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# Root endophytes along a primary succession gradient in northern Finland

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**Abstract** Primary successional vegetation gradients are characterized by changes in the soil microbial communities. However, information on possible shifts of the root endophytes along these gradients is scarce. The objective of the current study was to identify root endophytic fungi from a primary successional gradient on land uplift seashore of a geographically isolated island area. We applied a sequencing approach by amplifying the ITS region with fungal specific primers. We used mainly an isolate-based method, and to compare the abundance of culturable and unculturable endophytes, direct sequencing of one representative plant specimen *Deschampsia flexuosa* was also carried out. A total of 38 cultured endophytic strains were sequenced from *Empetrum nigrum* (Empetraceae), *Vaccinium vitis-idaea* (Ericaceae) and *Deschampsia flexuosa* (Poaceae). Out of these, 27 were identified as *Phialocephala fortinii*, three as *Mollisia minutella*, four as *Phialophora* sp., one as Ascomycetes sp. and three remained unidentified. The strains clustered into five clades in the phylogram, mostly irrespective of the successional stages and hosts from which they had been isolated. The early successional seashore dune ridge plants however, seemed to host a distinct fungal taxon, *Phialophora* sp. Culture-independent methods were applied on a root sample of a mid-successional *Deschampsia flexuosa* specimen and a total of 16 clones were randomly selected and sequenced. Out of 16 sequences, 13 were identified as unculturable strains and three showed closest similarity to a basidiomycete *Cortinarius callisteus*. The unculturable sequences were grouped into two main clades and were different from any culturable isolate in this study. Our results suggest that (i) *P. fortinii* dominates the isolate data at mid to late successional stages, (ii) roots of the ericaceous plants and the grass *Deschampsia flexuosa* are colonized by the same endophytic fungi in this ecosystem, and (iii) unculturable endophytes are common and potentially more abundant than the culturables. To our knowledge, this is the first report of the molecular phylogenies of the DSE in the mid-boreal zone and also the first report of the unculturable root endophytes of *D. flexuosa*.

**Keywords** Root endophytes · ITS sequences · Unculturables · *Phialocephala fortinii*

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## Introduction

Sandy shores of land uplift coasts provide an excellent example of primary succession on plant and microbial communities. On sandy seashores, the first vascular plants typically are separately-growing non-mycorrhizal ruderals, for example, members of Chenopodiaceae, Polygonaceae and Brassicaceae (Read 1989; Smith and Read 1997). They soon become replaced by arbuscular mycorrhizal (AM) perennial graminoids that grow on the dunes (Read 1989; Pennanen et al. 2001). Vegetation on the deflation basin beyond the dunes is comprised of patchily distributed ericoid mycorrhizal dwarf shrubs as well as sparse ECM (ectomycorrhizal) trees and few AM graminoid and forb species. Microbial and plant communities often show parallel development due to above- and belowground interactions during primary succession and formation of organic soil (Ohtonen et al. 1999; Aikio et al. 2000).

Generally, plant cover, intensity of mycorrhizal colonization in plant roots and the amount of fungal mycelium in soil increase, pH and soil disturbance decrease along with the development of soil organic layer towards the older stages of succession (Read 1989). Furthermore, as the bacterial phospholipid fatty acids (PLFAs) decrease in the soil organic matter, the fungal PLFAs increase along the succession of sandy shores (Pennanen et al. 2001).

A less-studied group of fungal colonizers along the primary successional gradient are the root endophytic fungi (Jumpponen 1999). Fungal endophytes are microbes that live inside the plant tissue without causing visible symptoms of disease (Schulz and Boyle 2005; Hyde and Soytong 2008). Root-associated endophytes are taxonomically diverse group of fungi (Tao et al. 2008; Zhu et al. 2008), and include the dark-septate endophyte (DSE)-complex (Jumpponen 1999; Mandyam and Jumpponen 2005; Arnold 2007). The function of root endophytes is largely unknown but mycorrhizal fungi are known to promote seed germination and stimulate the development and growth of protocorms, seedlings and juveniles (Tao et al. 2008; Zhu et al. 2008). According to the present knowledge, root endophytes include fungi with diverse ecological roles (Arnold 2007). The shifts in several fungal parameters along the primary successional gradient suggest that the communities of the root associated endophytes may also change.

Traditional methods to study endophytes are method-dependent and it has been suspected that the fungi isolated may not represent the full swath of fungi occurring as endophytes in plant tissues (Duong et al. 2006; Hyde and Soytong 2007, 2008). In recent years, multidisciplinary approaches have been initiated and the rapid development in molecular biology has had a major impact on fungal taxonomy (e.g. Shenoy et al. 2007; Thongkantha et al. 2009). The identification of endophytic fungi isolated through traditional methodology (i.e. culturing) has also considerably benefited from the development of the molecular tools (Hambleton and Currah 1997; Harney et al. 1997; Grünig et al. 2004; Menkis et al. 2004; Ashkannejhad and Horton 2006; Zhu et al. 2008), especially as these fungi typically remain sterile in culture (Addy et al. 2005; Sánchez Márquez et al. 2007, 2008). Despite the considerable progress, large gaps still exist in the knowledge of the diversity of these endophytes, especially in the boreal and tropical ecosystems (Mandyam and Jumpponen 2005; Schulz and Boyle 2005). More recently researchers have extracted DNA directly using DGGE, from plant tissues in order to establish the presence of endophytes (Duong et al. 2006; Hyde and Soytong 2008). These methods have proved to be labour intensive, but have shown that the endophytes revealed by direct sequencing often differ to those isolated using traditional methodology (Duong et al. 2008; Tao et al. 2008).

The goal of the current study was to identify root endophytic fungi from a primary successional gradient by a sequence-based approach and phylogenetic analysis. We isolated fungi using traditional methodology from the roots of two dwarf shrubs, *Empetrum nigrum* ssp. *hermaphroditum* (Empetraceae) and *Vaccinium vitis-idaea* (Ericaceae), and the graminoid *Deschampsia flexuosa* (Poaceae) from a land uplift shore of a geographically isolated island area. We aimed to compare the isolates from these plants growing within a close distance to each other, and to determine differences in the composition of isolates in relation to the stage of succession (early successional dune ridge, mid-successional deflation basin and late successional Scots pine forest). Since isolation of endophytes is method-dependent we also established the degree of overlap between the unculturable and culturable endophyte groups within one mid-successional *D. flexuosa* root system by direct sequencing. This method provided data on whether the culture method gave an accurate assessment of the endophytes present within roots of sand dune plants.

## Materials and methods

### Collection of plant material

The plant material for the endophytic fungal isolations was collected from the island of Hailuoto on the Bothnian Bay coast in Northern Finland (65°03′N, 24°36′E). The coast is very flat and strongly affected by post-glacial land uplift (ca. 7 mm per year) (Vermeer and Kakkuri 1988), constantly creating newly emerging non-vegetated zone on the shoreline and leading to a distinct succession of plant communities along the shore (Vartiainen 1980). The mineral soil in the seashore is sand with low total nitrogen content. Soil organic matter increases and soil pH decreases along the succession gradient towards late succession (Pennanen et al. 2001). The plant material was collected in early growing season, (June 5th, 2007) from five sites representing four kinds of habitats: Site 1: A sandy seashore dune ridge area with only occasional patches of *Empetrum nigrum* L. (subspecies *hermaphroditum*, referred to as *Empetrum nigrum* hereafter) and the grasses *Deschampsia flexuosa* L. and *Agrostis* spp.; Site 2: A mid-successional Scots pine forest on a deflation basin with sparsely located trees of age of 10–50 years and field layer characterized by patches of *Empetrum nigrum* and occasional *Vaccinium uliginosum* L., *Deschampsia flexuosa* and *Festuca* spp; Sites 3 and 4: Two separate sites of late-successional dry pine forest with field layer vegetation characterized by *Empetrum nigrum* and *Vaccinium vitis-idaea* L. ; Site 5: a late-successional oligotrophic pine (*Pinus sylvestris* L.) forest (*Cladina*-type (Kalliola 1973)) where the ground layer is characterized by mat-forming *Cladina*

lichens. We used soil borer (diameter 9 cm, to the depth of 15 cm) for sampling the roots of *E. nigrum* (Empetraceae) (sample code EN), *Vaccinium vitis-idaea* (Ericaceae) (sample code VV) and *Deschampsia flexuosa* (Poaceae) (sample code DF). The soil cores including the roots were kept in +4°C until the isolation. Altogether, we prepared duplicate isolate plates of 46 root systems (12 plates from Sites 1, 2 and 3 and five from Sites 4 and 5).

### Isolation of endophytic fungi

The fungi were isolated in June 8th–14th 2007 at the Botanical Museum, University of Oulu. The roots were carefully washed with tap water and surface sterilized either in 30% $H_2O_2$ or in 3.5% NaOCl, then rinsed in sterile $H_2O$, cut into 1 cm segments and plated (plates 9 cm in diameter) on malt extract agar (MEA, Difco) and potato dextrose (PDA, Difco) agar. The plates were incubated at room temperature in the dark and checked every second day for fungal growth. The strain was considered endophytic if it originated from the cutting edge of the root and had a relatively slow growth rate (about 10–15 mm of radial growth in 7 days). When endophytic fungal growth was observed, the mycelia were immediately transferred to a new plate. An isolate was transferred only when the probability of a good pure culture was considered high. Thus, when the strains originated very close to each other and in later stages, when they overgrew earlier strains, they were left untransferred and not calculated into the total number of the isolates.

### Fungal DNA extraction, PCR amplification of ITS region and electrophoresis

The pure cultures were used for the DNA extraction. DNA was extracted from 0.5 to 1.0 g fresh mycelia according to the method of Pirttilä et al. (2001). The target rDNA region including ITS1, ITS2 regions and 5.8S gene was amplified using primers ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). Amplifications were performed in a total reaction volume of 25 µl containing 2 mM of each dNTP, 2.5 mM $MgCl_2$, 5 pM of each primer, 1 unit of *Taq* DNA polymerase (Dynazyme, Finnzymes, Espoo, Finland) and 100 ng of template DNA. PCR amplifications were performed in a thermal cycler (PTC 200, MJ research, USA) with an initial denaturing step of 95°C for 3 min, followed by 40 amplification cycles of 94°C for 60 s, 50°C for 60 s, and 72°C for 120 s and a final extension step of 72°C for 10 min. The amplification products were separated by electrophoresis on 1.5% (*w*/*v*) agarose gel at 100 V for 2 h in 1× TAE buffer, stained with ethidium bromide (0.5 µg/ml) and visualized under 300 nm UV light and photographed. A 100 bp size marker (MBI Fermentas, Vilnius, Lithuania) was used as reference.

### Sequencing of fungal ITS region

Amplification products obtained from PCR reactions with unlabeled ITS primers (ITS1 and ITS4) were used for sequencing. Sequencing reactions were carried out using Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Extension products were then purified using ethanol/EDTA precipitation protocol and analyzed on a ABI 3100 *Avant* Genetic analyzer (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. DNA sequences obtained for each strain from each forward (ITS1) and reverse (ITS4) primer were inspected individually for quality. Both strands of the DNA were then assembled to produce a consensus sequence for each strain using Sequencher 4.7 software (Gene codes corporation, USA). The sequences were submitted to the NCBI (National Centre for Biotechnology and Information) and accession numbers were obtained.

### Root DNA extraction and PCR amplification

Total DNA was extracted from roots of *Deschampsia flexuosa* using the Omega SP plant mini kit (Bio-Tek) as per the manufacturer's protocol. Fungal Internal transcribed spacer region were amplified by PCR using primer pair ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4B (CAGGAGACTTGTACACGGTCCAG) (Gardes and Bruns 1993). The PCR reactions and thermal cycling conditions were the same as previously mentioned.

### Cloning, purification and sequencing

The primers ITS1F and ITS4B were used to amplify the unculturable fungi from the roots of *Deschampsia flexuosa*. The purified PCR product was cloned using InsTA clone PCR product cloning kit (MBI Fermentas, Germany), according to manufacturer's protocol. The DNA was transformed into *Escherichia coli* DH5α. Transformants were grown on LB plates containing 100 µg/ml of Ampicillin, Xgal (20 µg/ml) and Isopropyl b-D-1-thiogalactopyranoside (200 µg/ml). Single white colonies were streaked on LB Amp plates. Plasmid DNA was extracted using QiaPrep spin column (Quigen, miniprep kit, USA). Sixteen clones were randomly picked from ITS gene library for sequencing. The sequences were aligned and phylogenetically analysed along with closely matching GenBank and UNITE (http://unite.ut.ee/analysis.php) sequences.

### Molecular phylogenetic analysis

All sequences were compared with ITS sequences available in the GenBank and UNITE databases by BLASTn search. The

closest matches (more than 95% homology) in the GenBank sequences were also included in the clustal alignment and phylogenetic analysis. All sequences were aligned using Clustal X with default settings (Thompson et al. 1997). The phylogenetic analysis was performed by the Neighbour-joining method using Molecular Evolutionary Genetics Analysis (MEGA) (Tamura et al. 2007). The robustness of the phylogeny was tested by bootstrap analysis using 1,000 iterations. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option).

## Results

### Culturable fungal endophytes

Fifty pure isolates of endophytic fungi were obtained from 46 root systems. Out of these, six isolates originated from Site 1, 12 from Site 2, 18 from Site 3, seven from Site 4, and seven from Site 5 (data not shown). The average number of isolates per root system varied between 0.5 (Site 1) to 1.5 (Site 3). Out of these, 38 representative isolates were selected for the molecular identification study; three from *Vaccinium vitis-idaea*, 16 from *Empetrum nigrum*, and 19 from *Deschampsia flexuosa* respectively (Table 1). ITS sequences of these isolates including their accession numbers (EU314675–EU314712), original sampling sites, and the results of the BLAST searches [3] (http://www.ncbi.nlm.nih.gov/sites/entrez/) are listed in Table 1. Of the 38 isolates, 27 were found to be *Phialocephala fortinii*, three *Mollisia minutella*, four *Phialophora* sp., one Ascomycetes sp. and three unidentified endophyte species (matching closely, 95 and 97% respectively, to the sequence of *Epacris microphylla* root associated fungus).

The nucleotide frequencies were 0.237 (A), 0.241 (T), 0.262 (C), and 0.260 (G). The transition/transversion rate ratios were k1=2.156 (purines) and k2=2.394 (pyrimidines). The overall transition/transversion bias is $R$=1.198. Of 738 characters, 152 were conserved sites, 372 were parsimony informative, 446 were variable sites and 63 were singleton sites. The phylogram constructed using MEGA is shown in Fig. 1. Most of the clades were supported by high bootstrap values (>60%), only three of them with a lower, 52%, 54% and 56%, bootstrap support.

The phylogram of the 38 root endophytic fungi and 9 sequences from GenBank (close match to our sequences) were distributed into five clades, in which *Phialocephala fortinii* was present in two Clades (I and V) irrespective of the sites and hosts from which they had been isolated. Clades II and III contained *Mollisia minutella* isolates from three plant species. Four *Phialophora* sp isolates were clustered in the Clade IV along with a GenBank isolate. Clade V included isolates from all three hosts, *E. nigrum* (Empetraceae) (sample code EN), *Vaccinium vitis-idaea* (Ericaceae) (sample code VV) and *Deschampsia flexuosa* (Poaceae) (sample code DF). The one unidentified taxon and one ascomycete were clustered in the Clade II (bootstrap 92%) and two unidentified taxa were clustered in the Clade III (bootstrap 87%). Thus, based on the phylogenetic analysis, the unidentified isolates could be classified as *Mollisia minutella*. *Acephala applanata* was used as an outgroup in the analysis.

### Unculturable endophytic fungi

ITS sequences of the unculturable fungi including their accession numbers (FJ517587–FJ517602) and the results of the BLAST searches are listed in Table 2. Out of 16 clones, 13 matched with uncultured fungal accessions deposited to NCBI by research groups from Germany, Norway, Canada and USA. The sequences of three clones had the closest match with *Cortinarius callisteus* with 84, 91 and 100% sequence coverage (Clones 6, 8 and 13) and with 90, 95 and 94% homology. We also screened the UNITE database with a combined search (UNITE+INSD), as UNITE is a reliable database with authentic strains, and we found that three clones (UNC6, 8 and 13) had a close match with an uncultured fungal accession AM999704. Five clones (UNC7, 9, 11, 12 and 14), which had a close match with uncultured fungal accessions in the GenBank, were closely related to *Mycena galericulata* in the UNITE database. *Phialocephala fortinii* was used as an outgroup.

The nucleotide frequencies were 0.244 (A), 0.278 (T/U), 0.247 (C), and 0.23 (G). The transition/transversion rate ratios were $k_1$=3.607 (purines) and $k_2$=3.546 (pyrimidines). The overall transition/transversion bias is $R$=1.767, where $R = [A{*}G{*}k_1 + T{*}C{*}k_2]/[(A+G){*}(T+C)]$. There were a total of 261 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 and the consensus tree is shown in Fig. 2. All 16 unculturable fungi were distributed in two major clades. The first clade contained 8 unculturable fungi along with one GenBank accession, and the other clade contained 8 fungi along with four GenBank accessions.

## Discussion

The study of fungal endophytes using culturing techniques is method-dependent (Hyde and Soytong 2007, 2008) and therefore in this study we used both cultural methods and direct analysis of DNA from plants to compare the endophytes obtained. This approach has been adopted over

**Table 1** Database typing of root endophytic fungi isolated from *Empetrum nigrum* (EN), *Vaccinium vitis-idaea* (VV) and *Deschampsia flexuosa* (DF) from Hailuoto Primary successional seashore gradient. Strains marked with the same superscript originate from the same plant clone

| Sl. No. | Fungal isolate | Site description | Accession numbers | Closest match in the BLAST Incl. score* | Score | Accession numbers | Maximum identity (%) |
|---|---|---|---|---|---|---|---|
| 1 | **EN1** | late succession with *Cladina*-type forest site 5 | EU314675 | *Phialocephala fortinii* | 926 | EF093162 | 99 |
| 2 | **VV2**[1] | late succession with *Cladina*-type forest site 5 | EU314676 | *Phialocephala fortinii* | 909 | EF093162 | 98 |
| 3 | **VV3**[1] | late succession with *Cladina*-type forest site 5 | EU314677 | *Phialocephala fortinii* | 937 | EF093162 | 99 |
| 4 | **EN4**[2] | late succession with *Cladina*-type forest site 5 | EU314678 | *Mollisia minutella* | 857 | DQ008242 | 98 |
| 5 | **EN5**[2] | late succession with *Cladina*-type forest site 5 | EU314679 | *Mollisia minutella* | 863 | AJ430223 | 100 |
| 6 | **EN6** | late succession with EVT type forest site 4 | EU314680 | *Phialocephala fortinii* | 959 | EF093162 | 99 |
| 7 | **VV7** | late succession with EVT type forest site 4 | EU314681 | *Mollisia minutella* | 845 | DQ008242 | 98 |
| 8 | **EN8**[3] | mid successional deflation basin site 2 | EU314682 | *Phialocephala fortinii* | 937 | AY394921 | 100 |
| 9 | **EN9**[3] | mid successional deflation basin site 2 | EU314683 | *Phialocephala fortinii* | 937 | AY394921 | 100 |
| 10 | **EN10**[4] | late succession with EVT type forest site 3 | EU314684 | *Phialocephala fortinii* | 894 | AY078140 | 99 |
| 11 | **EN11**[4] | late succession with EVT type forest site 3 | EU314685 | *Phialocephala fortinii* | 935 | AY394921 | 99 |
| 12 | **EN12**[5] | late succession with EVT type forest site 3 | EU314686 | *Phialocephala fortinii* | 907 | AY394921 | 99 |
| 13 | **EN13**[3] | late succession with EVT type forest site 3 | EU314687 | *Phialocephala fortinii* | 928 | AY394915 | 99 |
| 14 | **EN14**[3] | late succession with EVT type forest site 3 | EU314688 | *Phialocephala fortinii* | 889 | AY078140 | 98 |
| 15 | **EN15**[4] | late succession with EVT type forest site 3 | EU314689 | *Phialocephala fortinii* | 926 | AY394921 | 99 |
| 16 | **EN16**[5] | late succession with EVT type forest site 3 | EU314690 | *Phialocephala fortinii* | 935 | EF446148 | 99 |
| 17 | **EN17**[5] | late succession with EVT type forest site 3 | EU314691 | *Phialocephala fortinii* | 918 | AY394921 | 98 |
| 18 | **EN18**[6] | late succession with EVT type forest site 3 | EU314692 | *Phialocephala fortinii* | 933 | EF446148 | 99 |
| 19 | **EN19**[6] | late succession with EVT type forest site 3 | EU314693 | *Phialocephala fortinii* | 915 | AY078138 | 99 |
| 20 | **DF20** | late succession with EVT type forest site 3 | EU314694 | *Phialocephala fortinii* | 902 | AY39492 | 98 |
| 21 | **DF21** | mid successional deflation basin site 2 | EU 314695 | *Phialocephala fortinii* | 896 | EF093162 | 99 |
| 22 | **DF22**[7] | late succession with EVT type forest site 3 | EU314696 | *Phialocephala fortinii* | 915 | EF093162 | 99 |
| 23 | **DF23**[7] | late succession with EVT type forest site 3 | EU314697 | *Phialocephala fortinii* | 937 | EF093162 | 99 |
| 24 | **DF24**[7] | late succession with EVT type forest site 3 | EU314698 | *Epacris microphylla* root associated fungus | 1022 | AY268196 | 97 |
| 25 | **DF25**[8] | mid successional deflation basin site 2 | EU314699 | *Phialocephala fortinii* | 922 | EF093162 | 99 |
| 26 | **DF26**[8] | mid successional deflation basin site 2 | EU314700 | *Phialocephala fortinii* | 924 | EF093162 | 99 |
| 27 | **DF27**[9] | late succession with EVT type forest site 3 | EU314701 | *Phialocephala fortinii* | 922 | AY078142 | 99 |
| 28 | **DF28**[9] | late succession with EVT type forest site 3 | EU314702 | *Phialocephala fortinii* | 915 | EF446148 | 99 |
| 29 | **DF29**[10] | mid successional deflation basin site 2 | EU314703 | *Phialocephala fortinii* | 905 | EF446148 | 99 |
| 30 | **DF30**[10] | mid successional deflation basin site 2 | EU314704 | *Phialocephala fortinii* | 920 | AY394921 | 99 |
| 31 | **DF31** | mid successional deflation basin site 2 | EU314705 | Ascomycete sp. | 869 | EF026054 | 97 |
| 32 | **DF32** | late succession with EVT type forest site 3 | EU314706 | *Phialocephala fortinii* | 928 | EF446148 | 99 |
| 33 | **DF33** | early successional dune ridge site 1 | EU314707 | *Phialophora sp.* | 776 | EF160066 | 95 |
| 34 | **DF34**[11] | early successional dune ridge site 1 | EU314708 | *Phialophora sp.* | 761 | EF160066 | 94 |
| 35 | **DF35**[11] | early successional dune ridge site 1 | EU314709 | *Phialophora sp.* | 784 | EF160066 | 95 |
| 36 | **DF36**[11] | early successional dune ridge site 1 | EU314710 | *Phialophora sp.* | 771 | EF160066 | 96 |
| 37 | **DF37** | early successional dune ridge site 1 | EU314711 | *Epacris pulchella* root associated fungus | 761 | AY627823 | 95 |
| 38 | **DF38** | mid successional deflation basin site 2 | EU314712 | *Epacris microphylla* root associated fungus | 1026 | AY268196 | 97 |

the last 4–5 years to identify endophytic mycelia sterilia by many authors (Wang et al. 2005; Promputtha et al. 2007; Tao et al. 2008), and extraction of whole DNA with methods to study the fungi based on the sequences presence have also been adopted (Wang et al. 2005; Rungjindamai et al. 2008; Seena et al. 2008). To our knowledge, this is the first report of the molecular phylogenies of the DSE in the mid-boreal zone and also the first report of the unculturable root endophytes of *D. flexuosa*.

## Culturable fungal endophytes

In this study, we obtained 50 endophytic isolates from a total of 46 root systems from (a successional gradient of a relatively small island approximately 30×30 km) a transect of a reasonably young land uplift history (approx. 50–300 years according to soil topography and average land uplift rate) (Alestalo 1979). The lowest number of isolates per root system was obtained from the early successional

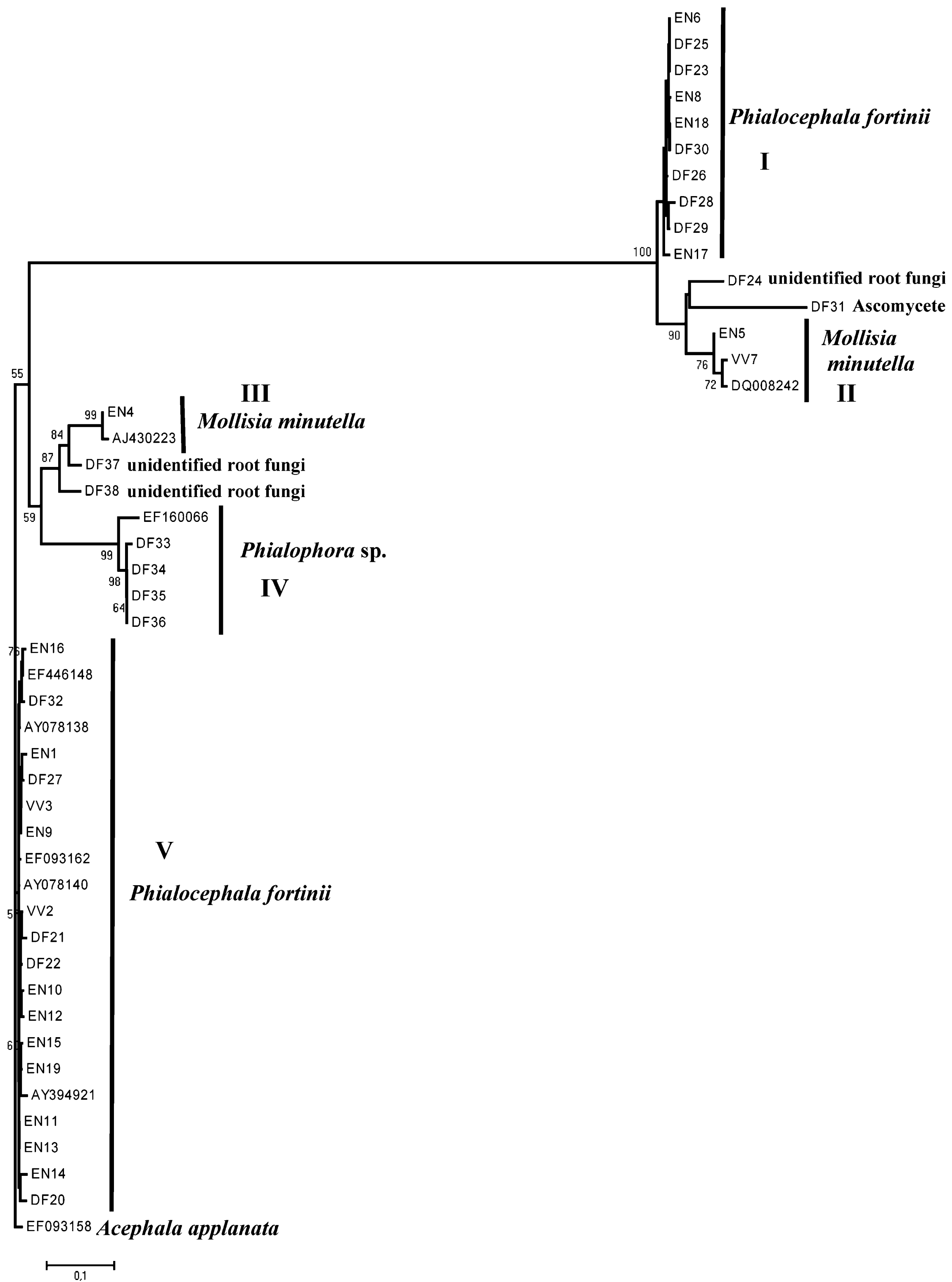


**Fig. 1** Neighbour-joining analysis of ITS and 5.8S rDNA sequences of root endophytic fungi. The tree was derived from sequences of 38 endophytic fungi isolated from different hosts *E. nigrum* (Empetraceae) (sample code EN), *Vaccinium vitis-idaea* (Ericaceae) (sample code VV) and *Deschampsia flexuosa* (Poaceae) (sample code DF) representing different stands and nine sequences were retrieved from GenBank. Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site, number of branches are bootstrap values (1,000 replicates, values below 50% are not shown)

**Table 2** Unculturable sequences closely matching to the GenBank sequences

| SL.NO | Accession number | GenBank closest match | location | Score | Accession number (Genbank) | Sequence coverage (%) | Max. identity (%) | UNITE database closest match | Acession number (UNITE) | E-value |
|---|---|---|---|---|---|---|---|---|---|---|
| UNC1 | FJ517587 | Uncultured fungus | Norway | 939 | AM999704 | 82 | 97 | Uncultured fungus | AM999704 | 0.0 |
| UNC2 | FJ517588 | Uncultured mycorrhizal fungus | Germany | 1116 | EF195594 | 93 | 98 | Uncultured fungus | DQ093748 | 0.0 |
| UNC3 | FJ517589 | Uncultured fungus | Oslo, Norway | 950 | AM999704 | 81 | 97 | Uncultured fungus | AM999704 | 0.0 |
| UNC4 | FJ517590 | Uncultured mycorrhizal fungus | Germany | 1109 | EF195594 | 92 | 98 | Uncultured fungus | DQ093748 | 0.0 |
| UNC5 | FJ517591 | Uncultured fungus | Oslo, Norway | 950 | AM999704 | 60 | 97 | Uncultured fungus | AM999704 | 0.0 |
| UNC6 | FJ517592 | *Cortinarius callisteus* | Kelowna, Canada | 930 | DQ097876 | 90 | 90 | Uncultured fungus | AM999704 | 0.0 |
| UNC7 | FJ517593 | Uncultured fungus | AK 99775, USA | 968 | EF434073 | 97 | 87 | *Mycena galericulata* | DQ404392 | 0.0 |
| UNC8 | FJ517594 | *Cortinarius callisteus* | Kelowna, Canada | 794 | DQ097876 | 100 | 95 | uncultured fungus | AM999704 | 0.0 |
| UNC9 | FJ517595 | Uncultured fungus | Alaska, USA | 977 | EU292528 | 97 | 87 | *Mycena galericulata* | DQ404392 | 0.0 |
| UNC10 | FJ517596 | Uncultured fungus | Oslo, Norway | 870 | AM999704 | 61 | 95 | uncultured fungus | AM999704 | 0.0 |
| UNC11 | FJ517597 | Uncultured fungus | Alaska, USA | 1127 | EF434073 | 97 | 90 | *Mycena galericulata* | DQ404392 | 0.0 |
| UNC12 | FJ517598 | Uncultured fungus | Alaska, USA | 1018 | EF434073 | 92 | 88 | *Mycena galericulata* | DQ404392 | 0.0 |
| UNC13 | FJ517599 | *Cortinarius callisteus* | Kelowna, Canada | 954 | DQ097876 | 84 | 94 | uncultured fungus | AM999704 | 0.0 |
| UNC14 | FJ517600 | Uncultured fungus | Alaska, USA | 950 | EF434073 | 97 | 87 | *Mycena galericulata* | DQ404392 | 0.0 |
| UNC15 | FJ517601 | Uncultured fungus | Goettingen, Germany | 1066 | EF195594 | 74 | 96 | uncultured fungus | DQ093748 | 0.0 |
| UNC16 | FJ517602 | Uncultured fungus | Oslo, Norway | 809 | AM999704 | 62 | 93 | uncultured fungus | AM999704 | 0.0 |

dune ridge (0.5 per root system) and highest from the late successional site (1.5 per root system). Although the goal of this study was not to monitor the absolute number of strains between different successional stages and the number of samples varied between sites, our result is in line with the reports on increased fungal abundance along with increasing soil successional age (Pennanen et al. 2001).

The sequencing of the isolates revealed that most of the fungal strains were conspecific with the dark septate endophyte *Phialocephala fortinii* (27 strains out of 38). *Phialocephala fortinii* has frequently been isolated from roots of numerous plant taxa worldwide (Jumpponen 1999; Menkis et al. 2004; Addy et al. 2005), including *Deschampsia flexuosa* (Zijlstra 2006). Jumpponen and Trappe (1998) have found *Phialocephala fortinii* common at a primary successional site on a glacier forefront. Our result is in line with these observations and suggests that *P. fortinii* is an ubiquitous endophyte in an isolate-based data and that it is also associated with primary successional sites.

In the phylogram, the *P. fortinii* strains grouped into two separate clades, mostly irrespective of the site or host plant species with the exception that at the early succession stage

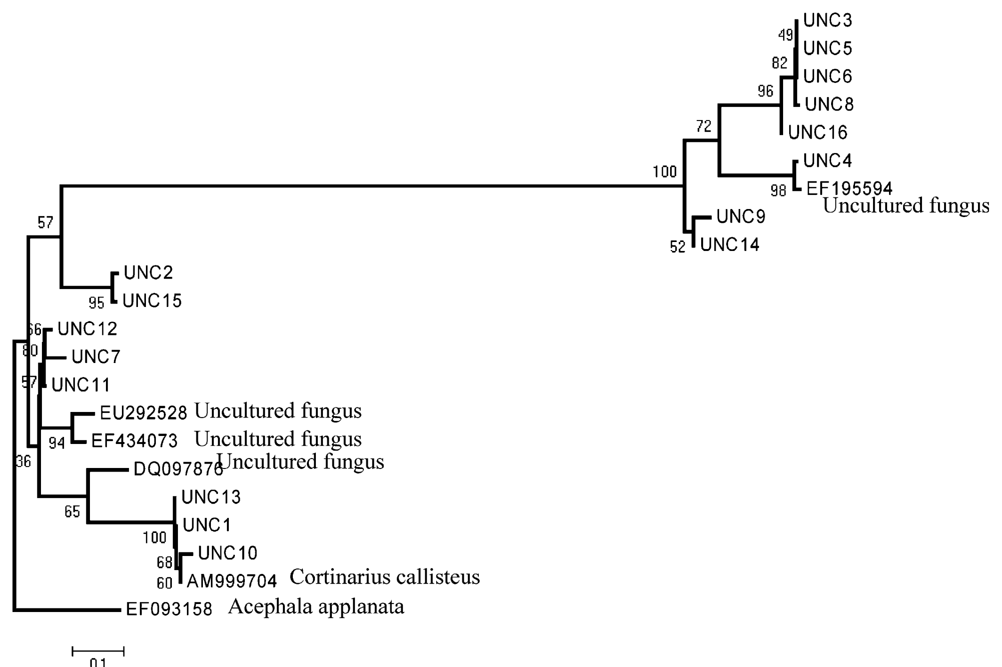


**Fig. 2** Neighbour-joining analysis of ITS and 5.8S rDNA sequences of unculturable fungi. The tree was derived from sequences of 16 endophytic unculturable fungi detected in *Deschampsia flexuosa* and from five sequences that were a close match with the unculturables were retrieved from GenBank. Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site, number of branches are bootstrap values (1,000 replicates, values below 50% are not shown)

of the sand dune ridge sites the species was absent. These clades were phylogenetically quite far from each other. It is probable, that *P. fortinii* includes several cryptic species (Grünig et al. 2004) that are spatially heterogeneous at the scale of a few square meters (Grünig et al. 2002; Piercey et al. 2004). These characteristics may explain the division of our *P. fortinii* sequences into two separate clades and the genetic diversity of our strains.

Isolates identified as *Mollisia minutella, Phialocephala fortinii* and *Phialophora* sp. were separated into different clades with a high bootstrap support (90–100%). The earlier findings have also shown *P. fortinii* and *Mollisia* to be phylogenetically closely related, but not conspecific (Vrålstad et al. 2002; Wang et al. 2006). In addition, *Mollisia* has been assumed to represent the teleomorph of the genus *Phialophora* (including *Cadophora*) (Sharples et al. 2000). The early successional stage at the dune ridge was characterized by the presence of *Phialophora* sp., while this taxon was absent from the sites of later successional stages. All these strains clustered together and were equally similar (94–96%) to the *Phialophora* sp. strain (EF160066) in the GenBank. The presence of *Phialophora* sp. and the absence of *P. fortinii* in these early successional dune ridges suggest that these root-associated fungi could differ in their ecology, for example in their dispersal and resource acquisition strategies. Due to the post-glacial land uplift phenomenon and the flat topography of the study area, new space for primary colonizers is continuously formed. Field observations on Bothnian Bay seashore (Aikio et al. 2000; Pennanen et al. 2001) suggest that competition increases along with increasing successional age not only among plants (Olff et al. 1993, Berendse et al. 1998), but also among fungal species according to the succession theory of mycorrhizal fungi (Read 1989; Schulz and Boyle 2005). Simultaneously, decrease in soil pH and accumulation of soil organic layer greatly affects nutrient availability, as especially nitrogen is increasingly incorporated into soil organic matter (Pennanen et al. 2001). Therefore, the abundance of *Phialophora* sp. in the early successional dune ridge could, hypothetically, indicate that it is able to effectively utilize inorganic nitrogen sources, is a relatively weak competitor and may have better dispersal abilities at colonizing recently emerged land. Consequently, compared to *Phialophora, P. fortinii* may perform better at utilizing organic nutrient sources (Caldwell et al. 2000), be a stronger competitor and have lower dispersal abilities. These hypotheses remain to be tested.

In one case the closely located *Empetrum* and *Deschampsia*-clones (clones growing as overlapped in the field) were most probably inhabited by the same fungal strain (strains EN16 and DF32). Vrålstad et al. (2002) and Zijlstra (2006) have reported helotialean fungi associated with dwarf shrubs to inhabit roots of grasses as well. Trees and ericaceous dwarf shrubs have also been shown to form mycorrhizae with the same helotialean fungi (Villareal-Ruiz et al. 2004; Vrålstad 2004) and Chambers et al. (2008) has reported an ericoid mycorrhizal isolate to form dark-septate endophyte-type colonization on a different host. Our result confirms that grasses and ericaceous dwarf shrubs can be colonized by the same endophytic fungi, also in the studied primary successional ecosystem.

### Unculturable isolates

A culture-independent approach by direct sequencing was used to detect the unculturable fungi in *Deschampsia flexuosa*. Our results indicate that unculturable fungi are common and numerous in the root system of a mid-successional grass species. This is in agreement with an earlier report (Vanderkoornhuyse et al. 2002). The ecological role of unculturable fungi in a root system could be similar to that of endophytes in general, varying from mutualistic to antagonistic (Saikkonen et al. 1998; Schulz and Boyle 2005). The sequences of unculturable fungi did not match with any of the culturable fungi obtained in the study. This is comparable with other studies on endophytes using culture dependent and direct sequencing approaches (Duong et al. 2006). This gives an idea of the negligible overlap between the unculturable and culturable groups of endophytes within a plant. Therefore, this study shows that isolated fungal endophytes appear to represent only a fraction of the total endophyte community and concurs with similar studies (Duong et al. 2006; Arnold 2007; Tao et al. 2008). The association of endophytes that can grow on laboratory media with those of their hosts are the most studied (Hyde and Soytong 2008; Huang et al. 2008), whereas the unculturable species may in fact represent equally intimate associations (Berch et al. 2002; Chelius and Triplett 2001; Hyde and Soytong 2007, 2008) an aspect that has barely been considered. Numerous publications on the abundance of *P. fortinii* as a root associate (Jumpponen 1999; Mandyam and Jumpponen 2005) suggest that it is favoured by the isolation method. The complete absence of *P. fortinii* in our unculturable endophyte data suggests that the abundance of *P. fortinii* may be overestimated due to the methodology used.

The direct sequencing method also indicates an association of *Deschampsia flexuosa* with the ectomycorrhizal fungus *Cortinarius callisteus* in our data. We consider this result to be highly questionable for three reasons (i) it was supported only by low sequence coverage (84 and 91%) (ii) the known ecology of this species does not match the conditions of our study site (Hansen and Knudsen 1992) or association with the graminoid host and (iii) the fruit bodies of this macrofungal species have never been observed at the study site

despite a ten-year monitoring study at the site (Karita Saravesi unpublished). Therefore, the sequence more likely represents another *Cortinarius* species. Only 350 out of 2,000 named species of *Cortinarius* are lodged in GenBank (Frøslev et al. 2007). In UNITE database, this sequence was identified as an uncultured fungus (AM999704).

A common saprotroph, *Mycena galericulata* (DQ404392 in UNITE), was detected in the roots of *Deschampsia flexuosa* by direct sequencing. This sequence closely matched five UNITE sequences (UNC7, UNC9, UNC11, UNC12, UNC14). This species may grow as a saprobe on graminoid roots. In addition, several *Mycena* species are also reported to have a capability to form a parasitic mycorrhizal association with non-photosynthetic orchids (Ogura-Tsujita et al. 2008).

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