Mycorrhiza (2005) 16: 33–41
DOI 10.1007/s00572-005-0011-z

ORIGINAL PAPER

Audrius Menkis · Rimvydas Vasiliauskas · Andrew F. S. Taylor · Jan Stenlid · Roger Finlay

Fungal communities in mycorrhizal roots of conifer seedlings in forest nurseries under different cultivation systems, assessed by morphotyping, direct sequencing and mycelial isolation

Received: 11 January 2005 / Accepted: 20 June 2005 / Published online: 22 September 2005

Abstract Fungi colonising root tips of *Pinus sylvestris* and *Picea abies* grown under four different seedling cultivation systems were assessed by morphotyping, direct sequencing and isolation methods. Roots were morphotyped using two approaches: (1) 10% of the whole root system from 30 seedlings of each species and (2) 20 randomly selected tips per plant from 300 seedlings of each species. The first approach yielded 15 morphotypes, the second yielded 27, including 18 new morphotypes. The overall community consisted of 33 morphotypes. The level of mycorrhizal colonisation of roots determined by each approach was about 50%. The cultivation system had a marked effect on the level of mycorrhizal colonisation. In pine, the highest level of colonisation (48%) was observed in bare-root systems, while in spruce, colonisation was highest in polyethylene rolls (71%). Direct internal transcribed spacer ribosomal DNA sequencing and isolation detected a total of 93 fungal taxa, including 27 mycorrhizal. A total of 71 (76.3%) fungi were identified at least to a genus level. The overlap between the two methods was low. Only 13 (13.9%) of taxa were both sequenced and isolated, 47 (50.5%) were detected exclusively by sequencing and 33 (35.5%) exclusively by isolation. All isolated mycorrhizal fungi were also detected by direct sequencing. Characteristic mycorrhizas were *Phialophora finlandia*, *Amphinema byssoides*, *Rhizopogon rubescens*, *Suillus luteus* and *Thelephora terrestris*. There was a moderate similarity in mycorrhizal communities between pine and spruce and among different cultivation systems.

Keywords Mycorrhizal fungi · Afforestation · Seedling cultivation systems

Electronic supplementary material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00572-005-0011-z and accessible for authorised users

A. Menkis (✉) · R. Vasiliauskas · A. F. S. Taylor · J. Stenlid · R. Finlay
Department Forest Mycology and Pathology,
Swedish University of Agricultural Sciences,
P.O. Box 7026, 750 07 Uppsala, Sweden
e-mail: Audrius.Menkis@mykopat.slu.se
Tel.: +46-18-672727
Fax: +46-18-673599

A. Menkis
Lithuanian Forest Research Institute,
Liepų 1, Girionys,
53101 Kaunas region, Lithuania

Introduction

The European Union is expected to spend nearly €6 billion on rural development of new member states during 2004–2006, and afforestation of abandoned agricultural land is an important issue in this context (The European Commission press release 2004). Large-scale afforestation of abandoned agricultural land is therefore expected to increase the demand for planting material, in particular of the conifers *Pinus sylvestris* and *Picea abies*, that are the most commonly used tree species for afforestation in central and northern Europe.

Fungal colonisation of root systems is an important factor in determining seedling vigour and, consequently, their quality (Smith and Read 1997). Several mycorrhizal fungi have been shown to have a positive impact on seedling health and productivity in forest nurseries (Jumpponen 2001; Sampagni et al. 1985; Sinclair et al. 1982; Stenström et al. 1997). Furthermore, following the transfer and outplanting of the seedlings into the field, mycorrhizal fungi promote survival, establishment and growth of young trees in newly established forest plantations (Kropp and Langlois 1990; Le Tacon et al. 1994; Perry et al. 1987; Stenström et al. 1990). The main mechanisms for this are thought to be enhanced uptake of water and nutrients (Smith and Read 1997), lengthened root life (Wilcox 1996) and protection against environmental stress factors such as drought, pathogens and heavy metal pollution (Chakravarty and Unestam 1985; Colpaert and Vanassche 1992; Morin et al. 1999; Ortega et al. 2004; Van Tichelen et al. 2001). Conversely, the presence of pathogenic fungi in seedling roots has adverse effects on their survival (Lilja et al. 1992).

Due to the absence of host trees, abandoned agricultural land may often lack any natural mycorrhizal inoculum, while pathogens may be present (Wilberforce et al. 2003). Consequently, failure in afforestation has previously been attributed to the absence of suitable mycorrhizal fungi (Bjorkman 1970; Marx 1980; Mikola 1970). One way of overcoming this problem would be to pre-inoculate seedlings with mycorrhizal fungi, and this possible option has been investigated in several recent studies (Scagel and Linderman 1998; Pera et al. 1999; Quoreshi and Timmer 2000; Baum et al. 2002; Dunabeitia et al. 2004; Teste et al. 2004). This would, however, require additional production efforts and costs. Alternatively, the promotion of natural mycorrhizal colonisation of plants under nursery conditions could also be a suitable solution (Cordell and Marx 1994). It is known that different cultivation systems may influence the diversity and relative abundance of root colonising fungi (Grogan et al. 1994; Lilja et al. 1992). Therefore, before selecting a cultivation system for seedling production, it would be desirable to know which systems promote mycorrhizal colonisation and by what taxa of fungi.

Previous studies on root-associated fungal communities in forest nurseries have mainly been done by morphotyping (morphological and anatomical identification) of mycorrhizal root tips (Grogan et al. 1994; Ursic et al. 1997), fungal isolation (Kope et al. 1996; Lilja et al. 1992) or a combination of both these methods (Danielson et al. 1984; Ursic and Peterson 1997). More recently, it has been demonstrated that direct sequencing of fungal DNA from root tips could be a powerful tool for identification of fungi (Egger 1995; Horton and Bruns 2001). The method has proved to be sensitive for the detection of potentially all root-inhabiting fungi, in particular, the taxa that are usually overlooked both by morphotyping and isolation, e.g. latent pathogens or slow-growing endophytes (Kernaghan et al. 2003).

The main aim of the present work was to investigate the extent of mycorrhizal colonisation and the fungal community structure in fine roots of *P. sylvestris* and *P. abies* seedlings under different nursery cultivation systems. Fungal community structure was determined by three different methods: morphotyping, direct DNA sequencing, and pure culture isolation followed by morphological identification or DNA sequencing.

Materials and methods

Site conditions and fieldwork

Pinus sylvestris and *Picea abies* seedlings were collected from six forest nurseries situated within a radius of 150 km in the south-western and central part of Lithuania (Table 1). Within the region, mean annual precipitation is about 660 mm, and the length of the growing season is about 190 days. Average temperature during the growth season is about 14°C. The investigated nurseries are owned by the State Forest Enterprise and produce plant material using standardised methods, including bare-root cultiva-

Table 1 Location of six forest nurseries in Lithuania where the influence of different cultivation systems upon the fungi associated with *P. sylvestris* and *P. abies* seedlings was investigated

Nursery	Location	Age (years)	No. of plants/root tips sampled	Cultivation system[a]	Mycorrhization (%)	
					Plants[b]	Roots[b]
P. sylvestris						
Dubrava	54°50′ N 24°06′ E	2	55/1506	Bare root	100 A	56.5 A
Kelme	55°39′ N 22°38′ E	2	55/1607	Bare root	98.2 A	49.6 B
Kulautuva	54°58′ N 23°38′ E	2	55/2084	Bare root	100 A	37.6 C
Veisejai	54°06′ N 23°44′ E	2	55/2041	Bare root	92.7 B	50.7 B
Varena	54°58′ N 24°30′ E	1	55/1874	Greenhouse	45.5 C	19.4 D
Tytuvenai	55°35′ N 23°05′ E	2	55/2593	Plastic trays	100 A	41.9 E
All *P. sylvestris*		1–2	330/11705	All systems	89.4	42.0
P. abies						
Dubrava	54°50′ N 24°06′ E	3	55/3522	Bare root	100 A	22.0 A
Kelme	55°39′ N 22°38′ E	3	55/1504	Bare root	100 A	43.0 B
Kulautuva	54°58′ N 23°38′ E	3	55/3966	Bare root	94.5 A	44.1 B
Veisejai	54°06′ N 23°44′ E	4	55/3346	Polyethylene rolls	96.4 A	57.8 C
Varena	54°58′ N 24°30′ E	4	55/3780	Polyethylene rolls	100 A	82.7 D
Tytuvenai	55°35′ N 23°05′ E	2	55/2343	Plastic trays	100 A	65.9 E
All *P. abies*		2–4	330/18461	All systems	98.5	53.0
All plants Investigated		1–4	660/30166	All systems	93.93	48.71

[a]Seedlings cultivated as bare root, growing in an open field nursery; greenhouse, bare rooted in the greenhouses; in plastic trays, containerized in interconnected plastic pots; in polyethylene rolls, in peat wrapped in a polyethylene roll

[b]Root tips with characteristic structures of mycorrhizal fungi were counted as mycorrhizal roots; plants with at least one mycorrhizal root tip were considered as mycorrhizal plants. Within a column of respective tree species, values followed by the same letter are not significantly different at $p \geq 0.05$

tion (outdoor & greenhouse), containerized plastic tray systems and polyethylene rolls. The soil in which bare-root seedlings were grown was derived from a sandy loam podzol. The substrate in plastic trays was a peat/sand mixture (3:1), while pure peat was used in polyethylene rolls and in the greenhouse cultivation systems. In nurseries, fertilizers are applied routinely at annual levels of 34–56 kg N ha^{-1} for bare-root seedlings in the field system and 0.11–0.15 kg N m^{-3} of substrate for bare-root greenhouse systems (Juska et al. 1982) as well as for both types of containers.

Samples were collected in April 2001 and 2002. The numbers of plants and their respective cultivation systems are shown in Table 1. After collecting, the root systems were excised from the stems, individually packed into plastic bags, transported to the laboratory and kept at 4°C for a maximum period of 4 weeks.

Morphotyping

The fungal communities associated with the root system were examined in two ways: (a) examination of a high number of root tips from a small number of plants (intensive sampling); this was done in 2001 and included approximately 10% (about 43–952 per plant) of all root tips from 25 seedlings of *P. sylvestris* [for five 1-year-old *P. sylvestris* seedlings from the greenhouse, 100% of the root tips (about 61–335 per plant) were morphotyped] and 30 seedlings of *P. abies* and (b) examination of a small number of root tips from a high number of plants (extensive sampling); this was done in 2002 and included 20 root tips from each of 300 seedlings of *P. sylvestris* and 300 of *P. abies*. The total numbers of plants and root tips morphotyped are presented in Table 1.

For intensive sampling, approximately 10% tips of the root systems from 55 studied plants were selected for the examination as follows. After washing the whole root system in tap water, it was cut into segments 20 mm in length, which were evenly dispensed in water in a 240×240×25 mm hyaline plastic dish on which a grid (100 squares in size 24×24 mm) was drawn. Ten squares in the grid (approximately 10% of the roots) were chosen at random, using coordinates derived from random number tables. All root tips present in the target squares were then selected for morphotyping. For the second approach, the root systems of another 600 seedlings were then washed as described above, and 20 single root tips from each plant were collected from different parts of the root system using forceps.

Mycorrhizal tips were identified by the presence of a mantle, external hyphae or rhizomorphs, the absence of root hairs, a slightly swollen apex and, in pine, dichotomous branching of the fine roots. Macroscopic features were examined using a Carl Zeiss Stemi 2000-C dissection microscope (Oberkochen, Germany). In the absence of macroscopic mycorrhizal features, sections were made of root tips using a razor blade to verify the presence of a Hartig net. Root squashes were used to examine the mantle, hyphae and rhizomorphs microscopically. These features were examined using a Carl Zeiss Axioplan microscope (Oberkochen) at 1,000× magnification. Each morphotype was accurately examined and thoroughly compared with available illustrative material in Agerer (1986–1988) and Agerer et al. (1996–1998). The morphotypes, which did not match any of those presented in cited material, were classed as unidentified, grouped accordingly to morphological characters and given the descriptive name (e.g. "brown", "carroty", "dark purple", etc.). Following the examination, up to five mycorrhizal root tips of each morphotype were taken from each root system and placed separately in 1.5-ml centrifuge tubes, labelled and stored at −40°C for direct DNA sequencing.

Direct sequencing

From each of 33 observed morphotypes, 1–22 individual root tips were selected for direct sequencing. More replicates were taken from more common morphotypes, to encompass, when available, both tree species and different cultivation systems. A total of 130 root tips were subjected to sequencing of the internal transcribed spacer (ITS) of the fungal ribosomal DNA (rDNA). Extraction of DNA, amplification and sequencing followed the method described by Rosling et al. (2003). The fungal-specific primer ITS1-F (Gardes and Bruns 1993) and universal primer ITS4 (White et al 1990) have been used for amplification by PCR. If only one DNA band was present per sample (confirming that all DNA came from one source only), the product was used for sequencing. Multiple-banded PCR products (up to four amplicons) were separated on 2% agarose gels and gel plugs were cored from the bands with pipette tips. Separated bands were re-amplified with universal primers ITS1 (internal to ITS1-F) and ITS4, and resulting single-banded products were sequenced in both directions using the same primers as for PCR amplification.

Isolation into pure culture

The isolation of fungal cultures was attempted from 8,535 individual mycorrhizal root tips collected during 2001 and included multiple representations of 12 out of the 15 morphotypes recognized. Three rare morphotypes represented by only one or two root tips (brownish, dark brown and pale reddish) were not used during isolation, since they were utilized during direct sequencing.

Before isolation, root tips were placed in 10×20 mm net bags (mesh size 0.2×0.2 mm), sterilised in 33% hydrogen peroxide for between 15 and 60 s and then rinsed three times in sterile deionised water (Danielson 1984; Sieber 2002). Tips were plated onto modified Melin Norkrans medium (Marx 1969) and incubated at room temperature in the dark. Dishes were checked daily and emerging cultures were transferred onto fresh agar medium. Fungal mycelia were examined under a Carl Zeiss

Axioplan microscope, equipped with 25× magnification, long distance objective, and were grouped accordingly to morphological characteristics. For identification, one to five representative cultures from each morphological group were ITS rDNA sequenced. Extraction of DNA, amplification and sequencing followed the method described by Rosling et al. (2003). Representatives from 15 sporulating cultures that had not been taxonomically defined by sequencing were sent for identification to the Central Bureau of Fungal Cultures (CBS) in Utrecht, the Netherlands. All strains sequenced in this work are deposited in the culture collection of the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala.

Identity of sequences

Databases at both GenBank (Altschul et al. 1997) and at the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala, were used to determine the identity of sequences. The criteria used for deciding on the taxon or genus for a given strain was its intra- and interspecific ITS sequence similarity to those present in the databases. Here, for each taxon, an individual approach was taken, as the extent of ITS variation differs from species to species or genus to genus. During the present work, however, in most cases, intraspecific ITS similarity for the sequenced fungi was 98–100%, and the similarity within genera varied between 90 and 97%.

Statistical analyses

Possible differences in mycorrhizal colonisation, morphotype/species richness and structure of fungal communities among the cultivation systems, nurseries and tree species were examined. The extent of colonisation was compared by chi-squared tests calculated from actual numbers of observations (presence/absence data) (Fowler et al. 2001). Morphotype richness was analysed by calculating their (species) accumulation curves (SACs) that show the relationship between the cumulative number of taxa found and the sampling intensity (Colwell and Coddington 1994). R computer language (Ihaka and Gentleman 1996) was used to calculate SACs. Fungal community structure was compared by calculating qualitative (S_S) Sorensen similarity indices (Magurran 1988).

Results

The amount of trees and roots examined during the present work in each cultivation system, the frequencies of observed mycelial morphotypes as well as fungal taxa detected by isolation and direct sequencing are presented in Electronic supplementary material.

Mycorrhizal colonisation and morphotypes

In both pine and spruce, 92.7–100% of studied plants were colonised by mycorrhizal fungi, and the differences between different nurseries and cultivation systems were usually not significant. The exception to this was greenhouse-cultivated pine, where only 45.5% of seedlings were mycorrhizal (Table 1). The level of mycorrhizal colonisation varied considerably between individual plants, and significant differences were observed between plants from different nurseries with the same cultivation system, as in bare-root pine and spruce, or in polyethylene rolls of spruce (Table 1). In all cases, the cultivation system had a profound impact on mycorrhizal colonisation of both tree species. In pine, mycorrhizal roots were most commonly encountered in plants from a bare-root cultivation system (3,466 mycorrhizal tips among 7,238 examined or 47.9%), followed by plants from plastic trays (1,087 among 2,593 or 41.9%) and the greenhouse (363 among 1,874 or 19.4%) (Table 1). All comparisons between the cultivation systems were highly statistically significant (chi-squared test, $p<0.0001$). In spruce, mycorrhizal roots were most commonly encountered in plants from polyethylene rolls (5,061 among 7,126 examined or 71.0%), followed by plants from plastic trays (1,545 among 2,343 or 65.9%) and the bare-root cultivation system (3,171 among 8,992 or 35.3%) (Table 1). All comparisons between the cultivation systems were also highly statistically significant (chi-squared test, $p<0.0001$). When grown under similar cultivation systems, pine and spruce were colonised by mycorrhizal fungi to differing extents. In bare-root systems, 47.9% of all pine roots and 35.3% of all spruce roots had mycorrhiza (chi-squared test, $p<0.0001$). The situation in plastic trays was reversed and 41.9% of pine roots were mycorrhizal, but 65.9% of spruce roots formed mycorrhiza (chi-squared test, $p<0.0001$).

A total of 33 distinct morphotypes were observed among the total of 30,166 root tips from 660 plants, studied during the present work, 14 of which were unique for pine, 7 for spruce and 12 (36.4%) were found on both tree species. The most common morphotypes on pine, both bare root and from plastic trays (cultivation systems that promoted the most mycorrhizal colonisation), were unidentified no. 20 ("yellow brown"), "*Thelephora*", "*Piceirhiza bicolorata*" and "*Suillus luteus*", observed on 12.7–94.5% of plants and up to 63.8% of root tips examined. In root systems of spruce from all three cultivation systems, the dominant morphotype was "*Amphinema*" (on 28.5–91.8% of plants, 10.2–80.5% root tips), followed by "*P. bicolorata*" and "*Thelephora*". The unidentified no. 20 ("yellow brown") morphotype was very common in bare-root and polyethylene roll cultivation systems but completely absent in plastic trays, where, by contrast, "*Laccaria*" was the second most dominant morphotype, which, in fact, was never observed in either bare roots or rolls. Identical morphotypes were often observed in different nurseries with a similar cultivation system. Sorensen indices of similarity (S_S) in bare-root

pine from four nurseries in all comparisons ranged between 0.50 and 0.89. In bare-root spruce from three nurseries, S_S was between 0.62 and 0.67, and in polyethylene roll-cultivated spruce from two nurseries, S_S was 0.77.

Extensive sampling of small numbers of root tips (20) from high numbers of plants (600) revealed significantly higher morphotype richness than intensive sampling of high numbers of root tips (about 500) from small numbers of plants (60), and 27 vs 15 morphotypes were found using these two sampling strategies (Fig. 1a). However, when the whole plant was taken as the sampling unit, both strategies revealed very similar morphotype richness, indicating that the 20 root tips taken per plant was sufficient to detect all of the taxa present (Fig. 1b). Only nine of the morphotypes (27.3%) were observed using both investigation strategies (Fig. 1a). Six morphotypes were found exclusively using intensive sampling (10% of the total) in 60 plants. The combination of both sampling strategies thus improved our overall ability to detect the actual fungal diversity. Flattening of the SACs in Fig. 1 indicates that our sampling effort was sufficient to detect a large proportion of the morphotypes present. The proportions of mycorrhizal roots recorded by each sampling approach (48.3 and 49.3%) were very similar.

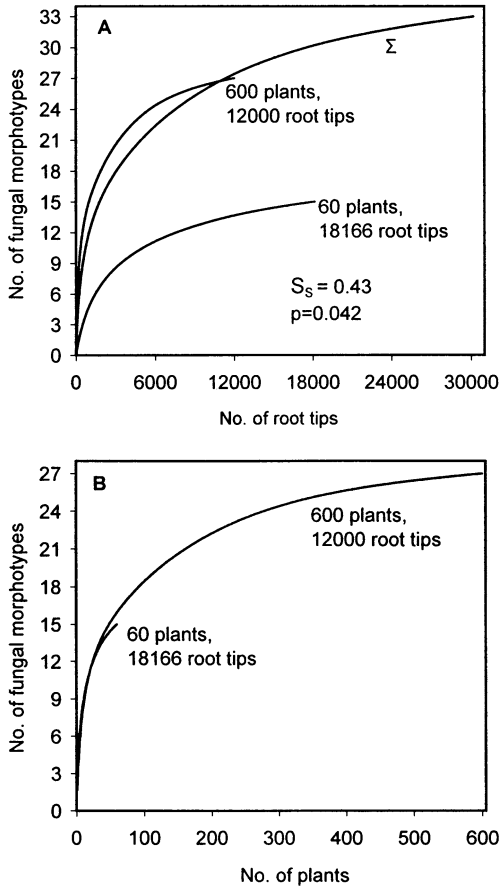

Fig. 1 Richness of mycorrhizal morphotypes of *P. sylvestris* and *P. abies* seedlings. The relationship between the cumulative number of morphotypes and **a** the number of root tips examined and **b** the number of plants examined. Community structure is compared by qualitative Sorensen similarity indices (S_S)

Detection of taxa by direct sequencing and isolation

Direct sequencing of 130 root tips with 33 distinct morphotypes revealed the presence of 60 fungal taxa, 27 of which were mycorrhizal. Pure culture isolation from 8,535 root tips with 12 morphotypes yielded 616 isolates representing 46 taxa, 8 of which were mycorrhizal. However, all eight of those were also detected by direct sequencing. Consequently, when pooled, direct sequencing and isolation detected a total of 93 fungal taxa, including 27 mycorrhizal (Fig. 2). The 7,919 tips that did not yield any culture (92.8%) remained sterile following surface sterilisation. A total of 71 (76.3%) fungi were identified at least to a genus level (Fig. 2). The overlap between the two methods was low, and the value of Sorensen index of qualitative similarity was 0.24. Only 13 (13.9%) of taxa were both sequenced and isolated, 47 (50.5%) were detected exclusively by sequencing and 33 (35.5%) exclusively by isolation.

The direct sequencing most frequently revealed the presence of mycorrhizal fungi, as the Basidiomycetes *S. luteus*, *Thelephora terrestris*, *Rhizopogon rubescens*, *Amphinema byssoides*, *Inocybe* sp.NS114 and Ascomycetes from the genera *Wilcoxina* and *Tuber*, and *Phialophora finlandia*. The isolations, by contrast, more frequently resulted in cultures of non-mycorrhizal Ascomycetes as *Phialocephala fortinii*, *Leptodontidium* spp. and *Oidiodendron* spp. Moreover, those were almost exclusively detected by isolation, as *Leptodontidium* spp. and *Oidiodendron* spp. were never sequenced directly from root tips, and *P. fortinii* was sequenced just once (0.5% among all sequences). On the other hand, also the isolations led to detection of mycorrhizal fungi, most common of which were *A. byssoides*, *P. finlandia* and *Hymenoscyphus* spp. (morphotype of *P. bicolorata*).

In species detection, the efficiency of isolation was much lower than that of direct sequencing. Thus, during the sequencing, a maximum of four fungal taxa were detected in just a single root tip, and on average, the method revealed the presence of 1.23–1.61 distinct fungi. By contrast, the detection of a similar number of taxa by isolation would require processing of 150–250 root tips; the success of isolation, expressed as the average number of cultures isolated from a single tip, was 0.03–0.09. The difference between those proportions, when compared by chi-squared test, was highly statistically significant ($p<0.0001$).

Single, distinct morphotypes commonly hosted several fungal taxa. In fact, there were only four morphotypes (12.1% of all observed) where just one taxon of fungus was detected, while the remaining 29 hosted 2–41 different taxa each. On average, from each single morphotype, direct sequencing as well as sequencing or morphological identtification of isolates revealed 6.5 fungal taxa. On the other hand, individual fungal taxa were also commonly detected in different mycorrhizal morphotypes. Thus, among the total 93 taxa in the present study, 46 (49.5%) were found in different morphotypes, inhabiting 2–19

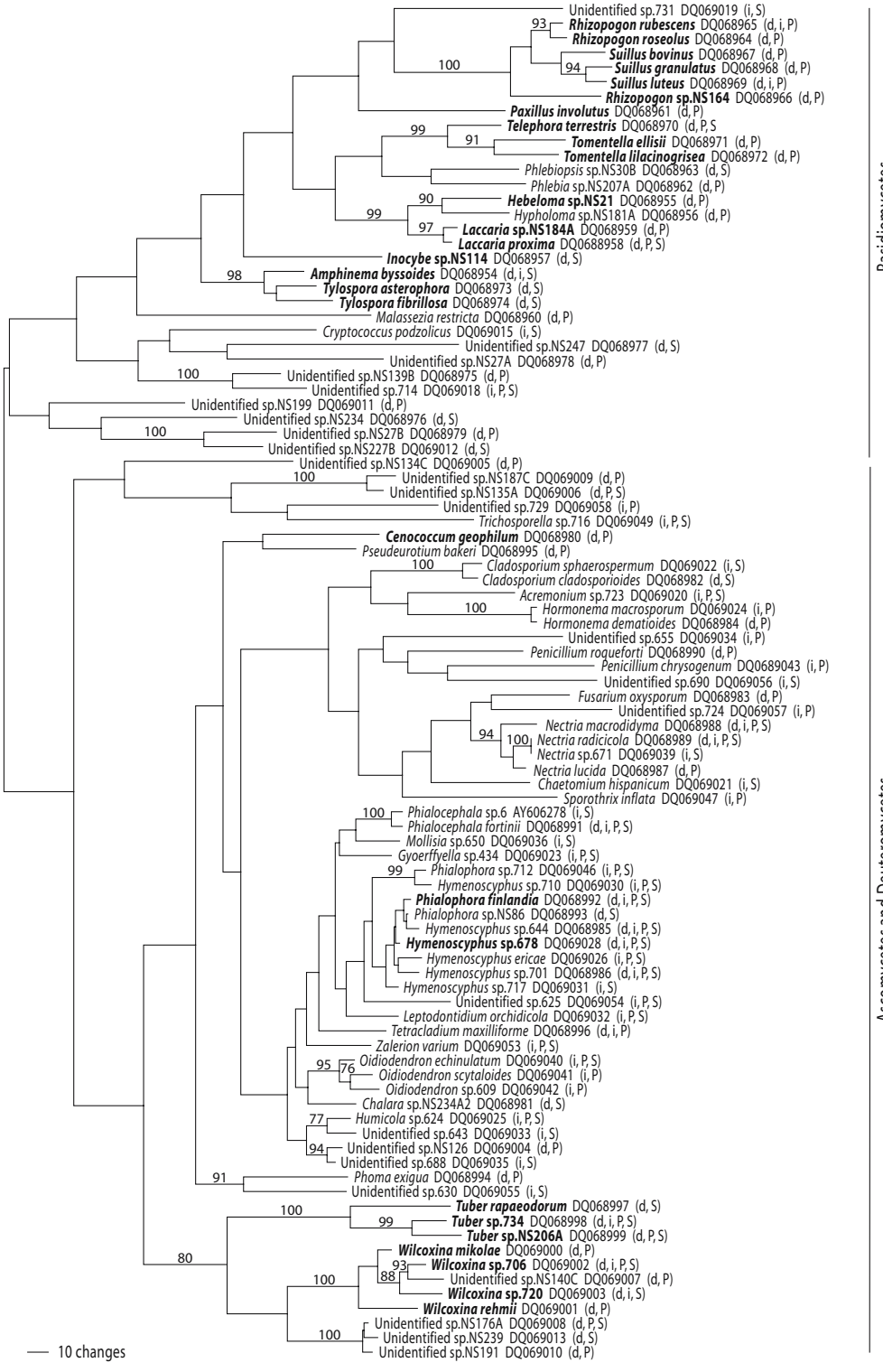

Fig. 2 Parsimonious tree of fungal ITS sequences from mycorrhizal root tips of *P. sylvestris* and *P. abies* seedlings, followed by GenBank accession number. Ectomycorrhizal taxa are labelled in *bold*. The detection method and host species are shown in *brackets* (*d* Direct sequencing, *i* isolation, *P* pine, *S* spruce). *Numbers* above the lines refer to bootstrap values >75% (10,000 fast replications). The tree was generated using the heuristic search and midpoint rooting options in PAUP (Swofford 2002)

morphotypes each. On average, an individual taxon there was likely to be found in 2.4 different morphotypes.

Despite the high diversity found within the morphotypes and the relatively wide range of morphotypes in which individual fungal taxa were detected, both direct sequencing and, to a lesser extent, isolation confirmed our morphotyping. There were 11 morphotypes among the total of 33, which were deemed to represent a certain fungal genus. In fact, the corresponding mycorrhizal taxa were sequenced or isolated from ten of them. Thus, *A. byssoides* and *S. luteus* were both sequenced and isolated from the respective "*Amphinema*" and "*S. luteus*" morphotypes. From the "*Cenococcum*" morphotype, we detected *Cenococcum geophilum* by sequencing; from "*Hebeloma*", *Hebeloma* sp.NS21; from "*Laccaria*", *Laccaria proxima*; from "*Rhizopogon*", *R. rubescens*; from "*Suillus*", *Suillus bovinus*

and *Suillus granulatus*; from "*Thelephora*", *T. terrestris*; from "*Tuber*", *Tuber* sp.734 (also isolated) and sp.NS206A and *Hymenoscyphus* sp.678 was isolated from the "*P. bicolorata*" morphotype, which is known to host *Hymenoscyphus* spp. (Vrålstad et al. 2000).

In one case (9.1%), our morphotyping apparently misidentified the fungal genus, since *R. rubescens* was sequenced from the morphotype "*Tricholoma*". On the other hand, despite the fact that in ten taxonomically defined morphotypes, the corresponding taxa were present, the proportion of root tips in which these taxa were identified by sequencing was not always high (33–100%). For the "*Amphinema*" morphotype, the "right" fungus was sequenced from 3 root tips out of 7; in "*Cenococcum*", from 1 root tip out of 1; in "*Hebeloma*", from 1 out of 1; in "*Laccaria*", from 2 out of 4; in "*Rhizopogon*", from 1 out of 1; in "*Suillus*", from 3 out of 9; in "*Thelephora*", from 5 out of 15; in "*S. luteus*", from 11 out of 13; and in "*Tuber*", from 5 out of 14. However, despite this mismatch, other mycorrhizal taxa were sequenced from misidentified root tips, showing that all these root tips were mycorrhizal.

Richness of taxa and community structure

In root systems of pine, a total of 68 taxa of fungi were found, including 21 mycorrhizal. In spruce, a total of 52 taxa were found, including 13 mycorrhizal. Consequently, species richness, and in particular, richness of mycorrhizal fungi, in pine was significantly higher than that in spruce (chi-squared tests, $p \leq 0.00014$). Out of 27 mycorrhizal fungi, 14 (52%) were unique for pine, 6 (22%) were unique for spruce, and 7 (26%) were common for both tree species (Fig. 2). When all fungi were pooled together, the trend remained the same, and, among all 93 taxa, 41 (44%) were unique for pine, 25 (27%) unique for spruce and 27 (29%) were found on both tree species (Fig. 2). When pine was compared to spruce, this resulted in moderately similar community structures of mycorrhizal and of all root-colonising fungi, as Sorensen qualitative indices were 0.41 and 0.45, respectively.

The species richness of mycorrhizal fungi was not correlated with the total species richness in a cultivation system, and the amount of mycorrhizal taxa in each tree species/system ranged from five to ten. Thus, in pine, the highest total number of taxa was found in plants from the bare-root system (total of 54 species, including 9 species mycorrhizal), followed by plants from plastic trays (20 including 10) and the greenhouse (15 including 7). In spruce, the highest total number of taxa was also found in plants from the bare-root system (29 including 5), then in plants from polyethylene rolls (28 including 7), with the lowest diversity observed in plastic trays (18 including 8).

In each tree species, there were two to six mycorrhizal fungi that were common between different cultivation systems. Due to low overall species abundance, this led to moderate similarities in mycorrhizal communities, and the Sorensen qualitative indices between cultivation systems were 0.35–0.54 for pine and 0.38–0.67 for spruce. On the other hand, the similar extent of variation was observed also within the bare-root system, which was the only one sampled at multiple sites (Table 1) and where the respective indices were 0.36–0.57 and 0.44–0.60. Yet, in quantitative terms, similarity between the mycorrhizal communities must have been higher due to dominance of *P. finlandia* in roots of both tree species under all cultivation systems. Thus, isolates of the fungus comprised 13–65 and 33–51% of all strains obtained from different systems of pine and spruce, respectively. Moreover, by direct sequencing, *P. finlandia* was also detected in each system of each tree species.

Discussion

The results of the present study showed that the extent of mycorrhizal colonisation of seedling roots in forest nurseries to a large extent depends on the cultivation system used. In pine, bare-root cultivation yielded seedlings with the highest degree of mycorrhizal colonisation, while plants in greenhouse cultivation systems were colonised to a much lower (40%) extent (Table 1). In spruce, plants cultivated in polyethylene rolls exhibited highest mycorrhizal colonisation, while in bare-roots, the level of colonisation was only half as high (Table 1). Moreover, our work has revealed the presence in forest nurseries of many mycorrhizal fungi that also form associations with trees under forest or field conditions (Fig. 2). The results therefore provide clear implications as to which cultivation system should be selected if the aim is to produce seedling material with a high extent of mycorrhizal colonisation, which in several cases has proven to increase seedling vitality (Genere 1995; Herrmann et al. 1992; Krasowski et al. 1999).

In the past, most of the community studies of mycorrhizal fungi were based exclusively on morphotyping (Fransson et al. 2000; Grogan et al. 1994; Ursic et al. 1997). However, it has already reported (Kåren and Nylund 1997; Wurzburger et al. 2001) that morphotyping on it own is not sufficient and that mismatching of mycorrhizal morphotypes with ITS types occurs. Our study showed that an individual fungal taxon may form mycorrhizal roots with different morphologies and that mycorrhizal roots with similar morphologies may be formed by different taxa. Occasionally, in mycorrhizal roots, we found fungal pathogens, as *Nectria radicicola* (teleomorph of *Cylindrocarpon destructans*) or *Fusarium oxysporum* (Fig. 2). This indicates that functionally different fungal taxa co-exist within mycorrhizal root tips.

In the present work, the combination of different sampling strategies and different detection methods had resulted in high estimates of fungal diversity. Certain morphotypes were revealed only by intensive root system analyses, while the others were only detected with increasing amounts of examined plants, although overall species richness was about the same under both strategies (Fig. 1). Moreover, in another study, it was found out that

the trade-off between sampling extensiveness and sampling intensity had only small effects on species richness in mycorrhizal community, but instead, the composition of species was influenced (Koide et al. 2005). By contrast, the combining direct sequencing with isolation has led to sharp increase in diversity of detected fungi, as the representatives of different ecological groups were often found by the different method. For example, the direct sequencing more commonly yielded mycorrhizal species, such as Basidiomycetes *Laccaria* spp., *Rhizopogon* spp., *Suillus* spp., *Tomentella* spp, *T. terrestris* and the Ascomycetes *P. finlandia* (Fig. 2). Those, except for the latter, were seldom or never isolated into pure culture. Isolation, on the other hand, more commonly resulted in cultures of the Ascomycetes, such as *Leptodontidium* spp., *Oidiodendron* spp., *P. fortinii* and also *P. finlandia*, but the representatives from two first genera were never sequenced (Fig. 2). The similar trend in detection of fungi from different ecological groups by direct sequencing and isolation was reported in related Canadian study (Kernaghan et al. 2003). One reason for this could be slow growth of some mycorrhizal species on artificial media.

In our material, *P. finlandia* was the most characteristic mycorrhizal fungus, as it was most commonly encountered on seedlings of both tree species in all cultivation systems. In several studies, the fungus was shown to form ecto- or ericoid mycorrhizas on trees and other plants (Wang and Wilcox 1985; Wilcox and Wang 1987a,b; Ursic and Peterson 1997; Vrålstad et al. 2002) and was reported to promote growth of infected seedlings (Wilcox and Wang 1987b). *P. finlandia* was frequently encountered in forest nurseries in Canada, where, in fact, also the other mycorrhizal fungi of the present work (e.g. from genera *Amphinema*, *Hebeloma*, *Thelephora*, *Tuber*) were common (Ursic and Peterson 1997; Ursic et al. 1997; Kernaghan et al. 2003). Moreover, *P. finlandia* was reported to form mycorrhiza with trees in north temperate forest (Tedersoo et al. 2003), indicating to certain ecological plasticity of the fungus. Therefore, its abundant mycorrhizas in studied nurseries might have a positive impact on vitality and establishment of outplanted seedlings.

Acknowledgements We thank the staff of Dubrava, Kaunas, Tytuvenai, Varena and Veisejai Forest Enterprises for providing plant material. This research was funded by The Royal Swedish Academy of Agriculture and Forestry (KSLA) and The Foundation for Strategic Environmental Research (MISTRA).

References

Agerer R (1986–1988) Colour atlas of ectomycorrhizae. Einhorn-Verlag, Schwäbisch Gmünd, München

Agerer R, Danielson RM, Egli S, Ingleby K, Luoma D, Treu R (1996–1998) Description of ectomycorrhizae. Einhorn-Verlag, Schwäbisch Gmünd, München

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Baum C, Stetter U, Makeschin F (2002) Growth response of *Populus trichocarpa* to inoculation by the ectomycorrhizal fungus *Laccaria laccata* in a pot and a field experiment. For Ecol Manag 163:1–8

Bjorkman E (1970) Forest tree mycorrhiza—the conditions for its formation and the significance for tree growth and afforestation. Plant Soil 32:589–610

Chakravarty P, Unestam T (1985) Role of mycorrhizal fungi in protecting damping-off of *Pinus sylvestris* L. seedlings. Physiological and genetical aspects of mycorrhizae. 1st European Symposium on Mycorrhizae, Dijon, pp 811–814

Colpaert JV, Vanassche JA (1992) Zinc toxicity in ectomycorrhizal *Pinus sylvestris*. Plant Soil 143:201–211

Cordell CE, Marx DH (1994) Effects of nursery cultural practices on management of specific ectomycorrhizae on bareroot tree seedlings. In: Pfleger FL, Linderman RG (eds) Mycorrhizae and plant health. The American Phytopathology Society, St Paul, MN, pp 133–151

Colwell RK, Coddington JA (1994) Estimating terrestrial biodiversity through extrapolation. Philos Trans R Soc Lond B Biol Sci 345:101–118

Danielson RM (1984) Ectomycorrhizal associations in jack pine stands in northeastern Alberta. Can J Bot 62:932–939

Danielson RM, Visser S, Parkinson D (1984) Production of ectomycorrhizae on container-grown jack pine seedlings. Can J For Res 14:33–36

Dunabeitia M, Rodriguez N, Salcedo I, Sarrionandia E (2004) Field mycorrhization and its influence on the establishment and development of the seedlings in a broadleaf plantation in the Basque country. For Ecol Manag 195:129–139

Egger KN (1995) Molecular analysis of ectomycorrhizal fungal communities. Can J Bot 73:S1415–S1422

Fowler J, Cohen L, Jarvis P (2001) Practical statistics for field Biology. Wiley, Chichester

Fransson PMA, Taylor AFS, Finlay RD (2000) Effects of continuous optimal fertilization upon Norway spruce ectomycorrhizal community. Tree Physiol 20:599–606

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

Genere B (1995) Early assessment of 2 plant types of *Laccaria laccata* S 238 N mycorrhizal Douglas fir seedlings in field trial. Ann Sci For 52:374–384

Grogan MH, O'Neill MJJ, Mitchell TD (1994) Mycorrhizal associations of Sitka spruce seedlings propagated in Irish tree nurseries. Eur J For Pathol 24:335–344

Herrmann S, Ritter T, Kottke I, Oberwinkler F (1992) Increase of forest plant performance (*Fagus silvatica* L and *Quercus robur* L) by controlled mycorrhization. Allg Forst-Jagdztg 163:72–79

Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Mol Ecol 10:1855–1871

Ihaka R, Gentleman R (1996) R: a language for data analysis and graphics. J Comput Graph Stat 5:299–314

Jumpponen A (2001) Dark septate endophytes—are they mycorrhizal? Mycorrhiza 11:207–211

Juska V, Baltrusaitiene V, Jasiukaitis L, Kirklys A, Veisreiderienе E (1982) Growth technology of plant material under controlled environment conditions. Raide, Kaunas (in Lithuanian)

Kåren O, Nylund JE (1997) Effects of ammonium sulphate on the community structure and biomass of ectomycorrhizal fungi in a Norway spruce in southwestern Sweden. Can J Bot 75:1628–1642

Kernaghan G, Sigler L, Khasa D (2003) Mycorrhizal and root endophytic fungi of containerized *Picea glauca* seedlings assessed by rDNA sequence analysis. Microb Ecol 45:128–136

Koide RT, Xu B, Sharda J (2005) Contrasting below-ground views of an ectomycorrhizal fungal community. New Phytol 166:251–262

Kope HH, Axelrood PE, Southerland J, Reddy MS (1996) Prevalence and incidence of the root-inhabiting fungi, *Fusarium*, *Cylindrocarpon* and *Pythium*, on container-grown Douglas-fir and spruce seedlings in British Columbia. New For 12:55–67

Krasowski M, Owens J, Tackaberry L, Massicotte H (1999) Above- and below-ground growth of white spruce seedlings with roots divided into different substrates with or without controlled-release fertilizer. Plant Soil 217:131–143

Kropp BR, Langlois CG (1990) Ectomycorrhizae in reforestation. Can J For Res 20:438–451

Le Tacon F, Alvarez IF, Bouchard D, Henrion B, Jackson MR, Luff S, Parlade IJ, Pera J, Stenström E, Villeneuve N, Walker C (1994) Variations in field response of forest trees to nursery ectomycorrhizal inoculation in Europe. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. CAB, Wallingford, pp 119–134

Lilja A, Lilja S, Poteri M, Ziren L (1992) Conifer seedling root fungi and root dieback in Finnish nurseries. Scand J For Res 7:547–556

Magurran AE (1988) Ecological diversity and its measurement. Princeton University Press, Princeton, NJ

Marx DH (1969) The influence of ectotrophic ectomycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to pathogenic fungi and soil bacteria. Phytopathology 59:153–163

Marx DH (1980) Ectomycorrhiza fungus inoculations: a tool to improve forestation practices. In: Mikola P (ed) Tropical mycorrhiza research. Oxford University Press, Oxford

Mikola P (1970) Mycorrhizal inoculation for afforestation. Int Rev For Res 3:123

Morin C, Samson J, Dessureault M (1999) Protection of black spruce seedlings against *Cylindrocladium* root rot with ectomycorrhizal fungi. Can J Bot 77:169–174

Ortega U, Dunabeitia M, Menendez S, Gonzalez-Murua C, Majada J (2004) Effectiveness of mycorrhizal inoculation in the nursery on growth and water relations of *Pinus radiata* in different water regimes. Tree Physiol 24:65–73

Pera J, Alvarez IF, Rincon A, Parlade J (1999) Field performance in northern Spain of Douglas-fir seedlings inoculated with ectomycorrhizal fungi. Mycorrhiza 9:77–84

Perry AD, Molina R, Amaranthus PM (1987) Mycorrhizae, mycorrhizospheres, and reforestation: current knowledge and research needs. Can J For Res 17:929–940

Quoreshi AM, Timmer VR (2000) Early outplanting performance of nutrient-loaded containerized black spruce seedlings inoculated with *Laccaria bicolor*: a bioassay study. Can J For Res 30:744–752

Rosling A, Landeweert R, Lindahl BD, Larsson KH, Kuyper TW, Taylor AFS, Finlay RD (2003) Vertical distribution of ectomycorrhizal fungal taxa in a podzol soil profile. New Phytol 159:775–783

Sampagni R, Perrin R, Le Tacon F (1985) Disease suppression and growth promotion of Norway spruce and Douglas-fir seedlings by the ectomycorrhizal fungus *Laccaria laccata* in forest nurseries. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Physiological and genetical aspects of mycorrhizae. 1st European Symposium on Mycorrhizae, Dijon, pp 799–806

Scagel CF, Linderman RG (1998) Influence of ectomycorrhizal fungal inoculation on growth and root IAA concentrations of transplanted conifers. Tree Physiol 18:739–747

Sieber TN (2002) Fungal root endophytes. In: Wasel Y, Eshel A, Kafkafi U (eds) Plant roots: the hidden half. Marcel Dekker, New York, pp 887–917

Sinclair WA, Sylvia DM, Larsen AO (1982) Disease suppression and growth promotion in Douglas-fir seedlings by the ectomycorrhizal fungus *Laccaria laccata*. Forest Sci 28:191–201

Smith SE, Read DJ (1997) Mycorrhizal Symbiosis, 2nd edn. Academy, London

Stenström E, Ek M, Unestam T (1990) Variation in field response of *Pinus sylvestris* to nursery inoculation with four different ectomycorrhizal fungi. Can J For Res 20:1796–1803

Stenström E, Damm E, Unestam T (1997) Le role des mycorhizes dans la protection des arbres forestiers contre les agents pathogenes du sol. Rev For Fr XLIX - no sp.:121–128

Swofford DL (2002) PAUP*: phylogenetic analysis using parsimony (* and other methods). Sinauer Associates, Sunderland, MA

Tedersoo L, Koljalg U, Hallenberg N, Larsson K-H (2003) Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. New Phytol 159:153–165

Teste FP, Schmidt MG, Berch SM, Bulmer C, Egger KN (2004) Effects of ectomycorrhizal inoculants on survival and growth of interior Douglas-fir seedlings on reforestation sites and partially rehabilitated landings. Can J For Res 34:2074–2088

The European Commission press release (2004) Enlargement and agriculture: EUR 5.76 billion for rural development in new member states. http://www.europa.eu.int/comm/agriculture/external/enlarge/press/index_en.htm

Ursic M, Peterson RL (1997) Morphological and anatomical characterization of ectomycorrhizas and ectendomycorrhizas on *Pinus strobus* seedlings in a southern Ontario nursery. Can J Bot 75:2057–2072

Ursic M, Peterson LR, Husband B (1997) Relative abundance of mycorrhizal fungi and frequency of root rot on *Pinus strobus* seedlings in southern Ontario nursery. Can J For Res 27:54–62

Van Tichelen KK, Colpaert JV, Vangronsveld J (2001) Ectomycorrhizal protection of *Pinus sylvestris* against copper toxicity. New Phytol 150:203–213

Vrålstad T, Fossheim T, Schumacher T (2000) *Piceirhiza bicolorata*—the ectomycorrhizal expression of the *Hymenoscyphus ericae* aggregate. New Phytol 145:549–563

Vrålstad T, Schumacher T, Taylor AFS (2002) Mycorrhizal synthesis between fungal strains of the *Hymenoscyphus ericae* aggregate and potential ectomycorrhizal and ericoid hosts. New Phytol 153:143–152

Wang CJK, Wilcox HE (1985) New species of ectendomycorrhizal and pseodomycorhizal fungi: *Phialophora finlandia*, *Chloridium paucisporum*, and *Phialocephala fortinii*. Mycologia 77:951–958

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

Wilberforce EM, Boddy L, Griffiths R, Griffith GW (2003) Agricultural management affects communities of culturable root-endophytic fungi in temperate grasslands. Soil Biol Biochem 35:1143–1154

Wilcox HE (1996) Mycorrhizae. In: Wasel Y, Eshel A, Kafkafi U (eds) Plant roots: the hidden half. Marcel Dekker, New York, pp 689–721

Wilcox HE, Wang CJK (1987a) Mycorrhizal and pathological associations of dematiacious fungi in roots of 7-month-old tree seedlings. Can J For Res 17:884–889

Wilcox HE, Wang CJK (1987b) Ectomycorrhizal and ectendomycorrhizal associations of *Phialophora finlandia* with *Pinus resinosa*, *Picea rubens*, and *Betula alleghaniensis*. Can J For Res 17:976–990

Wurzburger N, Bidartondo MI, Bledsoe CS (2001) Characterization of *Pinus* ectomycorrhizas from mixed conifer and pygmy forests using morphotyping and molecular methods. Can J Bot 79:1211–1216