needle pairs were randomly chosen for the isolation of mycota.

2.2 Isolation of Mycota from the Scots Pine Needles

All fungi were isolated from the needles using 2% malt extract agar (MEA). The needles were either placed directly or cut into five pieces and placed on the malt extract agar and incubated at room temperature for two weeks. Freshly emerging hyphae from the needles were sub-cultured into new plates until pure cultures were obtained. The samples were arranged according to season and latitudinal location. Due to the high number of individual cultures (3868), the pure cultures were classified in morphologically distinct groups based on the colony shape, height and colour of aerial hyphae, base colour, growth rate, surface texture and depth of growth into medium. Representatives of the different morphological classes were transferred to Petri plates containing MEA, pre-covered with cellophane membrane and the fungal isolates were allowed to grow for DNA isolation.

2.3 DNA Extraction

For isolation of total genomic DNA from the different fungi, a standard cetyl–trimethyl ammonium bromide (CTAB) method previously described (Chang et al. 1993) was used with some modifications. Briefly, pieces of hyphae harvested from the cellophane, was placed in 1.5 ml Eppendorf tube. Some pre-sterilized fine sand

was added followed by 100 µl of CTAB buffer. The sample was homogenized with a micropestle. After homogenization additional 500 µl CTAB buffer was added. Depending on the consistency of the sample, more CTAB buffer was added. The sample was incubated at 65 °C for 1 hour followed by the addition of 1 volume of chloroform: isoamyl alcohol (IAA) (24:1) and was centrifuged at 13 000 rpm for 15 minutes. The supernatant was transferred to a new 1.5 ml Eppendorf tube, 1 volume of chloroform: isoamyl alcohol (IAA) (24:1) was added and centrifuged at 13 000 rpm for 15 min. The upper phase was transferred to a new Eppendorf tube. The DNA was precipitated by adding two volumes of cold isopropanol, left on ice for 30 min and centrifuged for 20 minutes at 13 000 rpm. The pellet was washed by adding approximately 200 µl cold 70% ethanol at a room temperature. The pellet was re-suspended in 40 µl TE buffer (1 ml 1M TRIS-HCl, 0.2 ml 0.5 M EDTA pH 8).

2.4 PCR Amplification

The ITS regions were amplified using polymerase chain reaction (PCR) with primer pair, ITS1 and ITS4 (Gardes and Bruns 1993). Negative control was used to check for contaminations. The 25.0 µl reaction mixture included 20.2 µl PCR-quality water, 2.5 µl Biotools Buffer (10×), 0.5 µl of each primer (25 µM), 0.5 µl dNTPs (10 mM), 0.5 µl Biotools DNA polymerase (1 U/µl, –20 °C), and 0.8 µl DNA template. Cycling reactions were run on a PTC-100 Programmable Thermal Controller with the following conditions: 95 °C for 4 min; 39 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 50 s, and 72 °C for 7 min. Ethidium bromide was used to detect DNA bands on a 1% agarose gel and the visual detection was made by ultraviolet transillumination.

2.5 DNA Sequencing

The PCR products were cleaned and sequenced using either ITS1 or ITS4 primer at the Functional Biosciences, Inc. in Madison, USA (http://www.functionalbio.com/contact.htm).

2.6 BLAST Analysis and Sequence Identity

ITS rDNA sequences were obtained only for 50 representative isolates of morphotypes. These sequences were used for BLAST (Altschul et al. 1997) searches against GenBank / NCBI (Sayers et al. 2010) to provide taxonomic identification. The sequences were cleaned with an open source software utility (http://www.emerencia.org/FungalITSextractor.html) to extract the highly variable ITS1 subregion from fungal nuclear ITS sequences (Nilsson et al. 2010). The sequences with ≥97% similarity and the query coverage with ≥98% were set to constrict operational taxonomic units (OTUs) (Arnold and Lutzoni 2007) and the sequences were assigned to the matching species, taxon or order based on the closest BLAST matches and some morphological descriptions. The sequences were deposited to GenBank with the following accession numbers HM240795–HM240843.

2.7 Statistical Analysis

Simultaneous effects of season, latitude and needle age to fungal average frequencies were tested with a linear mixed model on SPSS 19 (Chicago, IL, USA). The latitude (North, Central and South), season (fall, winter and spring) and the year of the needle (1 or 2) were used as fixed factors. The site (inside the latitude) and needle pair was chosen as random factors. The tree was treated as random subject. With this test we performed also the pairwise comparison, which gave the p-values between factors.

2.8 Mycota Diversity Analyses

Species richness was calculated for the OTUs diversity analyses for the first and second year needles. Diversity across all geographical locations and seasons were calculated using Shannon-Wiener, Fisher's α, Chao-1 and Chao-2 indices. OTU richness between seasons and sites were estimated using the similarity indices: Morisita-Horn, Bray-Curtis, Classical Jaccard and Classical Sorensen. Chao et al. (2005) showed that Jaccard's and Sorensen's classic similarity esti-

mates function is poor when there are many rare species (e.g. singletons = isolated only once). In this study, due to high number of singletons only Morisita-Horn and Bray-Curtis similarity index estimates were considered for the outcome of the results. All analyses of the diversity were conducted with EstimateS Win820 version 8.0 (Colwell 2005). We analyzed all the combined isolations from 1st and 2nd year needle for diversity between the different seasons and sites.

3 Results

3.1 Diversity of the Mycota

A total of 810 Scots pine short shoots (1620 needles) were sampled for mycota. The 3868 fungal isolates were assigned to 68 OTUs (including both identified and morphologically distinct but unidentified types). The distribution of isolates among the 68 operational taxonomic units (OTUs) was divided into a few common taxa and many rare taxa (Table 1). Most of these OTUs (44) were placed in Ascomycota, with a small number in Basidiomycota (2) (Table 1). The fungal isolates were found predominantly in three classes within Ascomycota: Leotiomycetes (12 taxa), Sordariomycetes (11 taxa), Dothideomycetes (11 taxa), and with one taxon in Euritiomycetes (Table 1). One OTU was most common to all sites and seasons (*Hormonema dematioides* Lagerberg & Melin ~21% of all isolates), eleven other genera were quite common (2 to 10% of all isolates). These twelve commonly observed fungal species were considered as major components of the mycota (Tables 1, 2). The remaining identified genera, each of which accounted for less than 2% of the isolates, were considered to be rare (Table 1).

All the diversity indices suggest relatively high versatility of the mycota community (Table 3). The Shannon-Wiener's diversity index is expected to vary from 1.5 to 3.5 where 3.5 indicate the highest diversity. In the present study, the Shannon-Wiener's indices were between 2.97 to 3.22 (Table 3). Chao-1 formula estimates the number of missing species based on the number of singletons and doubletons in the sample. Estimated numbers of species varied from the observed number of species, suggesting that the mycota was under sampled, based on the singletons (Table 3). The diversity indices were always the highest in the South Finland and in the spring season (Table 3).

3.2 Needle Age

In every site the abundance and number of different species increased slightly with needle age (Table 3), but it was not statistically significant. Similar trend was observed in the different seasons with species diversity indices increasing with the needle age (Table 3).

3.3 Effect of Latitudinal Location

The species richness and frequencies of the different fungal OTUs increased from North to South Finland (Table 3). A total of 994, 1408 and 1466 fungi were isolated from the North, Central and South Finland, respectively (Fig. 1). Both diversity indices increased from the North to South Finland within 1st and 2nd year needles (Table 3). All the similarity indices (except the Morisita-Horn similarity index for 1st year needles) showed that the highest similarity were between Central and South Finland and lowest between North and Central or North and South Finland (Table 4). Significant differences were found between latitudes (Table 5) and the pairwise comparison revealed the statistical difference between North and South Finland (Table 6). The most frequently isolated fungal OTUs in North, Central and Southern Finland included *Hormonema dematioides* (nr. 1001), *Lophodermium conigenum* (Brunaud) Hilitz (nr. 1042), *Epicoccum* sp. (nr. 1003), *Alternaria* sp. (nr. 1004) and unidentified (nr. 1002) (Table 1, 2).

3.4 Seasonal Differences (Fall, Winter and Spring)

The number of different fungal OTUs increased from fall to winter and decreased again in spring (Table 3). However the frequencies of OTUs

Table 1. GenBank BLAST matches to the ITS1-sequence of representative isolates.

Isolate	GenBank accession no. of best matches	Mi(%) / Qc(%) [a]	Description of of best matches	Our definition of isolation	Site [b]	Season [c]	Freq.	Class [d]	Phylum [e]
1001	U50913	100/99	*Hormonema dematioides*	*Hormonema dematioides*	N C S	F W S	811	D	A
1002	AY971697	87/67	Fungal sp.	Unidentified	N C S	F W S	315		A
1003	HQ414592	100/100	*Epicoccum nigrum*	*Epicoccum* sp.	N C S	F W S	261	D	A
1004	EF110523	100/100	*Alternaria astragali*	*Alternaria* sp.	N C S	F W S	209	D	A
1005	AY971706	100/99	Fungal sp.	Unidentified	N C S	F W S	125	S	A
1007	EF432296	100/100	*Alternaria* sp.	*Alternaria* sp.	N C S	F W S	17	D	A
1009	AY561198	100/100	*Rosellinia* sp.	*Rosellinia* sp.	CS	F W S	40	S	A
1010	EU627019	87/100	Ascomycete sp.	Unidentified	N C S	F W S	28		A
1011			Unidentified	Unidentified	S	W	1		
1012			Unidentified	Unidentified	N S	F W	35		
1013	GU367606	97/100	*Phacidiopycnis washingtonensis*	Leotiomycetes sp.	C S	F W S	36	L	A
1014			Unidentified	Unidentified	C S	W S	13		
1015	AY560007	99/98	Foliar endophyte	Unidentified	S	F W S	18	D	A
1016	AY373898	100/100	*Penicillium brevicompactum*	*Penicillium* sp.	N S	F W S	95		A
1017			Unidentified	Unidentified	C S	F W S	31		
1019	AJ279479	99/100	*Arthrinium* sp.	*Arthrinium* sp.	S	W	2	S	A
1020	AB566283	98/100	*Arthrinium* sp.	*Arthrinium* sp.	N C S	F W S	49	S	A
1021	AF400263	98/100	*Trichoderma strictipilis*	*Trichoderma* sp.	N S	F W S	13	S	A
1022			Unidentified	Unidentified	N C S	F W S	70		
1024	GU931724	98/100	Uncultured Dothideomycetes	Unidentified	N C S	F W S	42		A
1025	AM262381	100/100	Fungal endophyte	Unidentified	N S	W	11		A
1026	AF377300	99/100	*Truncatella angustata*	*Truncatella* sp.	S	W S	16	S	A
1028			Unidentified	Unidentified	N C S	F W S	114		
1030	AB516665	100/100	*Dumontinia tuberosa*	Helotiales sp.	S	S	2	L	A
1031	AY616702	99/98	*Hypoxylon fuscum*	*Hypoxylon* sp.	C S	F W S	35	S	A
1032	GU985228	97/100	*Coniothyrium* sp.	Dothideomycetes sp.	N C S	F W S	80	D	A
1033			Unidentified	Unidentified	C S	W S	59		
1034	AF176975	95/100	*Daldinia petriniae*	*Daldinia* sp.	C S	W S	26	S	A
1035	Z81431	97/85	*Encoelia fascicularis*	Helotiales sp.	N C S	F W S	63	L	A
1036	FR668005	98/100	*Mollisia cinerea*	Leotiomycetes sp.	C S	W S	3	L	A
1037			Unidentified	Unidentified	N C S	F W S	12		
1038			Unidentified	Unidentified	N CS	F W S	51		
1039	GQ153091	98/100	*Dothideomycetes* sp.	Dothideales sp.	N S	F W S	17	D	A
1040	FJ196614	84/98	*Nectria* sp.	Hypocreales sp.	C S	W S	2	S	A
1042	HM060650	97/100	*Lophodermium conigenum*	*Lophodermium conigenum*	N C S	F W S	375	L	A

1043	EF530927	99/100	*Hypholoma capnoides*	*Hypholoma* sp.	N C S	F W	120		B
1044	GQ153108	96/97	Leotiomycetes sp.	Ascomycota sp.	C S	F W S	49	L	A
1046	AY546046	93/95	Fungal endophyte	Unidentified	C S	W	10		A
1048	AY775696	99/100	*Lophodermium pinastri*	*Lophodermium pinastri*	N C S	W S	109	L	A
1049	HQ540552	100/100	*Alternaria alternata*	*Alternaria* sp.	C S	F WS	51	D	A
1050	AY188379	89/95	*Tremella* sp.	Basidiomycete sp.	N C S	F W S	18		B
1051	EF420012	92/99	Fungal endophyte	Unidentified	S	W	8		A
1052	AY004787	98/100	*Drechslera biseptata*	*Drechslera* sp.	C S	F W S	2		A
1053	GU453167	95/100	Basidiomycota sp. BCE	Unidentified	N C S	F W S	25		
1054	GQ509269	93/100	Uncultured fungus	Unidentified	N C S	F W	31		
1055	AY354269	99/100	*Mollisia* sp.	Helotiales sp.	N C S	F W	14	L	A
1056	AY354270	99/100	*Mollisia* sp.	Helotiales sp.	S	W	6	L	A
1057	EU314678	98/98	*Mollisia minutella*	*Mollisia* sp.	N	F	12	L	A
1058			Unidentified	Unidentified	S	S	3		
1059	AM999582	100/100	Uncultured fungus	Unidentified	N C S	F W S	83		
1060	DQ069036	92/64	*Mollisia* sp.	Leotiomycetes sp.	S	S	2	L	A
1061	EF160121	91/66	*Neobarya* sp.	Sordariomycetes sp.	C S	W S	9	S	A
1063	AY259136	98/100	*Mollisia melaleuca*	Mollisia sp.	N C S	F W	27		A
1064			Unidentified	Unidentified	C S	F W S	37		
1065			Unidentified	Unidentified	S	S	4		
1066			Unidentified	Unidentified	S	S	2		
1067	FN665419	99/100	*Aureobasidium pullulans*	*Aureobasidium pullulans*	N C	W S	14	D	A
1068	HQ125358	99/100	Uncultured fungus	Unidentified	N	W	1		A
1069			Unidentified	Unidentified	N	W	1		
1070	FJ25190	100/100	*Phoma herbicola*	Dothideales sp.	N	W	1	D	A
1071	HQ161160	99/100	*Pleospora tarda*	Pleosporales sp.	N	W	1	D	A
1072	AY805568	99/100	*Lecytophora* sp.	Sordariomycetes sp.	N	W	1	S	A
1073			Unidentified	Unidentified	C	W	13		
1075			Unidentified	Unidentified	C	W	2		
1078			Unidentified	Unidentified	N	S	28		
1079			Unidentified	Unidentified	N	F S	19		
1080			Unidentified	Unidentified	N	S	11		
1081	HM849615	97/100	*Botryotinia fuckeliana*	*Botryotinia* sp.	N C S	F W S	67	L	A

[a] Mi, Max identity; Qc, Query coverage
[b] N, North; C, Central; S, South Finland
[c] F, Fall; W, Winter; S, Spring
[d] D, Dothiomycetes; S, Sordariomycetes; L, Leotiomycetes
[e] A, Ascomycetes; B, Basidiomycetes

Table 2. Major fungal OTUs and their observed frequencies in different sites and seasons from 1st and 2nd year needles.

Isolate	Our definition of the fungi	North		Central		South		Fall		Winter		Spring	
		1	2	1	2	1	2	1	2	1	2	1	2
1001	*Hormonema dematioides*	125	140	119	132	159	136	191	156	72	95	140	157
1042	*Lophodermium conigenum*	70	72	75	75	27	56	56	62	71	65	45	76
1002	Unidentified	49	31	53	55	75	52	85	76	35	30	57	32
1003	*Epicoccum* sp.	4	6	54	74	47	76	59	78	40	64	6	14
1004	*Alternaria* sp.	18	7	79	64	17	25	25	22	54	52	35	22
1005	Unidentified	26	19	26	22	19	18	50	45	18	8	3	6
1043	*Hypholoma* sp.	32	9	12	19	22	26	15	9	32	17	19	28
1028	Unidentified	9	24	6	26	19	30	0	10	21	48	13	22
1048	*Lophodermium pinastri*	8	19	25	42	9	6	0	0	26	34	16	33
1016	*Penicillium* sp.	35	25	13	4	5	13	5	12	7	1	41	29
1059	Unidentified	7	6	15	16	18	21	4	4	20	12	16	27
1032	Dothideomycetes sp.	19	17	0	0	14	30	0	0	4	8	29	39

1 = 1st year needle isolates frequency
2 = 2nd year needle isolates frequency

Table 3. Isolates diversity indices for the various sites and seasons.

Site/Season	Species richness	Abundance	Fisher's α	Shannon's (*H'*)	Chao 1	Chao 2
North [1)]	33	511	9.86	2.98	45.56	610.34
North [2)]	34	483	10.38	3.01	46.69	680.51
Central [1)]	37	668	11.2	3.15	56.49	60.09
Central [2)]	41	740	12.12	3.15	60.56	67.48
South [1)]	50	707	11.57	3.19	66.5	66.04
South [2)]	52	759	12.65	3.21	67	71
Fall [1)]	35	666	10.17	2.97	47.3	620.25
Fall [2)]	36	642	11.08	2.95	47.61	724.76
Winter [1)]	47	582	11.38	3.13	61.08	59.8
Winter [2)]	52	598	11.92	3.12	59.5	60.89
Spring [1)]	43	638	11.57	3.19	66.5	62.81
Spring [2)]	49	742	12.65	3.21	67	74
North [3)]	39	994	9.43	2.98	50.31	758.28
Central [3)]	43	1408	11.07	3.15	65.36	69.58
South [3)]	58	1466	11.72	3.22	69.88	75.33
Fall [3)]	40	1308	10.6	3.03	56.91	909.07
Winter [3)]	60	1180	11.39	3.15	66.24	66.06
Spring [3)]	50	1380	11.72	3.22	69.88	74.71

[1)] 1st year needles
[2)] 2nd year needles
[3)] 1st and 2nd year needle isolates combined

Table 4. Comparison of the different similarity index among geographical sites and seasons.

	SSa	SSb	SSc	MHa	MHb	MHc	BCa	BCb	BCc	Ja	Jb	Jc	SCa	SCb	SCc
North vs. Central	26	22	23	0.83	0.83	0.81	0.63	0.62	0.61	0.46	0.46	0.44	0.63	0.63	0.61
North vs. South	31	27	25	0.85	0.86	0.80	0.62	0.59	0.59	0.47	0.48	0.41	0.64	0.65	0.58
Central vs. South	41	34	36	0.90	0.86	0.91	0.72	0.67	0.73	0.68	0.64	0.63	0.81	0.78	0.77
Fall vs. Winter	39	30	34	0.79	0.73	0.82	0.57	0.52	0.58	0.64	0.58	0.63	0.78	0.73	0.77
Fall vs. Spring	34	27	30	0.84	0.85	0.81	0.60	0.58	0.58	0.61	0.53	0.55	0.76	0.69	0.71
Winter vs. Spring	42	32	39	0.83	0.79	0.82	0.61	0.56	0.60	0.62	0.55	0.63	0.76	0.71	0.77

SS = Species shared, MH = Morisita-Horn similarity index, BC = Bray-Curtis similarity index, J = Classical Jaccard's similarity index, SC = Classical Sorensen's similarity index
a = Combined estimate (1st and 2nd year needles isolates)
b = First year needle isolate estimates
c = Second year needle isolate estimates

Table 5. Type III tests of fixed effects [a)].

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	79.642	185.824	.000
Latitude	2	79.751	3.357	.040
Season	2	78.808	2.884	.062
Year	1	85.187	2.003	.161

[a)] Dependent variable: frequency.

Table 6. Pairwise comparison for latitude [a)].

Latitude [b)]	Latitude [b)]	Mean difference	Std. error	df	Sig. [c)]	Lower bound	Upper bound
1	2	–.796	.412	80.077	.057	–1.616	.024
	3	–1.016*	.413	79.279	.016	–1.838	–.193
2	1	.796	.412	80.077	.057	–.024	1.616
	3	–.220	.414	79.868	.597	–1.044	.604
3	1	1.016*	.413	79.279	.016	.193	1.838
	2	.220	.414	79.868	.597	–.604	1.044

Based on estimated marginal means
[a)] Dependent variable: frequency.
[b)] 1 = North, 2 = Central, 3 = South
[c)] Adjustment for multiple comparisons: least significant difference (equivalent to no adjustments).
* The mean difference is significant at the .05 level.

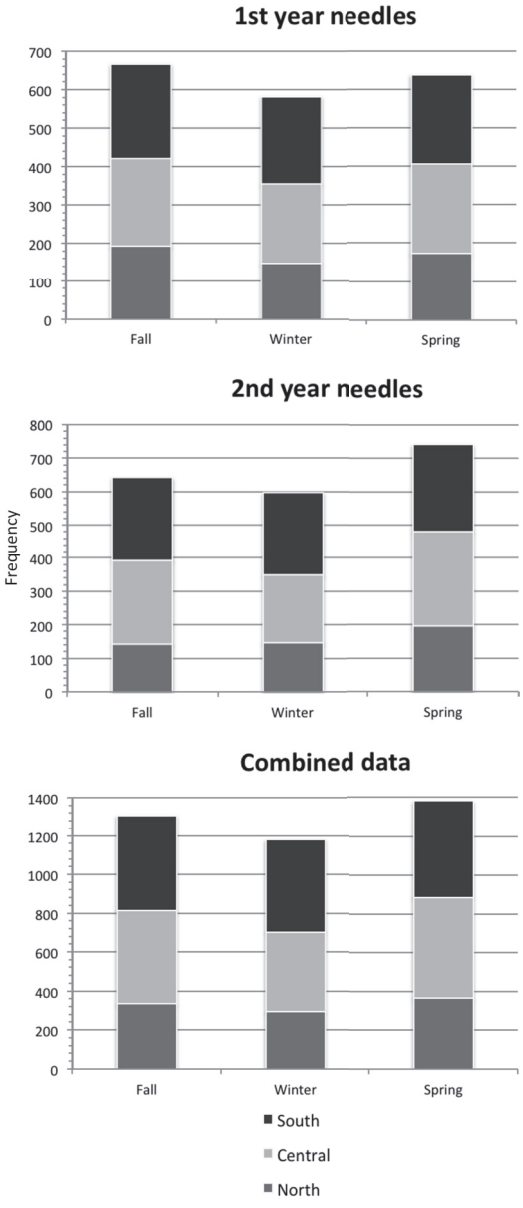

Fig. 1. The frequencies of isolations in the fall, winter and spring seasons in the different sites for 1st, 2nd year needles and combined data. Significant differences were found between sites for the combined data, between North and South Finland in 1st year needles and for North and Central and North and South Finland in the 2nd year old needles.

decreased from fall to winter increasing again in the spring (Table 3, Fig. 1). The number of fungal isolates in fall, winter and spring were 33.5% (1308 isolates), 30.5% (1180) and 36% (1380), respectively (Table 3, Fig. 1). The highest diversity indices for the seasons were in the spring for both needle ages as well as for the combined data (Table 3). The highest similarity between the seasons depended on the needle age; the indices showed the highest similarity between winter and spring for the second year needles and highest similarity between fall and spring for the first year old needles (Table 3). Statistical differences were not observed between seasons (Table 5). The most frequently isolated fungal OTUs in the fall, winter and spring seasons included *Hormonema dematioides* (nr. 1001), *Lophodermium conigenum* (nr. 1042), *Epicoccum* sp. (nr. 1003), *Coniothyrium* sp. (nr. 1032) and unidentified (nr. 1002) (Tables 1, 2).

4 Discussion

4.1 Diversity of the Mycota

In this study, the effect of geographical location, seasonal variation and age of the needles on the diversity and abundance of mycota of Scots pine (*P. sylvestris*) was investigated. Among the mycota, the class Leotiomycetes was found to be the most dominant in needles of *Pinus sylvestris* accounting for 27% of identified Ascomycetes (Table 1). Other authors have similarly reported that Leotiomycetes were the dominant component of the endophytes of *Pinus* spp. needles (Carroll and Carroll 1978, Kowalski 1993, Hata and Futai 1996, Ganley and Newcombe 2006). The most frequently reported fungal endophytes of Scots pine needles include *Lophodermium pinastri* and *Cyclaneusma minus* (Helander et al. 1994, Sieber 2007). *Lophodermium pinastri* is usually a primary colonizer of the symptomless needles of *Pinus* spp. and it is commonly recorded as a saprotroph in needles of Scots pine (Kowalski 1993, Helander et al. 1994, van Maanen and Gourbiére 2000) as well as from needles of other *Pinus* spp. (Carroll and Carroll 1978, Sieber et al. 1999, Botella and Diez 2011). *Lophodermium*

spp. (*L. pinastri* and *L. conigenum*) were the most common Leotiomycetes in this study. However it was not the most abundant isolate; this could be a consequence of competition between other species. As the most abundant isolate, the *Hormonema dematioides*, might have an antagonistic effect on the other common needle fungi reported in this study. *Hormonema dematioides* is frequently isolated from needles of various conifer species (*Picea mariana* (Mill.) BSP., Sokolski et al. 2007; *P. glauga* (Moench) Voess, Stefani and Berube 2006a; *Pinus nigra* Arn., Jurc et al. 1996; *P. cembra* L., *P. mugo* Turra and *Larix decidua* Mill., Schnell 1987). It has also been reported from needles of *Pinus sylvestris* (Kowalski 1993). Ganley and Newcombe (2006) noted that *Lophodermium* species were absent with higher occurrence of *Hormonema* and *Cladosporium* species, suggesting that these species could have some antagonist influence against *Lophodermium* species. *Hormonema* sp. has been reported to produce toxic secondary metabolites (Polishook et al. 1993). Ganley and Newcombe (2006) and Kowalski (1993) observed increased numbers of *Hormonema dematioides* specimens when fungi from the genera *Alternaria*, *Cladosporium* and/or *Epicoccum* were common. In this study, *Alternaria* and *Epicoccum* species were also one of the major fungal lineages observed. It is possible that these fungal species do promote the growth of *Hormonema* species at the expense of *Lophodermium* species. In Finland *Hormonema* spp. are frequently isolated from leaves of White Birch, *Betula pubescens* Ehrh., and Silver Birch, *B. pendula* Roth, (Helander et al. 2006, Helander et al. 2007) and also from Scots pine (Ranta et al. 1994, Helander 1995). *Hormonema* species appears to form common endophyte interactions with different host trees in Finland.

A common pathogenic isolate (*Cyclaneusma minus*) (Kowalski 1993, Helander et al. 1994) and endophyte (*Cenangium ferrucinosum*) (Helander 1995) of symptomless Scots pine needles were not observed in this study. These species have been reported as needle endophytes and the distribution are known to cover the northernmost regions of Finland where Scots pine grow (Helander et al. 1994, Helander 1995).

Interestingly, one of the reported OTUs *Hypholoma* sp. is a basidiomycete that usually grows on decaying wood. *Hypoxylun* sp. is another saprotroph recovered in this study. It has earlier been reported as a foliar endophyte of *P. glauca* (Stefani and Berube 2006b). *Hypoxylun fuscum* Fr. occurs as a pathogen or saprotroph on *Alnus incana* (L.) Moensch (Domanski and Kowalski 1987). Other isolates noted in this study such as *Penicillium* species are among the most common microfungi on decomposed wood (Crawford et al. 1990), although they are not considered as aggressive agents of wood decay (Seifert and Frisvad 2000). *Penicillium brevicompactum* Dierckx have been reported to degrade cellulose in vitro (Domsch et al. 1980) and it has been recorded from soft rot of timber (Seehan et al. 1975). *Botrytiana fuckeliania* (De Bary) Whetzel (teleomorph of *Botrytis cinerea* (De Bary) Whetzel) is the causal agent of grey mould diseases observed from many vegetable, ornamental crops and fruit, and it has a broad geographic distribution (Bulit and Dubos 1988). *Botrytis cinerea* have also been isolated from green symptomless needles of several *Pinus* spp. (Zamora et al. 2007). The presence of these saprotrophic or pathogenic fungal genera on symptomless pine needles cannot be explained but it is possible that they have a cryptic cycle as endophytes or epiphytes in conifer needles besides their known saprotrophic or pathogenic life stages on softwood or hardwood.

4.2 Needle Age

The older needles with longer exposure to conidia and spores were expected to be more likely to be colonized by fungi. In some coniferous trees as *Pinus tabulaeformis* Carr. and *Pinus strobes* L., several authors have reported that the infection rate tends to increase with needle age (Deckert and Peterson 2000, Guo and Wang 2008); similar results have been reported from some broadleaves (Herre et al. 2007, Osono 2008). Also the frequencies of fungi associated with needles of *P. sylvestris* have been reported to increase with needle age (Kowalski 1993, Helander et al. 1994). By contrast, in this study, no significant differences were observed in the frequencies of fungi isolated from first and second year needles. Hata et al. (1998) suggested that the frequency of *Pinus* spp. needle endophytes depends on increased chance

of infection, time of needle flush, improved habitat conditions and competition with other fungi. It is possible that greater differences could have been found if older needles were sampled, as the structure and physical barriers of the first and second year needle may not be very distinct.

4.3 Latitudinal or Geographical Distance

Many authors have reported increases in fungal diversity with decreasing latitude (Hawksworth 1991, 2001). In this study, diversity indices and abundance of fungi decreased at higher latitude (Table 3). Significant differences in frequencies of fungi were found between North and South Finland (Table 6). Sokolski et al. (2007) have noted that as the diversity of tree species surrounding *P. mariana* decreased from the southern region to the northern region, fungal endophyte diversity also decreased. The diversity indices for fungal isolates indicated that the similarity between sites were the highest between Central and South Finland and lowest between South and North Finland. Our results indicate that when latitude increases, the abundance and diversity of fungi decreases in the needles of Scots pine. By contrast, Higgins et al. (2007) have reported high diversity of endophytic fungi at higher latitude. Furthermore, latitude may not be directly involved in influencing fungal diversity; rather, indirect factors such as site characteristics, climate, humidity, abiotic and geographic structure may be more influential on the mycota.

4.4 Seasonal Variation

Guo and Wang (2008) have reported that seasons affect colonization and number of fungi associated with *Pinus tabulaeformis* needles in this order; spring>winter>autumn>summer. They indicated that the high precipitation could facilitate the dispersal of fungal spores in the spring time. Some other authors have also reported higher species richness in spring (Collado et al. 1999, Martín et al. 2004). Martín et al. (2004) observed that the diversity of fungal species was significantly higher in the spring, and they found that isolation frequencies for most of the dominant species were dependent on the season. Helander et al. (1994) on the other hand found that the age of needle affected the seasonal fungal frequencies. I**n** young Scots pine needles, the colonization by fungi increased during the summer, whereas **in** older needles no seasonal **variation** was detected. Zamora et al. (2007) observed that the species composition of the different fungi of needles of four different *Pinus* species were highest in the autumn versus spring season. In their study the autumn season had greater rainfall average which they implicated to promote the diversity of fungal assemblage. In this study the frequencies of fungal OTUs decreased from fall to winter and increased again in the spring. Species richness increased from fall to winter and decreased again in spring, leaving the highest richness indices pointing to spring season (Table 3). Significant statistical differences were not observed between frequencies between seasons (Table 5). The fluctuations in the frequencies were quite similar for each geographical location and between needle ages (Fig. 1). In our study, the highest rainfall was in the fall season, but the frequencies of fungi were the highest in spring and the species richness was highest in the winter. It seems that for the fungal colonization of needles, many factors beside the weather such as nutrient limitations, competitors/antagonists and temperature are influential.

4.5 Functional Significance of the High Number of Mycota

Many of the fungi identified in this study have been reported as endophytes by previous authors (Kowalski 1993, Helander et al. 1994, 2006, 2007, Helander 1995, van Maanen and Gourbière 2000, Stefani and Berube 2006a,b, Sokolski et al. 2007). Fungal endophytes that colonize inner grass leaf tissue are known to exert beneficial effects on the hosts through increased resistance to herbivores, pathogens, and drought (Kuldau and Bacon 2008, Saikkonen et al. 2010). Endophytic fungi colonisation may improve the host's ecological adaptability and enhance tolerance to environmental stresses by producing antimicrobial metabolites against phytopathogens (Schulz et al. 1995, 1998, Schulz and Boyle 2005). Arnold et al. (2003) have demonstrated that inoculation of endophyte-free

leaves with endophytes significantly decreases both leaf necrosis and leaf mortality when a tropical tree, Theobroma cacao L., seedlings were challenged with a major pathogen (Phytophthora sp.). Arnold et al. (2003) suggested that the capacity of diverse, horizontally transmitted endophytes of woody angiosperms could play an important role in host defense which was previously not properly recognized. Ganley et al. (2008) showed that fungal endophytes (commonly found in nature) can mediate resistance against pathogen (*Cronartium ribicola* J.C. Fisch) and thereby increase host fitness in *Pinus monticola* Douglas ex D. Don. They reported that resistance derived from endophytes is a form of induced resistance. Andersson et al. 2010 also demonstrated that leave endophytes of rubber tree (*Hevea brasiliensis* Müll. Arg.) have inhibitory activity on the growth of the causal agent of South American Leaf Blight, *Microcyclus ulei* (Henn.) Arx. These results suggest that fungal endophytes could play a vital role on host fitness against pathogens and pests (Arnold et al. 2003, Ganley et al. 2008, Andersson et al. 2010). In this study, the isolated mycota were very diverse and ranged from known endophytes with host adaptations to some latent saprotrophs and pathogens. It is possible that these plant-endophyte interactions have a beneficial effect to the host during extraneous stress situations.

4.6 Technical Considerations

In this study, the numbers of unculturable fungi were not taken into consideration which is likely to have led to under-estimation of the actual population of the needle mycota. Both culture-based and molecular methods have been established in order to study fungal population structure (Duong et al. 2006, Unterseher and Schnittler 2010). The problem with these methods is that many fast-growing fungi will be isolated in favour of unculturable or slow-growing fungi (Hyde and Soytong 2007, Hyde and Soytong 2008, Unterseher and Schnittler 2009). Many uncultureable fungi may escape detection as they are unable to grow in culture and are subsequently not isolated (Guo et al., 2001, Duong et al. 2006, Hyde and Soytong 2007, Tao et al. 2008). Molecular methods such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) or PCR product pyrosequencing could be used to overcome such limitations (Duong et al., 2006 Tao et al. 2008, Nilsson et al. 2009).

Finally, the results of the present study show that factors associated with latitudinal differences have an impact on the abundance of fungi.

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